Hypoxia-inducible regulatory networks and their roles in aging in C. elegans

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Hypoxia-inducible factor regulatory networks and their roles in aging in C. elegans

by

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CHAPTER 4. SWAN-1 interacts with EGL-9 to regulate HIF-1 activity in *C. elegans*

Abstract ................................................................................................................. 91
Introduction ............................................................................................................. 92
Materials and Methods .......................................................................................... 94
Results ..................................................................................................................... 99
  *ia50* allele activates HIF-1 activity in *vhl-1* mutants ........................................... 99
  *ia50* is a loss-of-function mutation in swan-1(F53C11.8) ....................................... 101
SWAN-1 suppresses HIF-1 activity via a VHL-1-independent pathway .................. 102
SWAN-1 interacts with EGL-9 to regulate HIF-1 activity ...................................... 103
DISCUSSION ......................................................................................................... 104
FIGURE LEGENDS ............................................................................................... 106
References ............................................................................................................. 108

CHAPTER 6. GENERAL CONCLUSION .................................................................... 121
SUMMARY ............................................................................................................. 121
GENERAL DISCUSSION ....................................................................................... 122
  HIF-1 modulates aging in *C. elegans* ............................................................... 122
  Hypoxia modulates aging in *C. elegans* ......................................................... 125
SWAN-1 regulates HIF-1 in *C. elegans* .............................................................. 128
REFERENCES ....................................................................................................... 130

Appendix I. Co-immunoprecipitation of HIF-1 interacting proteins from *Caenorhabditis elegans* ........................................................................................................ 134
FIGURE LEGENDS ............................................................................................... 138
REFERENCES ....................................................................................................... 139
  Table 1: Epitope-tagged HIF-1 transgenes ......................................................... 141
  Table 2: Mass-spec results ................................................................................. 141
APPENDIX II. ADDITIONAL RESULTS ......................................................................................... 143

Does HIF-1 over-expression regulate mitochondria to extend lifespan? ......................... 143

*hif-1* is involved in SIR2 pathway to modulate aging in *C. elegans.* ................................. 144

A role of autophagy in lifespan extension mediated by HIF-1 over-expression and hypoxia. ................................................................................................................................... 145

Figure Legends .......................................................................................................................... 145

References ................................................................................................................................... 146

Table 1: Effects of *pdhk-1* RNAi on the longevity of wildtype and HIF-1 over-expression
worms........................................................................................................................................... 149

Table 2: *hif-1* is involved in the longevity of SIR2 over-expression worms. ......................... 149

Table 3: Effects of *bec-1* RNAi on the longevity of HIF-1 over-expression worms. .............. 150

Table 4: Effects of *bec-1* RNAi on the longevity of hypoxia treatment.................................. 150

ACKNOWLEDGEMENTS ............................................................................................................. 152
CHAPTER 1. GENERAL INTRODUCTION

Molecular oxygen, as the final electron accepter of aerobic respiration, is essential for the survival of most metazoans. Oxygen deprivation (hypoxia) is often involved in development, homeostasis and many diseases. In order to adapt to hypoxia, multi-cellular organisms have evolved complex networks that regulate metabolic changes in both systemic and cellular levels to mediate changes in angiogenesis/vascular remodeling, glycolytic metabolism and cell proliferation (Hopkins and Powell 2001). Many of these metabolic changes are also involved in aging, suggesting that hypoxia response signaling may be intertwined with the pathways modulating aging. The hypoxia-inducible factor (HIF) transcription factor is a master regulator that mediates most of the transcriptional changes during hypoxia adaptation. Recent studies have suggested that HIF can influence replicative senescence in mammals (Katschinski 2006), but the roles of HIF in aging are not fully understood.

The C.elegans hif-1 gene is orthologous to mammalian HIF-1 α genes. C. elegans has proven to be a premier model for studying aging (Olsten et al. 2006) and also a powerful system for deciphering the regulation and function of HIF (Epstein et al. 2001; Shen et al. 2006). In this dissertation research, I studied the roles of HIF-1 and hypoxia response in organismal aging in C.elegans. My results established HIF-1 as an important modulator of aging in C.elegans. I discovered that both HIF-1 over-expression and hif-1 loss-of-function mutations extend life span. I further demonstrated that hypoxia treatment promotes longevity in C.elegans. My studies also provided evidence for HIF-1-independent pathways for hypoxia response to modulate aging. Finally, using forward genetic approaches, I identified a novel protein that regulates the activity of C. elegans HIF-1 in mechanisms distinct from the VHL-1-dependent proteosomal degradation pathway. I have divided the literature review into two major sections: aging and hypoxia.
LITERATURE REVIEW

AGING: LESSONS FROM STUDIES OF C. elegans

Why must we age? This is a question that interests most individuals at some time in their lives. Therefore, it is not surprising that aging is one of the most intriguing fields to the public. As human life expectancy continues to increase, age-related disorders, such as cancer, diabetes, cardiovascular and neurodegenerative diseases are among the biggest threats to human health. Understanding the molecular processes that govern aging holds the promise to develop novel treatments for these disorders.

In the past 20 years, studies of aging in invertebrate model organisms, including Caenorhabditis elegans, have provided key insights into the genes and gene networks that modulate aging. Since the discoveries in 1980s that mutations in single genes could significantly extend life span in C. elegans, hundreds of single-gene mutations have been found to increase longevity in nematodes, yeast and fruit flies. This is especially interesting because many of the pathways that modulate aging are conserved across species as diverse as nematodes and humans. There is great hope that these discoveries will translate to advances in human medicine, but major questions remain. For example, reduction-of-function mutations in daf-2, an insulin/IGF-1 receptor orthologue, double the life span in C.elegans (KENYON et al. 1993). Can we expect that interventions that inhibit insulin/IGF-1 pathway in humans will yield similar results? Also, in terms of deciphering how age-related diseases intersect with basic aging processes, how much we can learn from the model systems about age-related diseases in humans? In this literature review, I discuss some of the processes that are central to aging and review some of the important insights provided by studies in C.elegans.

The senescence of mitotic cells: when regeneration matters

Aging is a fundamental process with great natural diversity. Considering the longevity of nematodes (weeks) and mammals (years), it is fascinating to ask what enables humans to
live for decades, while nematodes live only for weeks? At present, a comprehensive answer to this question is yet to be found. However, cell replacement and regeneration may be major contributors. In mammals, many somatic tissues, to various degrees, have the capacity to replace the cell loss with proliferation of mitotic cells. This mechanism to maintain the integrity of tissues and organisms is totally absent in *C. elegans*, in which all somatic cells of adults are post-mitotic. The evolution of stem cells that renew, repair, and in some cases, regenerate somatic cells has enabled complex organisms, like humans, to live longer.

However, as demonstrated by Hayflick and colleagues more than 40 years ago, normal mitotic cells only had limited abilities to proliferate in culture (HAYFLICK 1965). In their classic experiment, they found that explanted human fibroblasts, after around 50 cell doubling, gradually lost their ability to divide. This phenomenon was termed as cellular senescence. Substantial evidence from recent studies suggested that cell senescence also occurs and is important to aging *in vivo* (CAMPISI and D'ADDA DI FAGAGNA 2007). Using senescence-associated markers, senescent cells were found in many renewable tissues in rodents, primates and humans, and their numbers increase with age (DIMRI et al. 1995; JEYAPALAN et al. 2007; KRISHNAMURTHY et al. 2004). It has been suggested that senescence-associated suppression of stem-cell proliferation and tissue regeneration may be a major cause of aging (JANZEN et al. 2006; KRISHNAMURTHY et al. 2006; MOLOFSKY et al. 2006).

Once cellular senescence was discovered, people began to ask what selective pressure might contain mitotic cells from unlimited replication. Although increases in post-reproductive longevity may provide little evolutionary benefit for most animal populations, increased ability to heal and repair tissues through unlimited cell replacement and regeneration would seem to provide huge advantage in fitness of natural selection. Yet evolution has selected the opposite. The predominant theory is that cellular senescence has evolved as a mechanism to suppress the tumor formation (BRAIG and SCHMITT 2006; CAMPISI 2001; SAGER 1991). Although cell proliferation is essential for tissue renewal in complex organisms such as mammals, it also increases the risks of acquiring and subsequently propagating mutations. Senescence limits the number of times each mitotic cells can replicate and limits tumor growth. The inextricable link between senescence and
tumor suppression is highlighted by the finding that transgenic mice with constitutively hyperactive forms of p53, a major tumor suppressor gene, were remarkably tumor-free, but also displayed accelerated aging phenotypes (Maier et al. 2004; Tyner et al. 2002).

The plasticity of aging in post-mitotic cells

Organismal aging is also determined by the lifespan of post-mitotic cells. Therefore, it is important to consider the aging process in post-mitotic cells. Post-mitotic cells are those that have irreversibly exited from the cell cycle and have undergone terminal differentiation. In mammals, post-mitotic cells include neurons, muscle cells, osteocytes and blood cells that comprise much of the brain, heart and skeletal muscle, bone and circulatory system. In addition, since many cells capable of mitosis (tissue specific stem cells or progenitor cells) are mostly quiescent, many of the factors that contribute to aging of post-mitotic cells are also applied to mitotic cells (Drummond-Barbosa 2008; Pan et al. 2007b). This allows post-mitotic cells as a good model to study aging at cellular level.

The lifespan of post-mitotic cells vary widely. For example, in adult C. elegans, where all somatic tissues are post-mitotic, aging is characterized by a progressive deterioration of muscle cells while the nervous system is largely preserved (Herndon et al. 2002). The comparison is more staggering if we consider human neurons, which can function throughout human life.

Neurodegenerative diseases, such as Alzheimer’s disease (AD) and Parkinson’s diseases (PD) are tightly linked to the aging of human neurons. The incidence of these diseases increases dramatically with age. Although the etiologies of these devastating diseases are not fully understood, they may be initiated by normal aging processes and accelerated by adverse genetic or environmental conditions (Mattson and Magnus 2006). Some of the factors that contribute to neurodegeneration also influence aging of other tissues, such as heart and muscle (Chien and Karsenty 2005). So there is real synergism between these related fields. Moreover, some risk factors of neurodegenerative diseases turn out to be major components in modulating aging in C.elegans (see detailed discussion below). Therefore, despite the huge difference between the longevity of human neurons and
C.elegans somatic tissues, some common themes in the basic biology of aging appear to be conserved.

**Mitochondria and oxidants: the enemy within**

According to the free radical theory of aging, macromolecular damages generated by reactive oxygen species (ROS) accumulate throughout the life and cause aging (BECKMAN and AMES 1998). Given that aerobic metabolism in mitochondria is the main source of ROS, mitochondria play uniquely important roles in aging as well as in neurodegenerative diseases.

Mitochondrial dysfunction has long been associated with the pathogenesis of PD. Impairment of mitochondrial respiration by inhibiting complex I in dopaminergic neurons results in increased ROS and oxidative damage to the cell (VILA and PRZEDBORSKI 2003). Oxidative damage may accrue and ultimately trigger mitochondria-dependent programmed cell death, which is critical to PD-related dopaminergic neurodegeneration (DAUER and PRZEDBORSKI 2003; VILA and PRZEDBORSKI 2003). Recent studies have demonstrated that many proteins involved in familiar forms of PD, such as PTEN-induced kinase 1, parkin, alpha-synuclein, directly or indirectly interact with mitochondria (VILA et al. 2008). Intriguingly, mitochondrial dynamics, the regional distribution of mitochondria within neurons, also appear to be important in neurodegenerative diseases. It has long been known that a large number of mitochondria localize at the pre-synaptic terminals. This regional enrichment of mitochondria reflects the high energy demand during synaptic activity, but may also restrict the ROS generation and molecular damage. In healthy neurons, mitochondria are actively transported to axon terminals. The impairment of mitochondrial axonal transport can be an important contributor to many neurodegenerative disorders (MAGRANE and MANFREDI 2009).

Perturbations of mitochondrial genes have profound impacts on aging of C. elegans. Some mutations of the respiratory chain in C.elegans, such as mev-1(succinate dehydrogenase cytochrome b), shorten lifespan presumably by increasing ROS levels (ISHII et al. 1998). Conversely, a mutation in isp-1, which encodes a component of respiratory chain complex III, extends life span (FENG et al. 2001). Likewise, knocking down more than
a dozen of mitochondrial components by RNAi in C. elegans increases lifespan, probably through reducing ROS generation (Dilllin et al. 2002b; Lee et al. 2003b). Indeed, Rea and Johnson noted that many of the mutations that increased longevity in C. elegans were predicted to divert cellular metabolism from the classic TCA and electron transport chain to alternative energy-producing pathway (Rea and Johnson 2003). However, it is worth noting that due to the lack of techniques to measure the ROS level in vivo, the causal relationship between ROS generation and altered lifespan in C.elegans still waits to be determined.

**Maintenance and repair programs: roles of stress response transcription factors**

ROS are by-products of respiration and other metabolic processes and also serve as signaling molecules (Finkel 2003). Although diverting from classic aerobic respiratory metabolism may decrease ROS generation and extend lifespan, it compromises the efficiency of energy production and may decrease the overall fitness of the organism. Therefore, organisms must adapt to certain levels of ROS and evolve processes to attenuate the damages that ROS cause. During evolution, maintenance and repair programs have been developed to counteract these damages, and increased ability to limit macromolecular damage may postpone aging.

Increased oxidative stress is associated with many neurodegenerative diseases, such as PD, AD, Huntington’s diseases and amyotrophic lateral sclerosis. In response to oxidative stress, the Nrf-2 transcription factor activates a group of anti-oxidant genes, including cystine/glutamate antiporter and glutathione peroxidase to protect cells from oxidative stress induced cell death (Kang et al. 2005; Qiang et al. 2004). Drug induced activation of Nrf-2 protects mice from insults of mitochondrial inhibition (Shih et al. 2005). Conversely, Nrf-2 deficient mice are significantly more vulnerable to oxidative stress induced by mitochondrial complex I and II inhibition (Calkins et al. 2005).

Some of the key regulators of macromolecular maintenance and repair have been shown to modulate post-mitotic aging. Some transcription factors, exemplified by DAF-16(FOXO), HSF-1(heat shock factor), SKN-1 (Nrf-2-like), orchestrate large sets of genes that modulate stress response, as well as overall metabolism (Hsu et al. 2003; Lee et al. 2003a; McElwee et al. 2003; Morley and Morimoto 2004; Murphy et al. 2003; Tullet
et al. 2008). Among these stress response genes are a handful of antioxidant enzymes, such as glutathione peroxidase, glutathione reductase, superoxide dismutase (SOD) and catalase. Another category of genes being activated by stress response transcription factors are chaperones that mediate protein homeostasis. Interestingly, while each antioxidant enzyme or chaperone may only make a minor contribution to longevity in C. elegans, mobilizing a cohort of these genes by activating stress response transcription factors dramatically increases lifespan. For example, using microarray analysis, Murphy et al. identified a group of stress response genes that are up-regulated in animals that carried a reduction-of-function mutation in daf-2. Of these, they focused on the genes that were positive regulated by DAF-16/FOXO. While a loss-of-function mutation of daf-16 completely abolishes the longevity of daf-2 mutant worms, knocking down individual daf-16-dependent stress response genes by RNAi shortened lifespan of daf-2 mutants between 10-20% (Murphy et al. 2003). Conversely, animals carrying additional hsf-1 gene copies lived ~40% longer than normal (Hsu et al. 2003) while over-expression of small heat-shock protein gene hsp-16, which is induced by HSF-1, extended life span ~ 10% (Walker and Lithgow 2003).

**Recycling and eliminating damaged molecules: a role of autophagy**

Accumulation of damaged proteins and organelles is a hallmark of aging and aging related diseases. Protein aggregations shared by neurodegenerative diseases disturb neural function by hampering axonal transport, synaptic integrity, and mitochondrial function (Rubinsztein 2006). Interestingly, autopsies of healthy old person’s brain revealed as many protein aggregates as in AD patients, emphasizing the common biology of normal aging and neurodegenerative disorders (Esiri 2001).

Autophagy, a cellular process whereby damaged macromolecules and organelles are degraded and recycled for ATP and reusable building blocks, provides another example that studies of neurodegeneration and C. elegans longevity have merged. An increasing body of evidence indicates that autophagy is a critical defensive mechanism in removing protein aggregates in neurons. Studies that used brain-specific conditional knockouts of autophagy-related genes (ATG) revealed that loss of autophagy elicits protein aggregate formation and neurodegeneration cascade (Hara et al. 2006; Komatsu et al. 2006).
Correspondingly, inducing autophagy holds a therapeutic promise to slow the neural loss in neurodegenerative disorders (BOSSY et al. 2008). It is also noteworthy that if pushed to the extreme, autophagy can be lethal to a cell, but at moderate levels, it may slow neural loss in neurodegenerative disorders (VENTRUTI and CUERVO 2007).


Organismal aging and lessons learned from long-lived mutant worms

The results of longevity study in C.elegans are not only informative to the aging of post-mitotic cells, but also provide insights into the aging in the organismal level. Using C.elegans as a model system, it has been demonstrated that mutations of single gene can delay aging. Some of these long-lived mutants are astonishing: down-regulating insulin/IGF-1 signaling (IIS) pathway routinely increases lifespan in nematodes twofold (KENYON 2005), and one severe loss-of-function mutation in age-1, which encodes the phosphatidylinositol 3-kinase catalytic subunit, was shown to increase lifespan tenfold (AYYADEVARA et al. 2008). It is probably unreasonable to believe that interventions to inhibit the evolutionarily conserved IIS pathway can also double the life span in humans, as complexity of signaling pathways increase during evolution (VIJG and CAMPISI 2008). However, understanding the mechanisms underpinning the dramatic impact of single gene mutations may give us sufficient knowledge to forestall degeneration in human life.

The first lesson we can learn from the long-lived mutant worm is that a single gene can orchestrate multiple mechanisms to modulate aging in cellular level. As discussed earlier, mitochondrial metabolism, stress response transcription factors and recycling and eliminating damaged proteins are major components in the cellular responses that modulate aging of
post-mitotic cells. Hypoporphic mutations of *daf-2* induce all of these responses (Figure 1). In *C. elegans*, the insulin/IGF receptor DAF-2 regulates a handful of transcription factors, including DAF-16, HSF-1, SKN-1, that mediate stress response. These transcription factors can mobilize a large number of genes that modulate oxidative stress and protein homeostasis (AN and BLACKWELL 2003; HSU et al. 2003; LEE et al. 2003a; McELWEE et al. 2003; MURPHY et al. 2003). DAF-16 also regulates a number of enzymes involved in mitochondrial metabolism in *C. elegans* (LEE et al. 2003a; McELWEE et al. 2003; MURPHY et al. 2003). Moreover, as discussed earlier, *daf-2* mutation induces autophagy, which is required for the life span extension in *daf-2* mutants (MELENDEZ et al. 2003).

Although cell autonomous processes like ROS neutralization and protein folding are central to post-mitotic aging, studies have revealed that some genes have non-cell-autonomous and tissue-specific roles. For example, many mutations in *C. elegans* that alter sensory perception extend lifespan (ALCEDO and KENYON 2004; APFELD and KENYON 1999). This longevity effect is largely *daf-16* dependant (ALCEDO and KENYON 2004; APFELD and KENYON 1999), indicating their association with insulin/IGF pathway. Apfeld et al, examined somatic mosaic animals and concluded that loss of *daf-2* function from only a few neurons was sufficient to extend lifespan (APFELD and KENYON 1998). Some sensory neurons may influence lifespan by producing insulin-like peptides (Li et al. 2003; MURPHY et al. 2003; PIERCE et al. 2001). Moreover, increasing DAF-16 activity in intestinal cells extends lifespan of worms ~50% (LIBINA et al. 2003). Interestingly, reduced IGF-1 signaling specifically in adipose tissue extends life span in mice (BLUHER et al. 2003). Therefore, tissue-specific intervention of particular signaling pathways might also slow aging in humans.

Studies of long-lived mutant worms also indicate that stochastic factors are important in aging. In standard culture conditions, the average adult lifespan of wild-type animals is 18-20 days. However, the first worm in a population may die at around 10 days, and the maximum lifespan is around 30 days. Extensive variability exists in age-related degeneration even among cells of the same type within an individual (HERNDON et al. 2002). Interestingly, senescence of mitotic cells also exhibit stochastic properties: senescent cells are present at early passage cultures and their percentage gradually increases with each serial passage in the
culture (CRISTOFALO and SHARF 1973). Even two cells derived from a single mitosis displayed variation in their ability to proliferate (JONES et al. 1985).

Conclusions

The prevalent theories on aging suggest that the aging results from the damage that is the natural consequence of normal metabolic processes and of less predictable stresses. The rates of damage and of cellular or tissue repair are influenced by environmental or genetic variables.

In this review, I discussed some common themes from studies that have analyzed the genetic basis of aging. Mutations that extend lifespan often 1) to decrease the production of ROS, the major source of molecular damages, mainly through modulating mitochondrial activity; 2) to increase cellular resistance to stresses, often by regulating some stress response transcription factors; 3) to eliminate damaged molecules and to reallocate energy resources to the maintenance and repair machinery from other cost processes, such as reproduction and growth. Importantly, these principle components are often orchestrated at cellular level as well as in tissue and organ system to regulate organismal aging.

Complex animals, like mammals, also recruit cell replacement to maintain the integrity of tissues and organisms, to counteract aging. However, this strategy also puts the animals in danger of cancer. Cellular senescence limits potential for tumor growth, but may also expedite aging.

In attempts to apply these strategies for human lifespan extension, however, it should be kept in mind that although many pro-aging pathways are evolutionarily conserved, the results of interventions that target these pathways may be different in different species as well as in different tissues. For example, while activation of DAF-16 dramatically increases lifespan in C. elegans, no correlation has been identified in other variants of FOXO genes to survival at older age in humans. Moreover, while activation of FOXO3 induces autophagy in muscle cells, it leads to apoptosis in neurons in mammals (GILLEY et al. 2003; LEHTINEN et al. 2006; MAMMUCARI et al. 2007; SHINODA et al. 2004). Nonetheless, a more complete understanding of aging mechanisms and their relationships to age-related diseases (i.e. cancer,
diabetes, cardiovascular and neurodegenerative diseases) will allow us to develop sophisticated and integrative interventions to increase human health.

HYPOXIA RESPONSE AND HYPOXIA-INDUCIBLE FACTOR

The survival of all metazoans depends on the utilization of molecular oxygen to maintain the intracellular bioenergetics. During development, homeostasis as well as many pathological conditions, such as tumor, ischemia and stroke, cellular oxygen levels often do not meet the physiological demand. This condition is termed as hypoxia. The intensive study of hypoxia began as early as 100 years ago when Schwarz and colleagues noticed that normal mammalian cells under hypoxia were less sensitive to irradiation than those irradiated in the presence of O$_2$ (CHURCHILL-DAVIDSON et al. 1955). This resistance effect was later also identified in human tumors, and varying O$_2$ concentration has since then been associated with significantly different effects on cellular processes (SIMON et al. 2008). Notably, hypoxia profoundly influences the properties of tumors, from proliferation and angiogenesis to irradiation and chemotherapy resistance. To understand the molecular mechanisms behind these effects has been the main focus of hypoxia research.

Discovery and molecular cloning of hypoxia-inducible factors

The cloning of hypoxia-inducible factor 1 (HIF-1) is the cornerstone in elucidating the mechanisms by which animal cells sense O$_2$ tension and regulate transcription in hypoxia (WANG et al. 1995). HIF-1 are heterodimeric transcription factor composed of a $\alpha$ and a $\beta$ subunit. Both subunits are members of the basic helix-loop-helix (bHLH) transcription factor family that contains PAS domain (the name PAS comes from the initial characterization of the domain in *Drosophila melanogaster* protein PER and SIM and the mammalian protein aryl hydrocarbon nuclear translocator (ARNT)). While HIF-1$\beta$ subunit, also known as ARNT, is generally expressed, the stability of HIF-1$\alpha$ subunit is tightly controlled by cellular oxygen level (JIANG et al. 1996).
The dimeric HIF-1 complex binds to conserved Hypoxia-Response-Element (HRE) (5'-G/ACGTG-3’) that is associated with numerous transcriptional target genes mediating hypoxia response. In mammals, HIF induces genes like VEGF and EPO, which are involved in systematic hypoxia response, such as angiogenesis/vascular remodeling and erythropoiesis. A large number of HIF target genes are associated with cellular hypoxia responses, such as metabolism, proliferation, migration and autophagy. (WOUTERS and KORITZINSKY 2008).

The complexity of HIF arises as the discovery of the HIF-1α paralogues. HIF-2α (also known as endothelial PAS domain protein 1) is also oxygen-regulated and can dimerize with HIF-1β. HIF-1α and HIF-2α activate transcription of an overlapping but distinct set of genes (WANG et al. 2005). Another parologue, HIF-3α was found to inhibit the transcriptional activity of HIF-1α by competing with HIF-1β (MAKINO et al. 2001).

The significance of the transcriptional changes regulated by HIF is highlighted by the tight association of HIF activation and cancer progression (GIACCIA et al. 2003; MAXWELL et al. 2001; SEMENZA 2003). Immunohistochemistry has revealed HIF accumulation in a broad array of human malignancies (MAXWELL et al. 2001). This accumulation has been associated with poor prognosis in breast cancer (BOS et al. 2001; SCHINDL et al. 2002), cervical cancer (BIRNER et al. 2000), ovarian cancer (BIRNER et al. 2001) and neuroblastoma (HOLMQUIST-MENGBIER et al. 2006). Conversely, downregulating HIF, either by inhibiting translation or promoting degradation, or inhibiting key HIF target genes, such as VEGFA, have proven to be promising interventions for anti-cancer therapies (GIACCIA et al. 2003; SEMENZA 2003).

Jiang et al. found HIF transcriptional complex was conserved in nematodes C. elegans (JIANG et al. 2001). Nematodes C.elegans consist of fewer than one thousand cells and do not have a complex circulatory system. The small body of the C.elegans enables the cells to obtain oxygen from diffusion. In natural habitat, C.elegans experience hypoxia when they burrow into soil searching for food. The identified C.elegans HIF complex consists of HIF-1 and AHA-1, which are C. elegans orthologues of HIF α and β subunits, respectively (JIANG et al. 2001; POWELL-COFFMAN et al. 1998). While AHA-1 expression did not change with oxygen level, HIF-1 was induced by hypoxia but quickly reduced upon reoxygenation (EPSTEIN et al. 2001; JIANG et al. 2001). HIF-1 regulates the majority of early transcriptional
changes in response to hypoxia (SHEN et al. 2005). Loss-of-function hif-1 mutant worms show decreased ability of hypoxia adaptation (JIANG et al. 2001). The conservation of HIF signaling makes C. elegans, with its powerful genetics, an attractive model in search of genes regulating HIF.

**Regulation of HIF**

**Oxygen-dependent degradation of HIF**

The discovery of EGLN prolyl hydroxylase family is a wonderful example to use C. elegans as a model system in dismantling the mechanism of HIF regulation. After the molecular characterization of the HIF1 complex in mammals, direct analysis of HIF1α subunit revealed that it is ubiquitinated and degraded by 26S proteasome in normoxia (HUANG et al. 1998; KALLIO et al. 1999; SALCEDA and CARO 1997). The next big step in elucidating the regulation of HIFα stability was the identification of the von Hippel-Lindau tumor suppressor (VHL) as the substrate recognition component of a ubiquitin ligase complex that targeted HIF1α for proteasomal degradation (COCKMAN et al. 2000; KAMURA et al. 2000; MAXWELL et al. 1999; OHH et al. 2000). However, one critical question remained: what was the oxygen sensor and how does it function to control HIF α stability?

To answer this question, the Ratcliffe group demonstrated that in C. elegans, the role of vhl-1, a homolog of VHL, in regulating HIF-1 stability was conserved (EPSTEIN et al. 2001). They then found that in a C. elegans mutant of egl-9, which encodes a 2-oxoglutarate dioxygenase, HIF-1 protein was constitutively stabilized (EPSTEIN et al. 2001). A family of egl-9 homologs, termed as prolyl hydroxylase domain (PHD) 1, PHD2 and PHD3, was subsequently identified in human and mice (EPSTEIN et al. 2001). Elegant biochemical studies demonstrated that the interaction between HIF-1α and VHL requires the hydroxylation of specific proline residue within the evolutionary conserved oxygen-dependent-degradation (ODD) domain in HIF-1α subunit (IVAN et al. 2001; JAAKKOLA et al. 2001; YU et al. 2001). PHDs were characterized as the enzymes that catalyze this reaction. Using oxygen and 2-oxoglutarate as co-substrate, iron and ascorbate as cofactors, PHDs sense the intracellular O2 tension as well as aerobic metabolites to regulate
the degradation of HIF-1α subunit (Bruick and McKnight 2001; Epstein et al. 2001; Ivan et al. 2002).

Interestingly, PHD2 and PHD3 genes contain hypoxia response elements and are induced by HIF-1 during hypoxia (Marxsen et al. 2004; Metzen et al. 2005; Stiehl et al. 2006). In C.elegans, expression of egl-9 mRNA was found to be induced by hypoxia in a hif-1-dependent manner (Shen et al. 2005). Therefore, HIF-1/PHD/pVHL forms a evolutionarily conserved negative feedback loop to regulate the oxygen homeostasis.

**Degradation independent regulation of HIF**

Regulation of HIF also occurs at levels independent of HIFα stability. For example, in mammals, Factor inhibiting HIF (FIH) mediates hydroxylation of an asparagines residue in the carboxy-terminal of the HIFα subunit, which blocks its interaction with transcription co-activator p300 (C/EBP). This results in the attenuation of HIF transcriptional activity (Lando et al. 2002). Interestingly, like PHDs, FIH is also an iron and 2-oxoglutarate dependent dioxygenase. This renders FIH a O2-senser to fine-tune the regulation of HIF activity (Mahon et al. 2001).

Apart from serving as an O2-sensor in oxygen-dependent degradation, VHL-independent functions of PHD in regulating HIF activity have been documented. To and Huang demonstrated that PHD2 could inhibit HIF-1 transcriptional activity in vhl-/- cells (To and Huang 2005). Moreover, there is evidence that in Hela cells PHD2 can recruit ING4 tumor suppressor to the promoter region of HIF-1 target gene to inhibit HIF-1 transcriptional activity (Ozer et al. 2005). The interaction between PHD and HIF alpha may be complex and need other co-factors, as demonstrated by Hopfer et al. that PHD3 required Morg1 (MAPK organizer 1), a WD-repeat protein, to repress HIF1 activity (Hopfer et al. 2006).

The degradation-independent role of EGL-9 is evidently conserved in *C.elegans*. When comparing the egl-9 -/- and vhl-1 -/- phenotypes, Shen et al. found that egl-9/- mutation had much stronger effect on the expression of HIF-1 target genes, while the HIF-1 protein level is quite similar in both mutants (Shen et al. 2006). Ratcliffe’s lab has shown that in contrary to
the fact that egl-9 RNAi increased the expression of a HIF target gene in vhl-1 mutant worms, the dioxygenase inhibitors did not, which suggest that some of the inhibition effect of EGL-9 may be independent of its catalytic activity (Bishop et al. 2004). Although homologs of genes involved in VHL-independent function of EGLNs, such as ING4 and Morg1, may exist in C. elegans genome, their roles in regulating HIF-1 activity have not been determined.

**Other hypoxic response signaling pathways**

Other pathways also contribute to hypoxia response, although they are not as well understood as the HIF pathway. More recently, two additional pathways have been proved to be involved in hypoxia response to influence gene expression and cell behavior in mammals.

The first is through the regulation of the kinase mammalian target of rapamycin (mTOR). mTOR, as part of mTOR complex (mTORC), integrates growth and metabolic signals and controls the translational machinery through its ability to phosphorylate ribosomal protein S6 kinase (p70S6K), eukaryotic elongation factor 2 kinase (eEF2 kinase) and eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) (Hay and Sonenberg 2004). Hypoxia inhibits mTOR through multiple pathways, and final outputs are reducing protein synthesis and inducing autophagy and/or apoptosis (for reviews, see Wouters and Koritzinsky 2008).

In addition to inhibit mTOR pathway, recent studies indicate that hypoxia can also induce endoplasmic reticulum (ER) stress and subsequently activate the unfolded protein response (UPR) (Ron and Walter 2007). Although little is known about the molecular mechanisms, hypoxia activates several ER stress sensors, such as PERK (PKR-like ER kinase) and IRE1 (inositol-requiring protein1) (Koumenis et al. 2002; Romero-Ramirez et al. 2004). Consequently, the effector pathways of UPR inhibit mRNA translation to prevent further accumulation of unfolded proteins in ER, and also induce ER-associated degradation (ERAD) and autophagy to remove misfolded proteins (Bernales et al. 2006; Koumenis et al. 2002; Ogata et al. 2006). Both TOR and UPR pathways are largely conserved in C. elegans (Jia et al. 2004; Shen et al. 2001). However, their roles in hypoxia response in C. elegans are still not clear.
Hypoxia response signalings other than HIF-1 pathway are present in *C.elegans*. Using cDNA microarray analysis, Shen et al. identified 110 hypoxia-regulated genes in *C.elegans*. Among these genes, 47 genes are regulated independently of *hif-1* function (SHEN et al. 2005). In the same study, the authors also found that *daf-16*, a forkhead transcription factor, are induced by hypoxia independently of *hif-1*. DAF-16 is well-known for its function in insulin/IGF-1 like signaling pathway to regulate dauer formation, stress resistance and longevity. Correspondingly, loss-of-function mutation of *daf-2*, an insulin/IGF-1 like receptor, has been found to increase the tolerance to severe hypoxia (<0.3% oxygen and high temperature) and anoxia (MENDENHALL et al. 2006; SCOTT et al. 2002). This hypoxia-tolerant phenotype is suppressed by mutations of *daf-16*. These data suggest that insulin/IGF-1 signaling may function to protect organisms from hypoxic stress in parallel to *hif-1* pathway.

Recently, Anderson et al. reported that a reduction-of-function mutation of *rrt-1*, an arginyl-transfer RNA synthetase, produces hypoxia resistance (ANDERSON et al. 2009). The mechanisms of this hypoxia-resistance phenotype appear to due to the attenuated translation rate, which may decrease the unfolded protein toxicity induced by hypoxia. In addition, using genome-wide RNAi screen, the same group identified 198 genes that when knocked down increase resistance to severe hypoxia (MABON et al. 2009). Although the normal function of these genes seem to increase hypoxia sensitivity, their roles during hypoxia response are not clear.

**The connection between hypoxia and aging**

Aging is associated with high incidence of stroke, cancer and ischemia, in which cases tissue or cells often experience hypoxia. It is an important question to ask how aging is connected to the decreased ability to cope with hypoxia in elderly. As the master regulator of transcriptional changes during hypoxia, does HIF play any roles in aging?

Recent studies suggested that HIF may be functionally involved in aging process. For example, it has been shown that hypoxia increased the replicative life span of fibroblasts and vascular smooth muscle cells *in vitro* (MINAMINO et al. 2001; PARRINELLO et al. 2003),
which may due to the upregulation of telomerase mediated by HIF-1 (NISHI et al. 2004). On the other hand, a reduced protein expression of HIF-1α with increasing age has been found in many tissues of mice and rat, including muscle, lung, kidney and brain (FRENKEL-DENKBERG et al. 1999; RIVARD et al. 2000; ROHRBACH et al. 2005). The reduced expression of HIF-1α in heart tissues from old human and mouse could be correlated with an increased expression of PHD3/EGLN3 (ROHRBACH et al. 2005). In addition, comparing the response to hypoxia of human young versus old fibroblast cells by microarray analysis demonstrated that many genes involved in angiogenesis, including HIF-1 target gene VEGF, was severely reduced in old fibroblasts (Kim et al. 2003).

Despite the emerging correlation between hypoxia and aging, some imperative questions remain unanswered. First, is reduced protein expression of HIF-1α a cause or a consequence of aging? Second, given that HIF-1 is associated with replicative senescence, does HIF-1 influence organismal aging? If the answer is yes, what is the mechanism and how does it connect to other signaling pathways that modulate aging?

**THESIS ORGANIZATION**

Hypoxia-inducible factor (HIF-1) transcription factor is the master regulator that mediates most of the transcriptional changes during hypoxia adaptation. In this dissertation, I studied roles of hif-1 and hypoxia signaling pathway in organismal aging in *C. elegans*. This thesis commences with a general introduction chapter which describes the background information related to this study. Chapter 1 starts with an extensive literature review of aging study. Major topics include: 1) Cell senescence of mitotic cells as the major contributors to human aging; 2) The plasticity of aging in post-mitotic cells in light of neurodegenerative diseases; 3) *C. elegans* as a model system to study post-mitotic aging; 4) Organismal aging and lessons learned from long-lived mutant worms. The second part of the review discusses the hypoxia response and hypoxia-inducible factor signaling, and their connections to aging and aging-related diseases.

The thesis is then organized into three research manuscripts. Chapter 2 is a copy of a paper submitted to *PLoS ONE*, and describes the role of hif-1 in modulating longevity in
C. elegans. In this paper, Yi Zhang generated the hif-1 transgenic animals, performed the life span assay and statistic analysis. Zhiyong Shao conducted the gene expression analysis in Figure 4C. Zhiwei Zhai contributed to part of the life span analysis of Figure 5D. Chuan Shen generated hif-1(ia07) allele, which is used in Figure 5.

The second paper is a manuscript being prepared for submission. This paper describes the extended life span induced by hypoxia in C. elegans and characterizes the roles of hif-1, daf-16 and endoplasmic reticulum (ER) stress response signaling in hypoxia mediated longevity. Yi Zhang made the primary observation and collected all the data for this manuscript.

The third paper is also a manuscript being prepared for submission. This paper describes the role of swan-1, a gene identified through forward genetic screen, in regulating HIF-1 activity. Yi Zhang performed the initial characterization of the allele and carried out the mapping. Yi Zhang and Zhiyong Shao analyzed the effect of swan-1 on HIF-1 stability and HIF-1 target genes.

The final part of this thesis is a general conclusion chapter that discusses the role of HIF-1 and hypoxia signaling pathway in regulating aging, and proposes potential experiments for future research. In addition, unpublished data including the co-immunoprecipitation experiments in searching for the proteins associated with HIF-1 in vivo and some life span assay results are organized in the appendix section.

**REFERENCE**


HARA, T., K. NAKAMURA, M. MATSUI, A. YAMAMOTO, Y. NAKAHARA et al., 2006
Suppression of basal autophagy in neural cells causes neurodegenerative disease in

HAY, N., and N. SONENBERG, 2004 Upstream and downstream of mTOR. Genes Dev 18:
1926-1945.

614-636.

HERNDON, L. A., P. J. SCHMEISSNER, J. M. DUDARONEK, P. A. BROWN, K. M. LISTNER et al.,
2002 Stochastic and genetic factors influence tissue-specific decline in ageing C.

HOLMQUIST-MENGELBIER, L., E. FREDLUND, T. LOFSTEDT, R. NOGUERA, S. NAVARRO et al.,
2006 Recruitment of HIF-1alpha and HIF-2alpha to common target genes is
differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive

HOPFER, U., H. HOPFER, K. JABLONSKI, R. A. STAHL and G. WOLF, 2006 The novel
WD-repeat protein Morg1 acts as a molecular scaffold for hypoxia-inducible factor

HOPKINS, S. R., and F. L. POWELL, 2001 Common themes of adaptation to hypoxia. Insights

HSU, A. L., C. T. MURPHY and C. KENYON, 2003 Regulation of aging and age-related disease

HUANG, L. E., J. GU, M. SCHAU and H. F. BUNN, 1998 Regulation of hypoxia-inducible factor
1alpha is mediated by an O2-dependent degradation domain via the

ISHII, N., M. FUJII, P. S. HARTMAN, M. TSUDA, K. YASUDA et al., 1998 A mutation in
succinate dehydrogenase cytochrome b causes oxidative stress and ageing in
IVAN, M., T. HABERBERGER, D. C. GERVASI, K. S. MICHELSON, V. GUNZLER et al., 2002
Biochemical purification and pharmacological inhibition of a mammalian prolyl
hydroxylase acting on hypoxia-inducible factor. Proc Natl Acad Sci U S A 99:
13459-13464.

IVAN, M., K. KONDO, H. YANG, W. KIM, J. VALIANDO et al., 2001 HIFalpha targeted for
VHL-mediated destruction by proline hydroxylation: implications for O2 sensing.

JAAKKOLA, P., D. R. MOLE, Y. M. TIAN, M. I. WILSON, J. GIELBERT et al., 2001 Targeting of
HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl

JANZEN, V., R. FORKERT, H. E. FLEMING, Y. SAITO, M. T. WARING et al., 2006 Stem-cell
ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature 443:
421-426.

JEYAPALAN, J. C., M. FERREIRA, J. M. SEDIVY and U. HERBIG, 2007 Accumulation of

JIA, K., D. CHEN and D. L. RIDDLE, 2004 The TOR pathway interacts with the insulin
signaling pathway to regulate C. elegans larval development, metabolism and life

JIA, K., and B. LEVINE, 2007 Autophagy is required for dietary restriction-mediated life span

JIANG, B. H., G. L. SEMENZA, C. BAUER and H. H. MARTI, 1996 Hypoxia-inducible factor 1
levels vary exponentially over a physiologically relevant range of O2 tension. Am J
Physiol 271: C1172-1180.

encodes a bHLH-PAS protein that is required for adaptation to hypoxia. Proc Natl
Acad Sci U S A 98: 7916-7921.


KRISHNAMURTHY, J., M. R. RAMSEY, K. L. LIGON, C. TORRICE, A. KOH et al., 2006

KRISHNAMURTHY, J., C. TORRICE, M. R. RAMSEY, G. I. KOVALEV, K. AL-REGAIEY et al.,


LEE, S. S., S. KENNEDY, A. C. TOLONEN and G. RUVKUN, 2003a DAF-16 target genes that

LEE, S. S., R. Y. LEE, A. G. FRASER, R. S. KAMATH, J. AHRINGER et al., 2003b A systematic

LEHTINEN, M. K., Z. YUAN, P. R. BOAG, Y. YANG, J. VILLEN et al., 2006 A conserved
MST-FOXO signaling pathway mediates oxidative-stress responses and extends life

LI, W., S. G. KENNEDY and G. RUVKUN, 2003 daf-28 encodes a C. elegans insulin
superfamily member that is regulated by environmental cues and acts in the DAF-2
signaling pathway. Genes Dev 17: 844-858.

LIBINA, N., J. R. BERMAN and C. KENYON, 2003 Tissue-specific activities of C. elegans

MABON, M. E., X. MAO, Y. JIAO, B. A. SCOTT and C. M. CROWDER, 2009 Systematic

 MAGRANE, J., and G. MANFREDI, 2009 Mitochondrial function, morphology, and axonal
transport in amyotrophic lateral sclerosis. Antioxid Redox Signal.


Marxsen, J. H., P. Stengel, K. Doege, P. Heikkinen, T. Jokilehto et al., 2004 Hypoxia-inducible factor-1 (HIF-1) promotes its degradation by induction of HIF-alpha-prolyl-4-hydroxylases. Biochem J 381: 761-767.


SALCEDA, S., and J. CARO, 1997 Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 272: 22642-22647.


SHEN, C., Z. SHAO and J. A. POWELL-COFFMAN, 2006 The Caenorhabditis elegans rhy-1 gene inhibits HIF-1 hypoxia-inducible factor activity in a negative feedback loop that does not include vhl-1. Genetics 174: 1205-1214.


TO, K. K., and L. E. HUANG, 2005 Suppression of hypoxia-inducible factor 1alpha (HIF-1alpha) transcriptional activity by the HIF prolyl hydroxylase EGLN1. J Biol Chem **280**: 38102-38107.


**FIGURE LEGEND**

**Figure 1.** A model of aging modulation in *C. elegans*. Aging results from the accumulative damages as the consequence of normal metabolism and of unpredictable stresses. Environmental and genetic variables modulate aging process through 1) decreasing ROS production by adopting alternative bio-energetic metabolism; 2) promoting stress resistance by activating key transcription factors; 3) reducing or removing damaged macro-molecules by suppressing translation or inducing autophagy.

**Figure 2.** Regulation of HIF-1 in *C. elegans*. The stability of HIF-1 protein is controlled by the evolutionarily conserved EGL-9/VHL-1 pathway. Under normoxic conditions, EGL-9 hydroxylates HIF-1 at Pro-621, which increases the affinity of HIF-1 for VHL-1, a ubiquitin E3 ligase. The ubiquitinated HIF-1 is then targeted for degradation by 26S proteasome. Hypoxia inhibits the HIF-1 prolyl hydroxylation. Accumulated HIF-1 dimerizes with its partner AHA-1 and binds to specific DNA recognition sequences in its target genes to direct changes in gene expression. In addition to regulating HIF-1 stability, EGL-9 also inhibits HIF-1 transcriptional activity. The molecular mechanism of this pathway is still obscure. RHY-1, a multi-pass transmembrane protein inhibits HIF-1 transcriptional activity through unknown mechanisms.
Figure 1. Mechanisms of aging modulation in *C. elegans*.

Figure 2. Regulation of HIF-1 in *C. elegans*. 
CHAPTER 2. The HIF-1 hypoxia-inducible factor modulates lifespan in *C. elegans*

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**ABSTRACT**

During normal development or during disease, animal cells experience hypoxic (low oxygen) conditions, and the hypoxia-inducible factor (HIF) transcription factors implement most of the critical changes in gene expression that enable animals to adapt to this stress. Here, we examine the roles of HIF-1 in post-mitotic aging. We examined the effects of *hif-1* loss-of-function mutations and of HIF-1 over-expression on longevity in *C. elegans*, a powerful genetic system in which adult somatic cells are post-mitotic. We constructed transgenic lines that expressed varying levels of HIF-1 protein and discovered a positive correlation between HIF-1 expression levels and longevity. The data further showed that HIF-1 acted in parallel to the SKN-1/ NRF and DAF-16/ FOXO transcription factors to promote longevity. HIF-1 over-expression also conferred increased resistance to heat and oxidative stress. We isolated and characterized additional *hif-1* mutations, and we found that each of 3 loss-of-function mutations conferred increased longevity in normal lab culture conditions, but, unlike HIF-1 over-expression, a *hif-1* deletion mutation did not extend the lifespan of *daf-16* or *skn-1* mutants. We conclude that HIF-1 over-expression and *hif-1* loss-of-function mutations promote longevity by different pathways. These data establish HIF-1 as one of the key stress-responsive transcription factors that modulate longevity in *C.*
elegans and advance our understanding of the regulatory networks that link oxygen homeostasis and aging.
Introduction

During development, homeostasis, and disease, cells integrate diverse environmental inputs and implement the appropriate changes in gene expression to survive stresses or execute developmental programs. Of the many environmental challenges that animals encounter, oxygen deprivation (hypoxia) is of particular concern. Oxygen is the final electron acceptor in cellular respiration, and is, therefore, necessary for metazoan life. Successful adaptation to hypoxia involves changes in genetic programs that modulate metabolism, cell death, growth, and cellular differentiation. Many of these processes also have roles in cellular aging. This suggests that the networks that govern oxygen homeostasis and aging may be intertwined, but the molecules and mechanisms that underpin these networks are not fully understood.

In animals as diverse as humans and C. elegans, the hypoxia-inducible factor (HIF) heterodimeric transcription factors are responsible for most of the hypoxia-induced changes in gene expression [1,2]. HIFs have been studied intensively by the biomedical research community, because HIFs have central roles in cancer and cardiovascular disease. Small molecules that inhibit HIF may be effective chemotherapeutic agents. Conversely, for patients in which injury, anemia, or ischemia has decreased oxygen supply to tissues, treatments that increase HIF levels may improve cell survival and stimulate angiogenesis.

HIF regulatory networks are acutely sensitive to oxygen. The HIF transcription complex is heterodimeric, and both subunits belong to the family of DNA-binding transcription factors that include basic-helix-loop-helix and PAS domains. HIFα stability is regulated by oxygen-dependent degradation. When oxygen levels are sufficiently high, HIFα is hydroxylated by the members of the EGL-9 / PHD family of Fe(II)- and 2-oxoglutarate-dependent dioxygenases. This modification allows VHL, a ubiquitin E3 ligase, to bind HIFα and target it for proteasomal degradation. In hypoxic conditions, HIFα is
stable, and the HIF complex activates a battery of genes that enable adaptation to low oxygen conditions. HIF stability and activity are influenced by reactive oxygen species, citric acid cycle intermediates, Fe(II) availability, and intracellular signaling events [1,3]. HIFs are, therefore, well-positioned to integrate information about oxygen availability and cellular redox and to effect changes that influence stress resistance.

Here, we test the hypothesis that HIF modulates post-mitotic aging. Previous studies in human cells had demonstrated that HIF promoted telomerase expression [4] and that this contributed to increases in the replicative lifespan of primary human lung fibroblasts in hypoxic conditions [5], but the roles of HIF in post-mitotic aging were not understood. The somatic cells of an adult C. elegans are post-mitotic, and the rate of aging can be modulated by genes that control insulin receptor signaling, mitochondrial function, protein folding, or response to reactive oxygen species and other stresses [6,7]. The C. elegans genome encodes a single HIF alpha subunit, HIF-1, and mutants lacking a functional hif-1 gene are viable in normal culture conditions [8,9]. In this study, we report that over-expression of HIF-1 protein extends the lifespan of C. elegans in a dose-dependent manner. Interestingly, we find that loss-of-function mutations in hif-1 gene can also increase C. elegans longevity and stress tolerance. The HIF-1 over-expression and hif-1 loss-of-function longevity phenotypes are not equivalent, as they differ in their dependence on daf-16/FOXO and skn-1/NRF2. These data provide insights to the intersection between oxygen homeostasis and aging, and they suggest that post-mitotic aging may be influenced by treatments or mutations that alter HIF-1 signaling.
RESULTS

HIF-1 over-expression causes dose-dependent extension of adult lifespan.

A small group of transcriptional regulators lying in the core of the stress response network, including DAF-16, SKN-1, SIR-2.1, PHA-4 and HSF-1, had been shown to extend lifespan when activated or over-expressed in transgenic C. elegans [10,11,12,13,14,15,16]. To determine whether HIF-1 had similar effects, we generated a series of transgenic animals with extra copies of hif-1 integrated into the genome. The hif-1 minigene is a fusion of genomic and cDNA sequences, and it includes a c-myc tag (Fig 1A). We confirmed that the epitope-tagged HIF-1 protein, like endogenous HIF-1, was over-expressed in animals that lacked vhl-1 or egl-9 (Fig 1B). Further, the transgene was able to restore expression of a HIF-1-dependent reporter, Pnhr-57:GFP, to a hif-1 deletion mutant (Fig 1C). In two independent lines (iais27 and iais28), the hif-1 integrated transgenes conferred moderate lifespan extension (22 or 23 days, p<0.0001, Tables 1 and S1).

To further test the hypothesis that HIF-1 over-expression increased lifespan in C. elegans, we constructed a transgene in which HIF-1 proline 621 was converted to glycine. In wild-type animals, HIF-1 proline 621 is hydroxylated by EGL-9, and this covalent modification had been shown to mediate binding of HIF-1 to VHL-1 [17]. Mutations in vhl-1 or egl-9 can also be used to inhibit HIF-1 degradation, but these mutations have other defects that can complicate interpretation of the phenotypes. The vhl-1(ok161) deletion causes some changes in extracellular matrices that are not suppressed by a mutation in hif-1 [18], and animals carrying strong loss-of-function mutations in egl-9 have an array of morphological and behavioral defects [19,20]. To understand the effects of HIF-1 over-expression, we generated transgenic animals carrying the hif-1 (P621G) expression
construct and analyzed 3 independently isolated lines (ials32, ials33, and ials34). As shown in Fig. 1D, the HIF-1(P621G) gain-of-function mutation released the protein from degradation via the egl-9/vhl-1 pathway. In these strains, the HIF-1 (P621G) protein was expressed at levels 3 – 14 times greater than HIF-1 expressed from transgenes without the stabilizing mutation (Fig 2A). The hif-1 (P621G) lines lived 20% - 34% longer than wild-type N2 worms (Fig. 2B, Table 1). Moreover, the mean adult lifespans of the five hif-1 transgenic strains were proportionately correlated with HIF-1 expression levels (Fig. 2C). These data established that HIF-1 over-expression caused dose-dependant lifespan extension.

**HIF-1 over-expression acts in parallel to DAF-16 and SKN-1 to extend lifespan.**

Mutations in the daf-2 insulin-like receptor gene increase nuclear localization of the DAF-16 FOXO transcription factor and dramatically increase longevity and resistance to heat or oxidative stress. Conversely, daf-16 loss-of-function mutants age quickly [10,13,21,22,23,24]. We considered the possibility that loss-of-function mutations in daf-16 might be epistatic to HIF-1 over-expression in longevity assays. As shown in Fig. 3A and Table 2, hif-1(P621G) transgenes increased the mean lifespan of daf-16 (mu86) mutants by up to 33%. This was very similar to the effect that HIF-1(P621G) had on wild-type animals (Table 1). This indicates that HIF-1 and DAF-16 were acting in parallel to influence post-mitotic aging.

SKN-1 is a transcription factor that mediates the phase II detoxification response during oxidative stress in *C. elegans* [25], and expression of constitutively active SKN-1 can extend lifespan via genetic pathways that are independent of DAF-16 [15]. We tested the hypothesis that a loss-of-function mutation in skn-1 could abolish the prolongevity effects of
HIF-1 over-expression. As shown in Fig 3B and Table 2, over-expression of HIF-1 through hif-1(P621G) transgenes extended the lifespan of skn-1(zu67) mutants by up to 25%.

We then asked whether the longevity effect of increased HIF-1 protein was independent of the DAF-2 insulin-like signaling pathway by crossing the hif-1(P621G) transgene to a long-lived daf-2 loss-of-function mutant. The hif-1 (P621G) transgene did not extend the lifespan of daf-2(e1370) worms (Fig. 3C, Table 2). Taken together, the genetic data indicate that increased dosage of HIF-1 promotes longevity via a pathway that acts in parallel to DAF-16 and SKN-1, but may be downstream of the DAF-2 insulin-like receptor.

Studies in mammalian cell lines have shown that the insulin receptor pathway can influence translation of HIF-1α, and recent studies in C. elegans have suggested that DAF-16 might regulate HIF-1 expression in some developmental or environmental contexts [26,27]. To directly test the hypothesis that DAF-2 regulated total HIF-1 protein levels, we compared the expression of epitope-tagged HIF-1 in wild-type animals and in daf-2 (e1370) mutants. We also assayed the expression of two HIF-1 targets: Pnhr-57:GFP and K10H10.2. Prior studies had shown that K10H10.2 mRNA levels were induced by hypoxia in wild-type animals in a hif-1-dependent manner and that mutations that caused over-expression of HIF-1 protein resulted in over-expression of K10H10.2 mRNA [2,20]. As shown in Fig 4A, the daf-2 (e1370) mutation did not have a significant effect on the expression levels of HIF-1 or HIF-1(P621G) proteins (Fig 4A) or on the expression of two HIF-1 targets that we assayed (Figs. 4B, 4C).

**Effects of hif-1 loss-of-function mutations on longevity.**

Prior studies did not clearly predict the effects a hif-1 loss-of-function mutation might have on longevity. HIF-1 had been shown to promote the expression of genes that inhibit formation of stress resistant dauer larvae. In accordance with this, hif-1 (ia04) mutants
arrest larval development as partial dauers when cultured at 27°C [2]. Since mutations that confer stress resistance and dauer formation are often correlated with longer lifespan, it was reasonable to expect that hif-1 mutants might age slowly. However, loss-of-function mutations in the daf-16 or skn-1 prolongevity genes are associated with premature aging [22,24,25], and our data in Fig. 2 had established that over-expression of HIF-1 extended lifespan. To address this question directly, we assayed the lifespans of wild-type animals and hif-1 (ia04) mutants. Under normal culture conditions (20°C), hif-1 (ia04) mutants lived approximately 20% longer than wild-type animals (24 days, compared to 20 days for wild-type N2) (Table 1). To further validate this finding, we isolated an additional loss-of-function allele in hif-1 [hif-1 (ia07), described in Figs 5A and 5B]. Sequence analysis predicts that hif-1 (ia07) should encode a truncated protein lacking a transcriptional activation domain. We also characterized hif-1 (ok2564), a deletion allele isolated by the C. elegans Genome Consortium (Figs 5A). Phenotypic analyses confirm that both hif-1 (ia07) and hif-1 (ok2564) decrease expression of HIF-1 targets (Fig 5C). Each of the hif-1 loss-of-function alleles increased the mean adult lifespan by at least 10% (Fig 5D, Table 1). hif-1 RNAi also extended the lifespan of wild-type animals (Table S2).

To understand whether the longevity phenotypes conferred by HIF-1 over-expression and hif-1 loss-of-function mutations had similar genetic underpinnings, we asked whether the hif-1 (ia04) mutation could extend the lifespan of daf-16- or skn-1-deficient mutants. As illustrated in Fig 5E and 5F, daf-16 (mu86) animals and daf-16 (mu86) hif-1 (ia04) double mutants had similarly short lifespans (~15 days), and there was not a significant difference between daf-2(e1370) and daf-2(e1370) hif-1(ia04) animals. We also determined that the hif-1 (ia04) deletion mutation did not extend the lifespan of skn-1(zu67) animals (Fig 5G, Table 2). These data suggest that hif-1 loss-of-function mutations extend life span via a mechanism that requires daf-16 and skn-1 function. Thus, the longevity phenotypes of HIF-1 over-expressing animals and hif-1 (ia04) animals can be distinguished by their requirements for daf-16 and skn-1 function.
Resistance to t-butyl-peroxide and heat stress.

Thermotolerance and stress resistance have been shown to be correlated with *C. elegans* longevity [28,29]. To determine whether changes in *hif-1* dosage that extend lifespan also conferred resistance to oxidative stress, we assessed relative resistance to t-butyl-peroxide. We assayed two loss-of-function alleles of *hif-1* and two lines that expressed the stabilized HIF-1 (P621G). All of these strains exhibited significantly higher rates of survival in the oxidizing agent compared to wild-type (Fig. 6A). We also tested the ability of these strains to withstand heat stress. Prior studies had determined that both *hif-1* (*ia04*) mutants and *egl-9*-deficient mutants were more resistant to heat stress than were wild-type animals [30]. We extended these studies and determined that, similar to the findings in peroxide treatment, loss-of-function mutations in *hif-1* or over-expression of HIF-1 increased the ability of the animals to survive at 35°C (Fig. 6B).

**DISCUSSION**

This study advances our understanding of the regulatory networks that link oxygen homeostasis and aging. Cells respond to oxygen deprivation by changing their patterns of gene expression to optimize energy production and mitigate cellular damage. Oxygen deprivation can extend the replicative lifespan of cells in culture [5], and *C. elegans* cultured in hypoxic conditions live longer. The hypoxia-inducible factors have been termed master regulators of oxygen homeostasis, and in *C. elegans*, HIF-1 is required for most of hypoxia-induced changes in gene expression [2]. Genes regulated by HIF-1 include genes involved in metabolism, cell signaling, transcription and translation. We show here that
over-expression of HIF-1 extends *C. elegans* lifespan in a dose-dependent manner. We hypothesize that multiple HIF-1 targets act in concert to slow cellular deterioration over time.

**Effects of HIF-1 over-expression**

When over-expressed, HIF-1 acts in parallel to the stress responsive transcription factors DAF-16 / FOXO and SKN-1 / NRF to promote longevity. In wild-type animals, DAF-16 and SKN-1 are inhibited by the sole *C. elegans* insulin-like growth factor receptor, DAF-2. Analogous to mammalian IGF signaling pathways, DAF-2 regulates phosphoinositide 3-kinase (PI3K) signaling. The effectors of PI3K include kinases (AKT-1, AKT-2, and SGK-1) that phosphorylate DAF-16 and SKN-1 to repress transcription factor nuclear localization [15,33,34]. As illustrated in Fig 3, HIF-1 over-expression can extend the lifespan of daf-16 or skn-1 loss-of-function mutants. Microarray studies have suggested that HIF-1 and DAF-16 may co-regulate some genes, but HIF-1 and DAF-16 clearly have independent functions as well [2,35]. The relationship between HIF-1 and DAF-2 is less clear. The genetic data leave open the possibility that the prolongevity functions of HIF-1 are regulated by DAF-2. DAF-2 and the PI3K pathway have been shown to have other DAF-16-independent functions, including regulation of SKN-1 nuclear localization [15]. Additionally, mutations in daf-2 were shown to confer resistance to some hypoxia-induced defects in neural development [27]. Here, we have demonstrated that mutations in daf-2 did not cause significant changes in whole animal levels of HIF-1 protein or in the expression of two HIF-1 targets that we assayed (Fig 4), but DAF-2 may have tissue-specific or stage-specific roles in HIF-1 regulation.

A recent independent study from the Kaeberlein research group provides added support for the conclusion that HIF-1 over-expression extends *C. elegans* lifespan. Mehta et al reported that *vhl-1* or *egl-9* RNAi increased *C. elegans* longevity and decreased the toxic effects of transgenes that expressed polyglutamine repeats or beta amyloid. Further, they
showed that *daf-16* RNAi did not completely suppress the longevity conferred by a *vhl-1* deletion mutation, and they found that *vhl-1* RNAi had no effect on the nuclear localization of DAF-16::GFP.

While moderate increases in HIF-1 protein expression promote longevity, dramatic over-expression of HIF-1 target genes can be detrimental. Dramatic over-expression of HIF-1 target genes in *C. elegans egl-9* mutants is associated with a range of morphological and behavioral defects [19,20,37]. We and others have shown that animals carrying a strong loss-of-function mutation in *egl-9* do not live longer than wild-type animals (Table 1 and ). This suggests animals treated with *egl-9* RNAi or animals carrying transgenes expressing stabilized HIF-1 benefit from increased HIF-1 function, but only to a point. Forcing gene expression beyond normal parameters can be deleterious. A similar pattern has been reported for yeast LAG1 and for *C. elegans SKN-1*. Moderate over-expression of SKN-1 extends life span, but over-expression from high copy transgenes is toxic [15].

**Effects of hif-1 loss-of-function**

*hif-1* loss-of-function mutations and *hif-1* RNAi can also extend lifespan, but HIF-1 over-expression and *hif-1* loss-of-function mutations exert their prolongevity effects through different pathways. We demonstrated that three independently isolated *hif-1* mutations extended *C. elegans* lifespan. The *hif-1 (ia07)* allele is predicted to encode a truncated HIF-1 protein that lacks a transcriptional activation domain, and the *hif-1(ia07)* phenotype is slightly more severe than the phenotypes of the *hif-1 (ia04)* and *hif-1 (ok2564)* deletion alleles (Fig 5A). In agreement with our findings, the Kapahi group determined that *hif-1* RNAi increased *C. elegans* lifespan, and they demonstrated that this effect was abrogated by dietary restriction . These data shown that the *hif-1* loss-of-function phenotype can be strongly influenced by the bacterial food.
There are also some intriguing differences between our findings and those from the other two groups that have recently examined the roles of HIF-1 in *C. elegans* aging. While we report and Chen et al. have reported that hif-1-deficient animals live longer when food is abundant, Mehta et al. did not detect differences between the lifespans of hif-1 (ia04) and wild-type animals in their experiments. This appears to reflect differences in assay conditions. Small differences in the bacterial food source, the temperature, or other conditions may alter the longevity phenotypes of hif-1-deficient animals. Chen et al. conducted their experiments at 25°C, and they assayed the effects of varying amounts of live bacterial food on plates containing FUdR and antibiotics. Mehta et al. performed their experiments at 20°C and fed the animals UV-treated bacteria on plates containing FUdR and antibiotics. All of the longevity assays that we report here were conducted in nutrient rich conditions in the absence of DNA synthesis inhibitors. Although our findings regarding the hif-1 loss-of-function phenotype largely agree with those of Chen et al., there are open questions about the role of daf-16 in hif-1-deficient animals. Chen et al. found that hif-1 RNAi could extend the mean lifespan of daf-16 (mgDf47) animals by 18%. In our experiments, double mutants carrying loss-of-function mutations in both hif-1 and daf-16 had similar lifespans to daf-16 (mu86) single mutants (Fig 3).

**HIF-1 and hormesis**

Why might apparently small differences in assay conditions influence the phenotypes of hif-1-deficient animals? The answer to this question may require a fuller understanding of the roles of HIF-1 in heat acclimation and hormesis. Wild-type *C. elegans* that have been incubated at 25°C are more thermotolerant than animals that have been incubated at 20°C. This process requires hif-1 function [30]. These data, when considered with the findings presented here, suggest that while loss-of-function mutations in
hif-1 or HIF-1 over-expression can increase thermal resistance (Fig 6 and [30]), hif-1-deficient animals are less able to adapt to or benefit from changes in the environment. *C. elegans* have been shown to be more sensitive to oxygen deprivation at high temperatures [40,41]. Future studies will investigate the ways in which culture conditions and environmental stresses can influence HIF-1 function and hif-1-deficient phenotypes and may discover additional roles for HIF-1 in aging, stress, and hormesis.

**Selective pressures to control HIF-1 signaling**

To our knowledge, this is the first case in a multicellular organism in which wild-type animals (grown in nutrient-rich conditions) have been shown to live shorter than either loss-of-function mutants or animals over-expressing the protein. However, in yeast, both loss-of-function mutations in the LAG1 longevity assurance gene and moderate over-expression of LAG1 can extend replicative lifespan. Recent studies in mammalian cells have shown that similar phenotypes can be caused by loss-of-function or gain-of-function mutations that force a signaling pathway outside of its normal range. For example, in models for TNF-induced apoptosis, kinase-dead and constitutively active MK2 mutants have been shown to have similar effects on apoptosis. When considering the complex roles of HIF-1 in aging, it is important to recognize that HIF-1 over-expression and *hif-1* loss-of-function phenotypes are qualitatively different and have distinct genetic requirements.

In the hundreds of millions of years since nematodes and mammals diverged from a common ancestor, some of the key molecules and feedback loops that modulated HIF signaling have been conserved [17,43]. The EGL-9 / VHL-1 pathway enables rapid up-regulation of HIF in hypoxic conditions, but the system is tightly controlled. In humans and in *C. elegans*, activation of HIF results in increased expression of the HIF prolyl
hydroxylases (PHD / EGL-9) [2,18]. This establishes a negative feedback loop to attenuate HIF expression. While either hif-1 depletion or HIF-1 over-expression increase longevity in optimal lab culture conditions, the conservation of HIF regulatory networks suggests that there is a strong selective pressure for keeping HIF signaling within a normal range, where HIF can effectively respond to subtle changes in the environment.

Small molecule inhibitors of the HIF prolyl hydroxylases are being developed as treatments for anemia or other pathologies that may be ameliorated by increased HIF expression. There is also great interest in pharmaceuticals that can inhibit HIF and may be effective in combinatorial treatments for certain cancers. We anticipate that drugs that target HIF may have widespread, dose-dependent impacts on stress resistance and on somatic aging.

**MATERIALS AND METHODS**

**C. elegans strains**

The mutant alleles were as follows: LGI: daf-16(mu86)lf; LGII: daf-2(e1370) reduction-of-function; LGV: hif-1(ia04)lf, hif-1(ia07)lf, hif-1(ok2564)lf, egl-9(sa307)lf; LGX: vhl-1(ok161)lf.

**hif-1 minigenes and generation of transgenic animals**

The pFOX1 and pFOX4 constructs encode wild-type HIF-1 and HIF-1(P621G), respectively. pFOX1 includes 5.2 kb genomic sequence 5’ to the hif-1 coding region. The first exon and first intron of hif-1 are fused to a cDNA fragment including exon 2 to exon 9 of hif-1 (for the predominant mRNA isoform: hif-1a). Five copies of c-myc epitope (5×myc)
(from clone CD3-128, Arabidopsis Biology Resources Center) were inserted C-terminal to hif-1 coding sequences, and this was followed by a stop codon and 400 bp of genomic sequence just 3’ to the hif-1 coding region. To create pFOX4, the P621G point mutation was introduced to the pFOX1 sequence. Microparticle bombardment was used to create C. elegans carrying integrated copies of each construct. The hif-1 constructs were introduced to hif-1 (ia04), unc-119(ed3) animals, and the unc-119 rescuing plasmid pPD#MM016b was the co-transformation marker. Animals carrying integrated copies of the transgenes were isolated and backcrossed at least four times prior to further characterization.

**Lifespan and stress analyses**

Longevity assays were conducted as described previously unless noted otherwise. Prior to life span assays, all strains were maintained at 20°C for at least two generations with abundant food. The OP50 bacterial food was grown in L-broth to a 600 nm optical density of approximately 0.4, and 250 microliters of live bacterial culture was spotted onto each 60 mm NGM plate. The plates did not contain FUdR or antibiotics. One to three days later, young adult hermaphrodites were allowed to lay eggs overnight on the bacterial food. The L4 larvae grown from these eggs were transferred to fresh plates. Each plate included 20 – 25 animals, and at least 50 animals were used in each independent trial. The worms were transferred to new plates every two days for the first two weeks and every six days thereafter. The data from individual experiments are listed in Table S1. Any experiments in which the assay conditions were modified are explicitly noted in the text and in the legends for Tables S2 and S3. Viability was scored every 2 days. Worms that crawled off the plates, burst at the vulva or died because progeny hatched in utero were excluded from final statistical analyses. Life span statistical analyses were carried out using JMP software (version 7.0) to
determine the means and percentiles. \( p \) values were calculated using the log-rank (Mantel-Cox) method.

In oxidative stress assays, young adult worms (one day after L4 molt) were transferred to NGM plates containing 7.2 mM \( t \)-butyl-peroxide (Sigma) and OP50 food. Animals were incubated at 20°C and were scored for survival at the time points shown. For thermal stress assays, young adult worms were incubated at 35°C on NGM plates seeded with OP50 and were scored for survival. At least 50 worms were tested in each trial and at least three independent trials were performed for each stress assay.

**Protein blots and Quantitative RT-PCR**

For each lane of an immunoblot, ten (for \( \text{Pnhr57:GFP} \)) to 100 (for the epitope-tagged HIF-1) L4 or young adult worms were assayed. Mouse anti-AHA-1 antibody (cell culture supernatant) was produced in ISU Hybridoma Facility and used at 1:100. Mouse anti-myc ascites (clone 9E10) from the Developmental Studies Hybridoma Bank was used at 1:1000. Mouse anti-GFP antibody (from Roche) was used at 1:1000.

Quantitative RT-PCR was performed as described previously [20]. \( \text{inf-1} \) is not regulated by hypoxia and was used as an input control. The standard curve method was used to analyze the expression levels. Student \( t \)-tests were used to determine the \( p \) value for protein expression and quantitative RT-PCR data.

**ACKNOWLEDGEMENTS**

The \( \text{hif-1} \) (\text{ok2564}) allele was provided by the \textit{C. elegans} Gene Knockout Consortium, and the \textit{daf-2} and \textit{daf-16} mutant strains were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources. This work was funded by grants from the National Institutes of Health (R01 GM078424) and the Iowa State
University Center for Integrated Animal Genomics. We thank P. Kapahi and M. Kaeberlein for discussions regarding the *hif-1* longevity phenotypes, and we are grateful to members of the Powell-Coffman, Coffman, and Essner labs and to individuals in the *C. elegans* research community for helpful suggestions and for comments on the manuscript.

**REFERENCES**


**FIGURE LEGENDS**

**Figure 1.** Characterization of the hif-1 and hif-1(P621G) transgenes. (A) Diagram of the minigenes that express epitope-tagged HIF-1. Genomic sequence including the hif-1 promoter sequence, exon 1, and intron 1, was fused to cDNA including exons 2-9 for the predominant hif-1 mRNA isoform (hif-1a). A myc epitope tag was inserted. A proline to glycine conversion at amino acid residue 621 prevents VHL-1-mediated degradation [17]. (B) Representative protein blots show that epitope-tagged HIF-1 is stabilized by loss-of-function mutations in vhl-1 or egl-9. (C) Western blots show the transgene restores HIF-1-mediated gene expression. Pnhr-57:GFP is a reporter for HIF-1 activity [20]. Its expression is dramatically reduced in hif-1(ia04) mutants and is restored by the ialIs28 integrated hif-1 transgene. (D) Oxygen-dependent degradation via the vhl-1 / egl-9 pathway is abolished when the HIF-1 proline residue 621 is mutated to glycine.

**Figure 2.** HIF-1 over-expression extends longevity in a dose-dependent manner. (A) Protein blots quantitate expression of HIF-1 and HIF-1(P621G) transgenes (tagged with the myc epitope). ialIs27 and ialIs28 contain integrated copies of the hif-1 minigene. ialIs32, ialIs33, and ialIs34 carry integrated copies of hif-1 (P621G). AHA-1 is orthologous to HIFβ / ARNT [46], and the abundance of AHA-1 does not vary significantly in these strains. (B) Strains carrying hif-1 transgenes live longer (p<0.0001). Proportion alive is plotted over time. (C) The mean adult lifespans of strains over-expressing HIF-1 are positively correlated with the HIF-1 expression levels. Error bars represent the standard errors of the means.

**Figure 3.** HIF-1 over-expression extends the lifespan of daf-16-deficient and skn-1-deficient mutants. (A, B) hif-1(P621G) transgenes (ialIs32 or ialIs34) extend the
lifespan of animals carrying loss-of-function mutations in (A) daf-16 \((p<0.0001)\) or (B) skn-1 \((p<0.0001)\). (C) The longevity of daf-2(e1370) animals carrying a hif-1 \((P621G)\) transgene is equivalent to that of daf-2(e1370) single mutants at 25°C.

Figure 4. The daf-2 insulin-like receptor does not regulate expression of HIF-1 protein or its targets. (A) A reduction of function mutation in daf-2 does not change the protein levels of epitope-tagged HIF-1 \((iaIs28)\) or HIF-1(P621G) \((iaIs32)\) in young adult animals \((p>0.2 \text{ from three independent experiments})\). (B,C) The expression of two HIF-1 targets were assayed in wild-type animals and in animals carrying a loss-of-function mutation in daf-2. (B) Pnhr-57:GFP expression was assayed by protein blots and was not significantly different in these genetic backgrounds \((p>0.2 \text{ from three independent experiments})\) (B) Quantitative RT-PCR revealed a marginal differences in the levels of K10H10.2 mRNA \((p = 0.09)\).

Figure 5. hif-1 loss-of-function mutations extend the lifespan of wild-type animals, via pathway(s) that require daf-16 and skn-1. (A,B) Characterization of hif-1 mutation alleles. (A) The hif-1 locus encodes 4 mRNA isoforms (www.wormbase.org). Exons are depicted as boxes, with coding sequences in black and untranslated regions in grey. The positions of two deletion alleles \((ia04\) and \(ok2564)\) and one nonsense mutation \((ia07)\) are shown. (B) The hif-1 \((ia07)\) point mutation introduces an early stop codon; (C) Protein blots show that all three mutations in hif-1 repress expression of the Pnhr-57::GFP reporter \([20]\). Expression was assayed in vhl-1 \((ok161)\) mutants, which are defective in oxygen-dependent degradation of HIF-1. (D) C. elegans carrying hif-1 loss-of-function mutations live longer than wild-type N2 \((p<0.0001)\). (E) hif-1\((ia04)\);daf-16\((mu86)\) double mutant animals do not live longer than daf-16\((mu86)\) single mutants. (F) daf-2 \((e1370)\) single mutants and daf-2 \((e1370)\); hif-1\((ia04)\) double mutants have equivalent lifespans at 25°C. (G) hif-1\((ia04)\);skn-1\((zu67)\) double mutant animals do not live longer than skn-1\((zu67)\) single mutants.
Figure 6. *hif-1* loss-of-function mutations and HIF-1 over-expression increase oxidative stress resistance and thermotolerance. (A, B) Resistance to (A) t-butyl peroxide or (B) heat stress was assayed in *hif-1* loss-of-function mutants and in animals over-expressing HIF-1. The *daf-2* (*e1370*) and *daf-16* (*mu86*) strains were included as a basis for comparison. Error bars represent the standard error of the means from at least 3 experiments. (*, *p*<0.0001 compared to wild type N2 worms using z-test).
Table 1. Over-expression of HIF-1 protein or \textit{hif-1} loss-of-function mutations extend the adult lifespan in \textit{C. elegans} in standard lab culture conditions.

<table>
<thead>
<tr>
<th>strain</th>
<th>life span mean ±S.E.</th>
<th>Percentage v. N2</th>
<th>n</th>
<th>p value*</th>
<th>number of exp</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 wild-type</td>
<td>20.0 ± 0.3</td>
<td></td>
<td>166</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>\textit{iais27} [Phif-1::hif-1::myc]</td>
<td>22.3 ± 0.4</td>
<td>12</td>
<td>109</td>
<td>&lt;0.0001</td>
<td>3</td>
</tr>
<tr>
<td>\textit{iais28} [Phif-1::hif-1::myc]</td>
<td>23.0 ± 0.3</td>
<td></td>
<td>131</td>
<td>&lt;0.0001</td>
<td>3</td>
</tr>
<tr>
<td>\textit{iais32} [Phif-1::hif-1(P621G)::myc]</td>
<td>25.9 ± 0.6</td>
<td>30</td>
<td>117</td>
<td>&lt;0.0001</td>
<td>3</td>
</tr>
<tr>
<td>\textit{iais33} [Phif-1::hif-1(P621G)::myc]</td>
<td>24.1 ± 0.5</td>
<td>20</td>
<td>125</td>
<td>&lt;0.0001</td>
<td>3</td>
</tr>
<tr>
<td>\textit{iais34} [Phif-1::hif-1(P621G)::myc]</td>
<td>26.7 ± 0.4</td>
<td>33</td>
<td>125</td>
<td>&lt;0.0001</td>
<td>3</td>
</tr>
<tr>
<td>\textit{hif-1}(ia04)</td>
<td>24.1 ± 0.5</td>
<td>20</td>
<td>147</td>
<td>&lt;0.0001</td>
<td>4</td>
</tr>
<tr>
<td>\textit{hif-1}(ia07)</td>
<td>27.0 ± 0.6</td>
<td>35</td>
<td>56</td>
<td>&lt;0.0001</td>
<td>2</td>
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<tr>
<td>\textit{hif-1}(ok2564)</td>
<td>22.4 ± 0.6</td>
<td>12</td>
<td>74</td>
<td>&lt;0.0001</td>
<td>2</td>
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<tr>
<td>\textit{egl-9}(sa307)</td>
<td>20.8 ± 0.3</td>
<td>4</td>
<td>126</td>
<td>0.2</td>
<td>4</td>
</tr>
<tr>
<td>\textit{egl-9}(sa307);hif-1(ia04)</td>
<td>19.2 ± 0.3</td>
<td>-4</td>
<td>134</td>
<td>0.3</td>
<td>3</td>
</tr>
</tbody>
</table>

* The longevity of each strain was compared to wild-type, and the \textit{p} values were calculated by log-rank tests.
Table 2. Effects of HIF-1 over-expression or hif-1 loss-of-function mutations on the longevity of daf-16 or skn-1 mutants.

<table>
<thead>
<tr>
<th>strain</th>
<th>Adult life span mean ± S.E.</th>
<th>Percentage v. control</th>
<th>n</th>
<th>p value</th>
<th>number of exp</th>
</tr>
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<tbody>
<tr>
<td>daf-16(mu86)</td>
<td>15.1 ± 0.2</td>
<td></td>
<td>191</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>daf-16(mu86); hif-1(ia04)</td>
<td>14.8 ± 0.3</td>
<td>-2</td>
<td>105</td>
<td>0.3†</td>
<td>3</td>
</tr>
<tr>
<td>daf-16(mu86); ials32</td>
<td>19.0 ± 0.4</td>
<td>27</td>
<td>77</td>
<td>&lt;0.0001*</td>
<td>2</td>
</tr>
<tr>
<td>daf-16(mu86); ials34</td>
<td>20.2 ± 0.4</td>
<td>33</td>
<td>78</td>
<td>&lt;0.0001*</td>
<td>2</td>
</tr>
<tr>
<td>skn-1(zu67)</td>
<td>15.4 ± 0.3</td>
<td></td>
<td>87</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>skn-1(zu67); hif-1(ia04)</td>
<td>14.8 ± 0.4</td>
<td>-4</td>
<td>76</td>
<td>0.5†</td>
<td>2</td>
</tr>
<tr>
<td>skn-1(zu67); ials32</td>
<td>19.2 ± 0.4</td>
<td>25</td>
<td>98</td>
<td>&lt;0.0001†</td>
<td>2</td>
</tr>
<tr>
<td>skn-1(zu67); ials34</td>
<td>17.6 ± 0.4</td>
<td>14</td>
<td>108</td>
<td>&lt;0.0001†</td>
<td>2</td>
</tr>
<tr>
<td>daf-2(e1370)</td>
<td>32.5 ± 1.1</td>
<td></td>
<td>76</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>daf-2(e1370); hif-1(ia04)</td>
<td>34.0 ± 1.2</td>
<td>5</td>
<td>75</td>
<td>0.2‡</td>
<td>3</td>
</tr>
<tr>
<td>daf-2(e1370); ials32</td>
<td>29.2 ± 1.8</td>
<td>-10</td>
<td>45</td>
<td>0.9‡</td>
<td>3</td>
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<tr>
<td>daf-2(e1370); ials34</td>
<td>30.2 ± 1.2</td>
<td>-7</td>
<td>65</td>
<td>0.9‡</td>
<td>3</td>
</tr>
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</table>

* The p value was calculated by log-rank test as a comparison to daf-16(mu86) worms.
† The p value was calculated by log-rank test as a comparison to skn-1(zu67) worms.
‡ The p value was calculated by log-rank test as a comparison to daf-2(e1370) worms.
Figure 1. Characterization of the hif-1 and hif-1(P621G) transgenes.

Figure 2. HIF-1 over-expression extends longevity in a dose-dependent manner.
Figure 3. HIF-1 over-expression extends the lifespan of *daf-16*-deficient and *skn-1*-deficient mutants.

Figure 4. The *daf-2* insulin-like receptor does not regulate expression of HIF-1 protein or its targets.
Figure 5. *hif-1* loss-of-function mutations extend the lifespan of wild-type animals, via pathway(s) that require *daf-16* and *skn-1*.

Figure 6. *hif-1* loss-of-function mutations and HIF-1 over-expression increase oxidative stress resistance and thermotolerance.
### Table S1: Data from individual lifespan experiments

<table>
<thead>
<tr>
<th>Genotype</th>
<th>life span mean ±S.E.</th>
<th>Percentage v. control</th>
<th>Maximum lifespan</th>
<th>n</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 wild-type</td>
<td>19.9 ± 0.5</td>
<td></td>
<td>26</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>N2 wild-type</td>
<td>20.3 ± 0.6</td>
<td></td>
<td>26</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>N2 wild-type</td>
<td>20.0 ± 0.6</td>
<td></td>
<td>26</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>N2 wild-type</td>
<td>20.4 ± 0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ials27 [Phif-1::hif-1::myc]</td>
<td>23.5 ± 0.5</td>
<td>18*</td>
<td>34</td>
<td>47</td>
<td>&lt;0.0001*</td>
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<tr>
<td>ials27 [Phif-1::hif-1::myc]</td>
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<td>7*</td>
<td>36</td>
<td>45</td>
<td>0.04*</td>
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<tr>
<td>ials27 [Phif-1::hif-1::myc]</td>
<td>21.4 ± 1.2</td>
<td>7*</td>
<td>36</td>
<td>17</td>
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<tr>
<td>ials28 [Phif-1::hif-1::myc]</td>
<td>24.6 ± 0.6</td>
<td>23*</td>
<td>36</td>
<td>46</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>ials28 [Phif-1::hif-1::myc]</td>
<td>22.0 ± 0.6</td>
<td>10*</td>
<td>28</td>
<td>40</td>
<td>0.003*</td>
</tr>
<tr>
<td>ials28 [Phif-1::hif-1::myc]</td>
<td>22.2 ± 0.4</td>
<td>11*</td>
<td>30</td>
<td>45</td>
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</tr>
<tr>
<td>ials32 [Phif-1::hif-1(P621G)::myc]</td>
<td>27.2 ± 0.7</td>
<td>36*</td>
<td>36</td>
<td>41</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>ials32 [Phif-1::hif-1(P621G)::myc]</td>
<td>27.0 ± 1.1</td>
<td>35*</td>
<td>38</td>
<td>39</td>
<td>&lt;0.0001*</td>
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<tr>
<td>ials32 [Phif-1::hif-1(P621G)::myc]</td>
<td>23.4 ± 0.9</td>
<td>17*</td>
<td>36</td>
<td>37</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>ials33 [Phif-1::hif-1(P621G)::myc]</td>
<td>25.3 ± 0.8</td>
<td>27*</td>
<td>36</td>
<td>42</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>ials33 [Phif-1::hif-1(P621G)::myc]</td>
<td>24.7 ± 1.2</td>
<td>23*</td>
<td>40</td>
<td>41</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>ials33 [Phif-1::hif-1(P621G)::myc]</td>
<td>22.2 ± 0.7</td>
<td>11*</td>
<td>34</td>
<td>42</td>
<td>0.001*</td>
</tr>
<tr>
<td>ials34 [Phif-1::hif-1(P621G)::myc]</td>
<td>28.3 ± 0.6</td>
<td>42*</td>
<td>38</td>
<td>36</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>ials34 [Phif-1::hif-1(P621G)::myc]</td>
<td>27.3 ± 0.8</td>
<td>37*</td>
<td>38</td>
<td>43</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>ials34 [Phif-1::hif-1(P621G)::myc]</td>
<td>24.9 ± 0.5</td>
<td>25*</td>
<td>34</td>
<td>46</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>hif-1(ia04)</td>
<td>25.7 ± 1.1</td>
<td>34*</td>
<td>34</td>
<td>27</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>hif-1(ia04)</td>
<td>24.2 ± 0.7</td>
<td>21*</td>
<td>34</td>
<td>43</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>hif-1(ia04)</td>
<td>25.6 ± 0.8</td>
<td>28*</td>
<td>32</td>
<td>44</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>hif-1(ia04)</td>
<td>21.5 ± 0.7</td>
<td>8*</td>
<td>32</td>
<td>33</td>
<td>0.002*</td>
</tr>
<tr>
<td>hif-1(ia07)</td>
<td>26.6 ± 1.0</td>
<td>33*</td>
<td>36</td>
<td>29</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>hif-1(ia07)</td>
<td>27.2 ± 0.9</td>
<td>36*</td>
<td>38</td>
<td>33</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>hif-1(ok2564)</td>
<td>22.8 ± 0.6</td>
<td>14*</td>
<td>30</td>
<td>43</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>hif-1(ok2564)</td>
<td>22.2 ± 0.7</td>
<td>11*</td>
<td>31</td>
<td>31</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>egl-9(sa307)</td>
<td>21.8 ± 0.7</td>
<td>9*</td>
<td>29</td>
<td>32</td>
<td>0.007*</td>
</tr>
<tr>
<td>egl-9(sa307)</td>
<td>21.5 ± 0.5</td>
<td>8*</td>
<td>26</td>
<td>27</td>
<td>0.007*</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
<td><strong>egl-9(sa307)</strong></td>
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<td>3*</td>
<td>30</td>
<td>34</td>
<td>0.45*</td>
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<tr>
<td><strong>egl-9(sa307)</strong></td>
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<td>-2*</td>
<td>28</td>
<td>38</td>
<td>0.41*</td>
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<tr>
<td><strong>egl-9(sa307); hif-1(ia04)</strong></td>
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<td>-7*</td>
<td>24</td>
<td>45</td>
<td>0.3*</td>
</tr>
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<td><strong>egl-9(sa307); hif-1(ia04)</strong></td>
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<td>4*</td>
<td>26</td>
<td>47</td>
<td>0.8*</td>
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<td>18.1</td>
<td>-9*</td>
<td>26</td>
<td>42</td>
<td>0.0003*</td>
</tr>
<tr>
<td><strong>daf-16(mu86)</strong></td>
<td>15.6</td>
<td>22</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>daf-16(mu86)</strong></td>
<td>15.4</td>
<td>20</td>
<td>37</td>
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<td></td>
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<tr>
<td><strong>daf-16(mu86)</strong></td>
<td>15.1</td>
<td>24</td>
<td>34</td>
<td></td>
<td></td>
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<tr>
<td><strong>daf-16(mu86)</strong></td>
<td>15.2</td>
<td>20</td>
<td>40</td>
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<tr>
<td><strong>daf-16(mu86)</strong></td>
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<td>-12†</td>
<td>20</td>
<td>39</td>
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<tr>
<td><strong>daf-16(mu86); hif-1(ia04)</strong></td>
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<td>-6†</td>
<td>20</td>
<td>33</td>
<td>0.14†</td>
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<tr>
<td><strong>daf-16(mu86); hif-1(ia04)</strong></td>
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<td>10†</td>
<td>20</td>
<td>30</td>
<td>0.11†</td>
</tr>
<tr>
<td><strong>daf-16(mu86); ials32</strong></td>
<td>19.8</td>
<td>30†</td>
<td>26</td>
<td>41</td>
<td>&lt;0.0001†</td>
</tr>
<tr>
<td><strong>daf-16(mu86); ials32</strong></td>
<td>18.1</td>
<td>28†</td>
<td>26</td>
<td>36</td>
<td>&lt;0.0001†</td>
</tr>
<tr>
<td><strong>daf-16(mu86); ials34</strong></td>
<td>20.3</td>
<td>33†</td>
<td>28</td>
<td>40</td>
<td>&lt;0.0001†</td>
</tr>
<tr>
<td><strong>daf-16(mu86); ials34</strong></td>
<td>20.2</td>
<td>33†</td>
<td>26</td>
<td>38</td>
<td>&lt;0.0001†</td>
</tr>
<tr>
<td><strong>skn-1(zu67)</strong></td>
<td>15.1</td>
<td>22</td>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>skn-1(zu67)</strong></td>
<td>16.1</td>
<td>26</td>
<td>29</td>
<td></td>
<td></td>
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<tr>
<td><strong>skn-1(zu67); hif-1(ia04)</strong></td>
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<td>-3†</td>
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<td>24</td>
<td>0.8†</td>
</tr>
<tr>
<td><strong>skn-1(zu67); ials32</strong></td>
<td>19.1</td>
<td>26†</td>
<td>28</td>
<td>58</td>
<td>&lt;0.0001†</td>
</tr>
<tr>
<td><strong>skn-1(zu67); ials32</strong></td>
<td>19.4</td>
<td>20†</td>
<td>30</td>
<td>40</td>
<td>&lt;0.0001†</td>
</tr>
<tr>
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<td>8†</td>
<td>26</td>
<td>49</td>
<td>0.002†</td>
</tr>
<tr>
<td><strong>skn-1(zu67); ials34</strong></td>
<td>18.7</td>
<td>16†</td>
<td>30</td>
<td>59</td>
<td>&lt;0.0001†</td>
</tr>
<tr>
<td><strong>daf-2(e1370)</strong></td>
<td>29.6</td>
<td>50</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>daf-2(e1370)</strong></td>
<td>32.6</td>
<td>48</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>daf-2(e1370)</strong></td>
<td>34.9</td>
<td>50</td>
<td>29</td>
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</tr>
<tr>
<td><strong>daf-2(e1370); hif-1(ia04)</strong></td>
<td>28.7</td>
<td>-3¥</td>
<td>54</td>
<td>15</td>
<td>0.77¥</td>
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<tr>
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<td>9¥</td>
<td>54</td>
<td>30</td>
<td>0.18¥</td>
</tr>
<tr>
<td><strong>daf-2(e1370); hif-1(ia04)</strong></td>
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<td>5¥</td>
<td>54</td>
<td>30</td>
<td>0.9¥</td>
</tr>
<tr>
<td><strong>daf-2(e1370); ials32</strong></td>
<td>35.8</td>
<td>16¥</td>
<td>58</td>
<td>17</td>
<td>0.007¥</td>
</tr>
<tr>
<td><strong>daf-2(e1370); ials32</strong></td>
<td>25.2</td>
<td>-16¥</td>
<td>40</td>
<td>28</td>
<td>0.6¥</td>
</tr>
<tr>
<td><strong>daf-2(e1370); ials34</strong></td>
<td>31.5</td>
<td>3¥</td>
<td>54</td>
<td>21</td>
<td>0.6¥</td>
</tr>
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<td><strong>daf-2(e1370); ials34</strong></td>
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<td>2¥</td>
<td>52</td>
<td>23</td>
<td>0.6¥</td>
</tr>
<tr>
<td><strong>daf-2(e1370); ials34</strong></td>
<td>27.8</td>
<td>-10¥</td>
<td>42</td>
<td>21</td>
<td>0.42¥</td>
</tr>
</tbody>
</table>
* The longevity of was compared to wild-type N2, and the p values were calculated by log-rank tests.
† The longevity of was compared to daf-16(mu86) worms, and the p values were calculated by log-rank tests.
‡ The longevity of was compared to skn-1(zu67) worms, and the p values were calculated by log-rank tests.
¥ The longevity of was compared to daf-2(e1370) worms, and the p values were calculated by log-rank tests.

All of the experiments in Table S1 were conducted at 20°C on live bacterial food, as described in the Materials and Methods of the main text.
Table S2: Effects of *hif*-1 RNAi on the longevity of wildtype worms.

<table>
<thead>
<tr>
<th>strain</th>
<th>Adult life span mean ±S.E.</th>
<th>Maximum lifespan</th>
<th>Percentage v. control RNAi</th>
<th>n</th>
<th>p(^{†}) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 (L4440 RNAi)</td>
<td>16.5 ± 0.7</td>
<td>28</td>
<td></td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>N2 (L4440 RNAi)</td>
<td>17.3 ± 0.7</td>
<td>28</td>
<td></td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>N2 (L4440 RNAi)</td>
<td>16.7 ± 0.5</td>
<td>26</td>
<td></td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>N2 (hif-1 RNAi)</td>
<td>19.5 ± 0.8</td>
<td>30</td>
<td>15</td>
<td>35</td>
<td>0.009</td>
</tr>
<tr>
<td>N2 (hif-1 RNAi)</td>
<td>19.6 ± 0.9</td>
<td>32</td>
<td>16</td>
<td>43</td>
<td>0.0008</td>
</tr>
<tr>
<td>N2 (hif-1 RNAi)</td>
<td>19.0 ± 0.7</td>
<td>28</td>
<td>12</td>
<td>39</td>
<td>0.02</td>
</tr>
</tbody>
</table>

These lifespan assays were performed at 20°C.

\(^{†}\) The *p* value was calculated by log-rank test as a comparison to N2 worms treated with control RNAi (L4440).

**Supplemental Methods for Table S2.**

*hif*-1 RNAi construct and lifespan analyses on RNAi bacterial food: To generate the *hif*-1 RNAi construct, a 1.4 kb *hif*-1 cDNA fragment was excised from pHJ06 (Jiang et al. 2001 PNAS 98: 7916) and was inserted into the pPD129.36 (L4440) vector at the EcoRV and XhoI sites. The *hif*-1 RNAi construct was transformed into HT115 bacterial strain. RNAi plates used in life span assay were prepared with 5mM IPTG and with no FuDR or antibiotics (Kamath et al 2001 Genome Biol 2: RESEARCH0002). The lifespan assays were performed at 20°C. Young adult hermaphrodites which had been maintained at 20°C on NGM plates and OP50 bacterial food were transferred to RNAi plates and allowed to lay eggs overnight. The L4 larvae grown from these eggs were transferred to fresh plates. Each plate included 20 – 25 animals, and at least 50 animals were used in each independent trial. The worms were transferred to new RNAi plates every two days for the first two weeks and every six days thereafter. Viability was scored every 2 days. Worms that crawled off the
plates, burst at the vulva or died because progeny hatched in utero were excluded from final statistical analyses. Life span statistical analyses were carried out using JMP software (version 7.0) to determine the means and percentiles.
Table S3. Lifespan assays at 25 ºC on UV-irradiated bacterial food (OP50).

<table>
<thead>
<tr>
<th>strain</th>
<th>Adult life span mean ±S.E.</th>
<th>Maximum lifespan</th>
<th>Percentage v. N2</th>
<th>n</th>
<th>p* value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>15.9 ± 0.5</td>
<td>22</td>
<td></td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>15.3 ± 0.5</td>
<td>20</td>
<td></td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>hif-1(ia04)</td>
<td>17.5 ± 0.4</td>
<td>22</td>
<td>13</td>
<td>33</td>
<td>0.001</td>
</tr>
<tr>
<td>hif-1(ia04)</td>
<td>17.3 ± 0.4</td>
<td>26</td>
<td>11</td>
<td>72</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

* The p value was calculated by log-rank test as a comparison to N2 worms.

Supplemental Methods for Table S3.

To kill the bacterial food, standard NGM plates spotted with OP50 bacteria as described in the main text were exposed to UV light (302nm) for 15 minutes using a Benchtop UV transilluminator system (UVP). The death of UV irradiated bacterial food was confirmed by streaking the bacteria on LB agar plates. For the experiments in Table S3, young adult hermaphrodites which had been maintained at 20ºC on NGM plates spotted with live OP50 bacterial food were allowed to lay eggs overnight on the UV-irradiated bacterial food. The L4 larvae grown from these eggs at 20ºC were transferred to fresh UV-irradiated plates and removed to 25ºC to perform the lifespan assays as described in the main text.
CHAPTER 3. HIF -1-INDEPENDENT HYPOXIA RESPONSE SIGNALING MEDIATES AGING IN C.ELEGANS

Abstract

Hypoxia is associated with animal development and with the pathology of many age-related diseases, such as cancer, stroke and ischemia. Hypoxia signaling is important for animals to adapt to low oxygen tension. The components of hypoxia signaling are potential targets for the treatment of age-related diseases. However, the effects of hypoxia and the roles of hypoxia signaling in organismal aging are not well understood. Here we report that hypoxia slows aging in the nematode Caenorhabditis elegans. This prolongevity effect is not determined during development, but is exerted in adult life. In Chapter 2, we have shown that over-expression of HIF-1 increases longevity. Here we demonstrate that hypoxia can also extend life span through hif-1-independent pathways. Hypoxia promotes longevity in mechanisms distinct from the insulin-like signaling pathway. We present evidence that hypoxia induces inositol-requiring protein 1 (ire-1)-mediated unfolded protein response (UPR), and ire-1 is required for hypoxia-mediated lifespan extension. Thus, ire-1-mediated UPR is involved in the hif-1-independent hypoxia response that slows aging in C. elegans.
INTRODUCTION

Oxygen availability influences many cellular processes including energy metabolism, proliferation and cell death. Oxygen deprivation (hypoxia) is one of the common conditions shared by cells during many human diseases, such as cancer, stroke and ischemia. Interestingly, the incidence of these disorders increases with aging, which suggests that the networks governing oxygen homeostasis and aging may be intertwined. However, the interrelationship between hypoxia response signaling pathways and aging is not well understood.

Hypoxia-inducible factor (HIF) transcription factors regulate most of the transcriptional changes during oxygen homeostasis in animals (Semenza 2007; Shen et al. 2005). The heterodimeric HIF complex is composed of two subunits, both of which contain basic-helix-loop-helix and PAS domains. HIFα stability is acutely regulated by cellular oxygen levels and is dedicated to oxygen homeostasis. In normoxia, HIFα proteins are hydroxylated at specific proline residues by the EGLN family of 2-oxoglutarate dioxygenases. Hydroxylated HIFα is then degraded through the von-Hippel-Lindau (pVHL)-mediated proteosome pathway. Hypoxia suppresses the hydroxylation reaction and thus stabilizes HIFα (Epstein et al. 2001). The HIF complex then activates a group of genes to facilitate hypoxia adaptation. In C. elegans, hif-1 encodes the ortholog of HIF alpha and is required for hypoxia adaptation during development (Jiang et al. 2001; Padilla et al. 2002). C. elegans hif-1 is associated with heat stress response (Treinin et al. 2003), oxygen sensing behavior (Chang and Bargmann 2008) and neuronal development (Pocock and Hobert 2008).

We and others have established that hif-1 is one of the key stress response transcription factors that modulate aging in C. elegans (Chen et al. 2009; Mehta et al. 2009; Zhang et al. 2009). Honda et al showed that hypoxia (1% oxygen) increased lifespan in C. elegans, while
worms cultured in hyperoxia live shorter (HONDA et al. 1993). However, the role of hif-1 in regulating aging during hypoxia is elusive. In this study, we investigate the role of hypoxia and hypoxia signaling pathways in organismal aging in *C. elegans*. Although stabilization of HIF-1 protein promotes longevity, we demonstrate that hypoxia can extend adult lifespan in the absence of *hif-1*. Further, we show that the *ire-1*-mediated unfolded protein response is required for the pro-longevity effect of hypoxia.

**RESULTS**

**HIF-1 is not necessary for hypoxia-mediated lifespan extension.**

The nematode *C. elegans* has a broad range of oxygen tolerance, and this is likely due in part to its small body size. It can maintain near-normal metabolic rates at oxygen levels as low as 2 percent (VAN VOORHIES and WARD 2000). To explore the effects of hypoxia on aging in *C. elegans*, we first assayed the lifespan of worms cultured in 1% O₂. We found that hypoxia treatment extended the mean lifespan of *C. elegans* by approximately 17% (Fig 1B and Table 1), indicating that oxygen deprivation and the resulting changes in cellular signaling and metabolism mediate a beneficial effect and delay aging. The hypoxia-inducible factor (HIF-1) pathway mediates most of the transcriptional changes in response to hypoxia in *C. elegans* (SHEN et al. 2005). In normoxia, HIF-1 protein is degraded through the evolutionarily conserved EGL-9/VHL-1 pathway. Hypoxia suppresses this degradation pathway and thus induces HIF-1 accumulation and activity. Our previous studies have shown that HIF-1 over-expression was sufficient to increases lifespan in *C. elegans* (ZHANG et al. 2009). We considered the possibility that HIF-1 over-expression was the sole mechanism by which hypoxia slowed aging. To test this hypothesis, we assayed the lifespan of *hif-1* mutant worms in hypoxia. *hif-1* loss-of-function mutants have a superficially normal phenotype but
fail to adapt to hypoxia during development (Jiang et al. 2001). To avoid developmental defects in hypoxia, we raised the hif-1 mutant worms through the young adult stage in normal culture conditions before shifting to hypoxia and determining their lifespan. To our surprise, as shown in Figure 2A and Table 1, hypoxia treatment extended the adult lifespan of hif-1(ia04) worms by approximately 18%.

We also asked whether hypoxia treatment during embryonic and larval stages would induce changes that would prolong adult lifespan. To test this hypothesis, we applied hypoxia to animals during embryogenesis and larval development. After they reached the young adult stage, we shifted the worms to normoxia and assayed their lifespan. These worms did not live longer when compared to worms without hypoxia treatment (Fig. 1C and Table 1). In contrast, the worms treated with hypoxia only after they reached young adult stage in normoxia lived 17% longer (Fig. 1D and Table 1). These studies showed that worms that are cultured in 1% oxygen during their adult lives do not receive further longevity effects from hypoxia treatment during embryonic and larval development. Since hypoxia treatment during adulthood increased longevity to similar degrees as during whole lifespan, in the following experiments, adulthood hypoxia treatment was employed in the lifespan assay.

We further tested the effect of hypoxia on the transgenic worms carrying extra copies of the hif-1(P621G) transgene. Our prior work has demonstrated that the transgenic HIF-1(P621G) protein was stabilized in normoxia and that animals expressing HIF-1(P621G) exhibited increased longevity (Zhang et al. 2009). As shown in Figure 2B, hypoxia treatment further extended the lifespan of long-lived HIF-1 over-expression transgenic worms by approximately 17%. Taken together, these results support a model in which hypoxia promotes longevity via parallel pathways. HIF-1 stabilization increases longevity and stress resistance, and hypoxia also increases C. elegans adult lifespan through a HIF-1–independent pathway.
Hypoxia modulates longevity by a mechanism distinct from insulin-like signaling.

DAF-2 insulin-like signaling is a well-established pathway that regulates longevity in *C. elegans*. Reduction-of-function mutations in the daf-2 insulin like receptor induce the nuclear localization of DAF-16/FOXO and dramatically increase lifespan and stress resistance. Conversely, loss-of-function mutations in daf-16 shorten lifespan (KENYON et al. 1993; KIMURA et al. 1997; LIN et al. 2001; OGG et al. 1997). Previous studies found that daf-16 mRNA expression was induced by hypoxia in a hif-1-independent manner (SHEN et al. 2005). We tested whether daf-16 was required for life span extension mediated by hypoxia. As illustrated in Figure 3A and Table 1, hypoxia extended the mean lifespan of daf-16(mu86) mutants by 28%. This indicates that daf-16 is not required for hypoxia-induced longevity in *C. elegans*.

The heat-shock factor (HSF-1) transcription factor activates heat-shock proteins in response to heat and other forms of stress. Like DAF-16, HSF-1 is regulated by the DAF-2 insulin-like receptor. Loss-of-function mutations in hsf-1 suppress lifespan extension in daf-2-deficient animals (HSU et al. 2003; MORLEY and MORIMOTO 2004). Earlier microarray studies by our research group demonstrated that several heat shock proteins were induced by hypoxia via hif-1-independent pathways (SHEN et al. 2005). It was, therefore, important to test the hypothesis that hsf-1 was required for hypoxia-induced lifespan extension. As illustrated in Figure 2B and Table 1, hypoxia increased lifespan of hsf-1(sy441) mutants by 24%.

SKN-1 mediates the phase-II detoxification pathway during oxidative stress (AN and BLACKWELL 2003), and mutation of daf-2 has been shown to increase nuclear localization of SKN-1 to extend longevity (TULLET et al. 2008). We tested the hypothesis that a loss-of-function mutation in skn-1 could abolish the pro-longevity effects of hypoxia.
treatment. As shown in Figure 2C and Table 1, hypoxia extended the lifespan of skn-1(zu67) mutants by up to 25%.

Having determined that DAF-16, HSF-1, and SKN-1, the three major stress-responsive transcription factors regulated by DAF-2, were not required for hypoxia-induced longevity, we tested the hypothesis that daf-2 mutants benefited from hypoxia. Indeed, as shown in Figure 2D, hypoxia treatment further extended the lifespan of long-lived daf-2(e1370) mutant worms. Taken together, these observations indicated that hypoxia induces longevity in mechanisms distinct from insulin-like signaling pathway.

**IRE-1 mediated unfolded protein response is required for hypoxia-mediated lifespan extension.**

In mammals, hypoxia has been shown to cause accumulation of unfolded protein in the endoplasmic reticulum (ER) and thereby activate the unfolded protein response (UPR) through three signaling pathway mediated by IRE1, PERK, and ATF6 (RON and WALTER 2007). The Phsp-4::GFP transgene has been shown to be a reliable reporter of activation of the UPR in C. elegans (CALFON et al. 2002; YONEDA et al. 2004), and we asked whether hypoxia would induce Phsp-4::GFP expression. Indeed, hypoxia significantly induced the expression of Phsp-4::GFP (Figure 4D), consistent with the expectation that hypoxia would elicit the UPR in C. elegans.

We investigated the role of ire-1-mediated UPR in hypoxia-induced lifespan extension. ire-1 encodes an ER transmembrane protein that senses misfolded proteins in the ER lumen and activates the downstream transcription factor XBP-1 through alternative splicing. Activated XBP1 then regulates target gene expression to cope with ER stress (CALFON et al. 2002). As shown in Figure 4H, the induction of Phsp-4::GFP expression was completely abolished by a loss-of-function mutation in ire-1 or xbp-1, suggesting that ire-1 signaling is
essential for UPR induced by hypoxia (ANDERSON et al. 2009). As shown in Figure 5A and Table 1, a loss-of-function mutation in \textit{ire-1} completely abrogated the pro-longevity effect of hypoxia. Additionally, animals a carrying loss-of-function mutation in \textit{xbp-1} did not live longer in hypoxia (Figure 5B and Table1). In contrast, hypoxia treatment extended lifespan of mutants of \textit{pek-1}, which encodes the \textit{C.elegans} PERK homolog that functions in parallel to \textit{ire-1} to regulate UPR. Taken together, these results suggested that \textit{ire-1}-mediated UPR was required for the hypoxia-mediated lifespan extension, but \textit{pek-1} did not have a critical role.

To understand the relationship between UPR and HIF-1 in hypoxia-induced longevity, we asked whether \textit{ire-1} was required for hypoxia-induced lifespan extension in animals over-expressing HIF-1. As shown in Figure 6A, 6B and Table 1, reducing \textit{ire-1} or \textit{xbp-1} expression by RNAi did not abolish the pro-longevity effect induced by HIF-1 over-expression. These results support a model in which \textit{ire-1}-mediated UPR functions in parallel to HIF-1 over-expression to modulate aging in hypoxia.

**DISCUSSION**

Numerous studies have suggested that the genes and pathways that modulate aging intersect with the signaling networks in response to adverse environmental conditions. The availability of oxygen, which is the final electron acceptor of aerobic respiration, is critical for the survival of all metazoans. Our study and previous studies demonstrated that hypoxia extends lifespan in \textit{C.elegans}. Although hypoxia responsive signaling pathways, such as HIF-1, have been studied extensively in mammals, and many components of the pathways are conserved in \textit{C.elegans}, their roles in hypoxia-mediated lifespan extension are unknown. We found that \textit{hif-1}, which controls most of the transcriptional changes during hypoxia adaptation, is not essential for hypoxia-mediated lifespan extension. In addition, hypoxia
increases longevity in mechanisms distinct from insulin like signaling pathway. Instead, we found that hypoxia-activated UPR is required for the pro-longevity effect of hypoxia. These results suggested that hypoxia modulates aging through orchestrating multiple genetic pathways.

Our data showed that hypoxia treatment in adulthood is necessary and sufficient for hypoxia-mediate lifespan extension. It has been clear that perturbation of lifespan-regulatory network during adulthood can modulate aging (Curran and Ruvkun 2007; Dillin et al. 2002a; Hansen et al. 2008; Hansen et al. 2007). Conversely, lifespan extension achieved by down-regulating some mitochondrial genes was specified during development, and inhibiting respiratory-chain components during adulthood only did not affect lifespan (Dillin et al. 2002b). Hypoxia treatment during development resulted in neuronal migration defect and altered behavior in C.elegans (Chang and Bargmann 2008; Pocock and Hobert 2008). Our results suggested that lifespan extension mediated by hypoxia can be dissociated from its roles in developmental specification.

HIF-1 is a key regulator in hypoxia adaptation and is involved in many cellular processes, including glucose metabolism, angiogenesis and cell death. Recent studies established hif-1 as an important modulator of stress resistance and aging in C.elegans (Chen et al. 2009; Mehta et al. 2009). Thus, it is a surprise that loss-of-function mutation of hif-1 does not abolish the hypoxia-mediated longevity effect. We hypothesize that induction of HIF-1 over-expression acts synergistically with other oxygen-sensitive signaling pathway(s) to modulate aging in hypoxia. We also found that the worms lived a longer life in 10% oxygen (unpublished data). On the other hand, it has been reported that hyperoxia (60% oxygen) shortened the lifespan. Therefore, it is possible that oxygen modulates aging through mechanisms that do not involve hif-1.

Recent studies indicated that ER stress and protein homeostasis are important in aging (Cohen et al. 2006; Steinkraus et al. 2008; Viswanathan et al. 2005). Accumulation of unfolded/misfolded proteins in the ER lumen induces unfolded protein response through
three signaling pathways mediated by IRE1, PERK, and ATF6 (RON and WALTER 2007). We found that hypoxia induces ER stress and unfolded protein response in C.elegans. Our work demonstrated that lifespan extension by hypoxia in C.elegans requires functional signaling of IRE-1 and XBP-1. The fact that loss-of-function mutation of ire-1 or xbp-1 entirely abolished the pro-longevity effects of hypoxia underscores the importance of protein homeostasis during hypoxia adaptation.

Our results indicate that IRE-1 and XBP-1 act in parallel to HIF-1 over-expression to modulate aging in C.elegans. A recent study from Kapahi research group demonstrated that loss-of-function mutation of hif-1 increased the lifespan in rich-food condition. The longevity effect of hif-1 mutant was dependent on functional ire-1-mediated UPR (CHEN et al. 2009). These results supported the hypothesis that HIF-1 over-expression and loss-of-function mutation act in different pathways to modulate aging in C.elegans.

Our study underlines the complex interaction of signaling pathways that links oxygen homeostasis and aging. While multiple oxygen-sensing pathways, such as HIF-1, DAF-16 and UPR are all induced by hypoxia in C.elegans, their roles in lifespan extension are distinct. Importantly, many components of oxygen-sensing pathways are potential therapeutic targets for treatment of hypoxia-related diseases, such as stroke, ischemia and cancer. Our results suggest that intervention of unfolded protein response pathway may prove particularly efficient in certain pathological conditions.

MATERIALS AND METHODS
C. elegans strains

The mutant alleles were as follows: LGI: daf-16(mu86)lf; LGII: daf-2(e1370) reduction-of-function, ire-1(v33)lf; LGIII: xbp-1(zc12)lf; LGIV: skn-1(zu67)lf; LGV: hif-1(ia04)lf; LGX: pek-1(ok275)lf.

Life span assays

All strains were maintained at 20°C for at least two generations prior to a life span assay. In order to avoid effects of hypoxia on food, standard NGM plates (60mm NGM plates spotted with 250 ul OP50 bacteria and allowed to grow at room temperature overnight) were irradiated by UV light. For each trial, at least 50 synchronized L4 or young adult worms were picked on UV irradiated plates at 25 to 30 worms per plate and cultured at 25°C thereafter. Worms were removed to new plates every 2 days, and viability was scored. Worms that crawled off the plates, burst at the vulva or died because progeny hatched in utero were excluded from final statistical analyses. Life span statistical analyses were carried out using JMP software (version 7.0) to determine the means and percentiles. p values were calculated using the log-rank (Mantel-Cox) method.

Hypoxia treatment

Hypoxia (1% oxygen) was achieved by mixing room air and nitrogen in hypoxia chamber. UV irradiated NGM plates with experiment animals were placed in hypoxia chamber for 15 minutes to reach hypoxia. The plates were enclosed in air-tight polypropylene jars (Nalgene) balanced with 1% oxygen, and cultured in incubator (Precision) at 25°C. The worms were censored every two days and removed to new NGM plates. The procedure of inspection and transfer was performed at room air within 10 minutes.
Microscopy

To measure the expression of Phsp-4::GFP under normoxia or hypoxia, age synchronized L4 larvae of SJ4005 zcIs4[Phsp-4::GFP] or SJ30 ire-1(zc14), zcIs4[Phsp-4::GFP] were incubated in room air or hypoxia (1% oxygen) for 4 hours. The worms were allowed to recover for 2 hours before they were mounted on agar pads and imaged. Pictures were taken at DIC microscopy or fluorescence microscopy on a Nikon E800 microscope connected to a Spot RT digital camera (Roche Diagnostics).

REFERENCES


**FIGURE LEGENDS**

**Figure 1. Hypoxia signaling functions during adulthood to modulate aging in C.elegans.** The proportion of animals remaining alive is plotted against adult animal age. Lifespan values are given in Table 1; assays were carried out in 1% oxygen or room air at 25°C. (A) Schematic of the experimental protocol. Wild-type N2 worms were treated with hypoxia (1% oxygen) since embryo (B), or during embryonic and larvae stage (C) or adulthood (D) only. (B) Wild type N2 worms live longer in hypoxia ($p<0.0001$). (C) N2 worms develop to young adult in hypoxia do not live longer in normoxia ($p>0.8$). (D) Hypoxia treatment during adulthood extends lifespan of N2 worms ($p<0.0001$).

**Figure 2. HIF-1 is not required for hypoxia-mediated lifespan extension.** Hypoxia treatment (1% oxygen) since L4/young adult stage increases longevity of (A) hif-$1$($ia04$) mutants or (B) animals carrying extra copies of hif-$1$(P621G) transgene. The proportion of animals remaining alive is plotted against adult animal age. Lifespan values are given in Table 1; assays were carried out in 1% oxygen or room air at 25°C.

**Figure 3. Hypoxia modulates longevity in a mechanism distinct from insulin-like signaling to extend lifespan.** Survival curves of mutant worms exposed to hypoxia during adulthood are shown, with proportion of animals alive plotted against adult age. Lifespan values are given in Table 1; assays were carried out in 1% oxygen or room air at 25°C. (A-C) Hypoxia treatment (1% oxygen) since L4/young adult stage extends lifespan of animals carrying loss-of-function mutation in (A) daf-$16$ ($p<0.0001$), (B) hsf-$1$ ($p<0.0001$), or (C) skn-$1$ ($p<0.0001$). (D) Hypoxia further increases the lifespan of long-lived daf-$2$ mutants ($p<0.0001$).
**Figure 4. Hypoxia induces unfolded protein response.** (A-H) Phsp::GFP expression in L4 *C. elegans* after 4 hours exposure in normoxia or hypoxia (1% oxygen). Scale bar, 100 μm.

**Figure 5. IRE-1 mediated unfolded protein response is required for hypoxia longevity effect.** (A-B) Loss-of-function mutation of *ire-1(v33)* (A) or *xbp-1(zc12)* (B) abolishes lifespan extension mediated by hypoxia. (C) Animals carrying loss-of-function mutation of *pek-1(ok275)* live longer in hypoxia. Survival curves of mutant worms exposed to hypoxia during adulthood are shown, with proportion of animals alive plotted against adult age. Lifespan values are given in Table1; assays were carried out in 1% oxygen or room air at 25°C.

**Figure 6. IRE-1 mediated unfolded protein response acts in parallel to HIF-1 over-expression to modulate aging.** Animals carrying *hif-1(P621G)* transgene live longer than wild-type worms when treated with control RNAi (A), *ire-1* RNAi (B) or *xbp-1* RNAi (C). The proportion of animals alive is plotted against adult age. Lifespan values are given in Table2; assays were carried out in room air at 25°C.

**Figure 7. Genetic models depicting lifespan modulation by hypoxia.** HIF-1 and UPR are both induced by hypoxia and both extend longevity. Alternatively, HIF-1 and an unknown pathway extend longevity in hypoxia, but animals that are defective for UPR components suffer more from hypoxic stress, offsetting the benefits of hypoxia.
Table 1. Statistic analysis of life span data for replicate experiments in the manuscript.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hypoxia* life span mean ±S.E. (n)</th>
<th>Nomoxia life span mean ±S.E. (n)</th>
<th>Percentage v. normoxia</th>
<th>p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 (hypoxia whole life)</td>
<td>19.5 ± 0.4 (33)</td>
<td>15.4 ± 0.5 (25)</td>
<td>27</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>N2 (hypoxia whole life)</td>
<td>18.9 ± 0.4 (61)</td>
<td>17.3 ± 0.3 (51)</td>
<td>10</td>
<td>0.0005</td>
</tr>
<tr>
<td>N2 (adult hypoxia)</td>
<td>18.5 ± 0.3 (57)</td>
<td>16.4 ± 0.3 (45)</td>
<td>13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>N2 (adult hypoxia)</td>
<td>18.8 ± 0.4 (38)</td>
<td>15.9 ± 0.5 (30)</td>
<td>18</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>N2 (larvae hypoxia)</td>
<td>17.3 ± 0.3 (60)</td>
<td>17.3 ± 0.3 (51)</td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td>N2 (larvae hypoxia)</td>
<td>16.1 ± 0.3 (63)</td>
<td>16.4 ± 0.3 (45)</td>
<td>-2</td>
<td>0.82</td>
</tr>
<tr>
<td>hif-1(ia04)</td>
<td>19.9 ± 0.4 (50)</td>
<td>17.5 ± 0.4 (33)</td>
<td>14</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>hif-1(ia04)</td>
<td>21.9 ± 0.4 (68)</td>
<td>17.3 ± 0.4 (72)</td>
<td>27</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>hif-1(ia04)</td>
<td>20.2 ± 0.3 (53)</td>
<td>17.9 ± 0.3 (57)</td>
<td>13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ials32 <a href="P621G">Phif-1::hif-1</a>::myc</td>
<td>23.3 ± 0.5 (52)</td>
<td>20.1 ± 0.5 (48)</td>
<td>17</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>daf-16(mu86)</td>
<td>15.6 ± 0.3 (60)</td>
<td>11.3 ± 0.3 (39)</td>
<td>38</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>daf-16(mu86)</td>
<td>16.2 ± 0.4 (57)</td>
<td>12.7 ± 0.4 (55)</td>
<td>28</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>daf-16(mu86);hif-1(ia04)</td>
<td>15.4 ± 0.4 (56)</td>
<td>12.0 ± 0.3 (54)</td>
<td>28</td>
<td>&lt;0.0001</td>
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<tr>
<td>hsf-1(sy441)</td>
<td>12.8 ± 0.3 (49)</td>
<td>10.3 ± 0.2 (40)</td>
<td>24</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>hsf-1(sy441)</td>
<td>12.9 ± 0.3 (57)</td>
<td>10.3 ± 0.3 (56)</td>
<td>24</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>skn-1(zu67)</td>
<td>18.6 ± 0.4 (50)</td>
<td>15.1 ± 0.4 (53)</td>
<td>23</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>skn-1(zu67)</td>
<td>18.5 ± 0.4 (55)</td>
<td>14.8 ± 0.4 (47)</td>
<td>25</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>daf-2(e1370)</td>
<td>39.2 ± 1.1 (58)</td>
<td>33.2 ± 0.9 (55)</td>
<td>18</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ire-1(v33)</td>
<td>12.1 ± 0.5 (49)</td>
<td>12.0 ± 0.6 (44)</td>
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<td>0.83</td>
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<td>ire-1(v33)</td>
<td>11.2 ± 0.4 (47)</td>
<td>11.3 ± 0.4 (30)</td>
<td>-1</td>
<td>0.84</td>
</tr>
<tr>
<td>xbp-1(zc12)</td>
<td>12.9 ± 0.6 (28)</td>
<td>13.5 ± 0.4 (37)</td>
<td>-4</td>
<td>0.65</td>
</tr>
<tr>
<td>xbp-1(zc12)</td>
<td>11.9 ± 0.4 (46)</td>
<td>12.2 ± 0.4 (42)</td>
<td>-2</td>
<td>0.69</td>
</tr>
<tr>
<td>pek-1(ok275)</td>
<td>19.5 ± 0.4 (45)</td>
<td>16.4 ± 0.5 (43)</td>
<td>19</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pek-1(ok275)</td>
<td>18.7 ± 0.5 (42)</td>
<td>15.5 ± 0.5 (41)</td>
<td>21</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Unless indicated otherwise, animals were grown in room air at 20° C till L4/young adult stage before being subjected to hypoxia (1% oxygen) treatment at 25° C.

† The longevity of each strain was compared to wild-type, and the p values were calculated by log-rank tests.
Table 2: Effects of *ire-1* and *xbp-1* RNAi on the longevity of HIF-1 over-expression worms.

<table>
<thead>
<tr>
<th>strain</th>
<th>Adult life span mean ±S.E.</th>
<th>Maximum lifespan</th>
<th>Percentage v. control</th>
<th>n</th>
<th><em>p</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N2 (L4440 RNAi)</em></td>
<td>13.3 ± 0.4</td>
<td>20</td>
<td></td>
<td>56</td>
<td></td>
</tr>
<tr>
<td><em>lals32 (L4440 RNAi)</em></td>
<td>18.5 ± 0.4</td>
<td>24</td>
<td>27</td>
<td>51</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><em>N2 (ire-1 RNAi)</em></td>
<td>11.1 ± 0.4</td>
<td>18</td>
<td></td>
<td>57</td>
<td></td>
</tr>
<tr>
<td><em>lals32 (ire-1 RNAi)</em></td>
<td>14.6 ± 0.4</td>
<td>20</td>
<td>33</td>
<td>53</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><em>N2 (xbp-1 RNAi)</em></td>
<td>10.1 ± 0.4</td>
<td>18</td>
<td></td>
<td>47</td>
<td></td>
</tr>
<tr>
<td><em>lals32 (xbp-1 RNAi)</em></td>
<td>15.7 ± 0.4</td>
<td>22</td>
<td>51</td>
<td>53</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* The longevity of each strain was compared to wild-type, and the *p* values were calculated by log-rank tests.
Figure 1. Hypoxia signaling functions during adulthood to modulate aging in *C. elegans*.

Figure 2. *hif-1* is not essential for hypoxia-mediated lifespan extension.
Figure 3. Hypoxia modulates longevity by a mechanism distinct from insulin/IGF-1-like signaling.

Figure 4. Hypoxia induces Phsp-4::GFP, a UPR marker in a ire-1 dependent manner.
Figure 5. *ire-1* mediated unfolded protein response is required for hypoxia-modulated longevity effect.
Figure 6. *ire-1* mediated UPR acts in parallel to HIF-1 over-expression to modulate aging.

Figure 7. Genetic models depicting lifespan modulation by hypoxia.
CHAPTER 4. SWAN-1 interacts with EGL-9 to regulate
HIF-1 activity in C. elegans

A manuscript in preparation
Yi Zhang, Zhiyong Shao, Qi Ye and Jo Anne Powell-Coffman

Abstract

Hypoxia-inducible factor (HIF) is the transcription factor that mediates the key transcriptional changes to enable oxygen homeostasis in animals. The α subunit of the heterodimeric HIF complex is acutely regulated by cellular oxygen levels and thus dictates oxygen-dependent transcriptional regulation. C. elegans HIF-1 is the ortholog of the mammalian HIFα subunit. HIF-1 protein is degraded in an oxygen-dependent manner via the evolutionarily conserved EGL-9/VHL-1 pathway. Recent studies demonstrated that EGL-9 regulates both HIF-1 protein stability and HIF-1 activity. However, the molecular basis of how EGL-9 regulates HIF-1 transcriptional activity is still unclear. In order to identify the genes that repress HIF-1 activity, we carried out a forward genetic screen to search for mutants that over-expressed a HIF-1 target reporter gene. One of the alleles identified in this screen was mapped to swan-1, which encodes a WD repeat protein. Loss-of-function swan-1 mutations increase HIF-1 activity but have little effect on HIF-1 protein levels. We present evidence that SWAN-1 binds directly to EGL-9. Therefore, our results establish SWAN-1 as a novel regulator of HIF-1 that suppresses HIF-1 activity in parallel to the VHL-1 mediated degradation pathway.
Introduction

During development, homeostasis and diseases, aerobic organisms often experience low cellular oxygen conditions (hypoxia). In mammals, the hypoxia-inducible factor (HIF) transcription factor is the master regulator of transcriptional changes to enable the cellular and systemic adaptation to hypoxia (SEMENZA 2007b). HIF target genes are important in many hypoxia adaptation processes, including erythropoiesis, angiogenesis/vascular remodeling, glycolytic/mitochondrial metabolism and cell proliferation/survival. Hypoxia is involved in many human diseases, such as cancer, myocardial ischemia and stroke. Inhibitors of HIF or HIF target genes have been used as cancer chemotherapeutic agents, since HIF over-expression is often associated with survival of tumor cells in hypoxic microenvironments. Conversely, interventions that promote HIF activity may benefit patients afflicted with cardiovascular disorders. Therefore, elucidating the networks that regulate HIF may help develop novel therapeutic approaches to treat hypoxia-related diseases.

The heterodimeric HIF-1 complex is composed of two subunits, both of which contain basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domains that mediate dimerization and DNA binding. While the HIF-1 beta subunit can form complexes with other transcription factors, HIF-1α is sensitive to cellular oxygen levels and thus dictates HIF activity. Under normoxic conditions, HIF-1α is hydroxylated at proline residue(s) in the evolutionarily conserved oxygen-dependent-degradation (ODD) domain by the EGLN family (also known as PHD or HPH family) of 2-oxoglutarate dioxygenases which utilize O2 as a co-substrate. Hydroxylated HIF-1α is bound by the von Hippel-Lindau protein (pVHL), which in turn recruits a ubiquitin protein ligase complex that targets HIF-1α for proteasomal degradation. When the oxygen concentration is limited (hypoxia), this suppresses the hydroxylation reaction and hence stabilizes HIF-1α (EPSTEIN et al. 2001).
The nematode *Caenorhabditis elegans* has proven to be a powerful model to decipher the evolutionarily conserved signaling pathways that regulate hypoxia response and hypoxia-inducible factor (Epstein et al. 2001; Jiang et al. 2001; Shen et al. 2006). *C. elegans* *hif-1*, *aha-1*, *egl-9*, and *vhl-1* genes are orthologous to mammalian HIF-1α, HIF-1β, EGLN, and VHL genes, respectively (Epstein et al. 2001; Jiang et al. 2001; Powell-Coffman et al. 1998). The stability of HIF-1 protein is controlled by the evolutionarily conserved EGL-9/VHL-1 pathway (Epstein et al. 2001).

Prior studies demonstrated that in addition to modulating stability, there are mechanisms regulating transcriptional activity of HIF-1 in *C. elegans*. Many treatments or mutations that eliminate oxygen-dependent degradation of HIF-1 protein do not abolish EGL-9-mediated repression of HIF-1 transcriptional activity (Bishop et al. 2004; Shao et al. 2009; Shen et al. 2006). These data suggested that EGL-9 acts as an oxygen sensor in the oxygen-dependent degradation pathway and also attenuates HIF-1 activity independent of VHL-1 (Shen et al. 2006). In addition, loss-of-function mutations in the *rhy-1* gene, whose expression is regulated by HIF-1, cause a modest increase of HIF-1 protein, but promote expression of some HIF-1 target genes at levels much higher than in *vhl-1* mutants, suggesting that RHY-1 functions in a negative feedback loop to inhibit HIF-1 transcriptional activity (Shen et al. 2006). Thus, the genetic data suggest that HIF-1 transcriptional activity is repressed by both RHY-1, a transmembrane protein, and EGL-9. This pathway(s) does not require EGL-9 hydroxylase activity (Shao et al. 2009), but the molecular mechanisms are largely unknown.

In this study, we identify a novel regulator of HIF-1 from an EMS mediated enhancer screen designed to identify mutations that promoted HIF-1 activity (Shen et al. 2006). We identified new alleles of *egl-9* and *rhy-1* and the *ia50* allele, which mapped to a region of the genome that had not been previously implicated in HIF-1 regulation. The *ia50* allele mapped to *swan-1* (F53C11.8), a gene that encodes a WD-repeat protein of the AN11 family (Yang et al. 2006). We further characterized *ia50* and a deletion allele of *swan-1*, and we
found that swan-1 was required to repress expression of HIF-1 targets but had little effects on HIF-1 protein levels. Double mutants lacking vhl-1 and swan-1 function have a higher expression level of HIF-1 target genes than either single mutant. Using yeast two-hybrid and pull down assays, we showed that SWAN-1 physically interacted with EGL-9. These data support a model in which SWAN-1 inhibits HIF-1 localization or transcriptional activity.

**Materials and Methods**

**Culture of *C. elegans* and strains**

All strains were maintained at 20°C on nematode-growth-medium (NGM) plates spotted with OP50 bacteria unless otherwise noted (BRENNER 1974). The wild type strain used for all experiments were N2 Bristol (BRENNER 1974). The mutant alleles were as follows: LGII: rhy-1(ok1402) If, LGV: hif-1(ia04) If, swan-1(ok267) If, egl-9(sa307) If; LGX: vhl-1(ok161) If. To build the mapping strain ZG196, the Hawaiian strain CB4856 (DAVIS et al. 2005) was crossed to ZG119 iaIs07[Pnhr-57::gfp + unc-119(+)] IV; vhl-1(ok161) X. The F2 hermaphrodites from this cross that exhibited strong GFP expression were then crossed back to the Hawaiian strain. The procedure was repeated 6 times until all the single nucleotide polymorphisms used in this study were confirmed as the Hawaiian pattern.

**EMS mutagenesis screen**

The forward genetic screen was performed as described previously (SHEN et al. 2006). Briefly, ZG119 iaIs07[Pnhr-57::gfp + unc-119(+)] IV; vhl-1(ok161) X animals were mutagenized with EMS, and the F2 progeny were screened for dramatically increased expression of the Pnhr-57:: GFP reporter (SHEN et al. 2006) using fluorescent stereo microscopy. The ia50 mutant allele was outcrossed twice to the parental strain prior to any further mapping or characterization.

**Mapping of ia50 to the swan-1 locus**
Chromosome and interval mapping were performed as described by Davis et al. (Davis et al. 2005) with some modification. Briefly, ZG196 males (carrying SNPs from the Hawaiian strain) were crossed to ZG359 ia50; iaIs07[Pnhr-57:: gfp + unc-119(+)] IV; vhl-1(ok161) X. Fifty F2 animals exhibiting the vhl-1, swan-1 double mutant phenotype (of strong GFP expression in hypodermis, intestine and pharynx) and fifty animals exhibiting the vhl-1 single mutant phenotype (strong GFP expression only in intestine) were picked into separate tubes, and genomic DNA was prepared. Analysis of the divergent SNPs showed enrichment of Bristol bands in mutant lanes and an enrichment of Hawaiian bands in non-mutant lanes for SNPs lying between -5 and +13 mu on chromosome V. For interval mapping, individual self-progeny of ZG359/ZG196 F1 hermaphrodites (described above) with the swan-1, vhl-1 double mutant phenotype were picked into a 96-well plate to prepare the genomic DNA. Analysis of four SNPs (-5, +1, +6, +13) (Davis et al. 2005) indicated 27/96 mutants were Hawaiian at one or more SNPs. Of those 27 mutants, 25/27 had the Hawaiian SNP at -5 mu; 8/27 had the Hawaiian SNP at +1 mu; none had the Hawaiian SNP at +6 mu; and 2/27 had the Hawaiian SNP at +13 mu. This placed ia50 close to +6 mu. We then used SNP R10D6(+5.83 mu) and pkP5086(+6.42 mu) to do three point mapping. Among 960 worms over-expressing the reporter examined, 3/960 had the Hawaiian SNP at +5.83 mu, and 9/960 had the Hawaiian SNP at +6.42 mu. Of note, all mutants that carried the Hawaiian SNP at +5.83 mu also carried the Hawaiian SNP at +6.42 mu. This placed ia50 at +5.67 mu.

Transgenic animals

Bacterial strains containing cosmids used for transformation were kindly provided by the Sanger Institute (United Kingdom). The swan-1 plasmids were gifts from Dr. Erik Lundquist at University of Kansas (Yang et al. 2006). Plasmid or cosmid DNA was extracted from 4 ml bacteria media using a mini-prep plasmid preparation kit (Qiagen) and were confirmed by restriction enzyme digestion. To generate extrachromosomal arrays of transgenes, DNA (30 ng/µL plasmid DNA or 10 ng/µL cosmid DNA) was microinjected into young adult ia50;vhl-1(ok161);Pnhr-57::GFP mutant hermaphrodite germlines, following
standard procedures (EPSTEIN 1995). The *rol-6* plasmid pRF4 was used as the coinjection marker (MELLO *et al.* 1991).

**RNA interference**

RNAi was performed as previously described (ZHANG *et al.* 2009). Bacterial strains containing RNAi construct were from Ahringer library (KAMATH *et al.* 2001).

**Egg-laying defect assay**

To quantify the egg-laying defect, late L4 wild-type or mutant worms were removed to NGM plates spotted with OP50 bacteria. After culture at 20 °C for 24 hours, the worms were inspected under microscopy (400×) to count the number of eggs held in utero. At least 10 worms were inspected for each strain. Student t-test was used to calculate the p-value.

**Brood size assay**

Synchronized worms were obtained from overnight egg laying and allowed to develop at 20°C. Individual L4 animals were removed to NGM plates with single worms per plate. The worms were allowed to lay eggs for 24 hours. On subsequent days each worms was transferred to a new NGM plate and allowed to lay eggs for 24 hours until no eggs were produced. The number of viable progeny was scored by counting hatched larvae after 48 hours.

**Western blots:**

To assay *Pnhr-57::GFP* or *HIF-1::HA* expression, 40 – 100 worms from synchronized populations of L4 animals or young adults were boiled in 1x SDS buffer [50mM Tris-HCl PH6.8, 10%(v/v) glycerol, 2% (w/v) SDS, 100mM DTT, 0.1% (w/v) bromphenol blue] for 10 minutes before loading to a 10-12% polyacrylamide gel for electrophoresis. After separation, proteins were transferred to nitrocellulose membranes. The blots were probed with monoclonal antibodies: GFP antibody (from Roche) at 1:1000 dilution; HA antibody (from Cell Signaling Technology, clone 6E2) at 1:1000 dilution; Mouse anti-myc ascites (clone 9E10) from the Developmental Studies Hybridoma Bank at 1:1000 dilution, or AHA-1 antibody (produced in ISU Hybridoma Facility) (JIANG *et al.* 2001) at 1:100
dilution. The secondary antibody (goat anti-mouse IgG+IgM from Bio-rad) was diluted 1:3000. The relative intensity of protein bands were quantified by Image J software. The statistical significance of differences was assessed by t-tests.

**Quantitative RT-PCR:**

Total RNA was isolated from synchronized populations of L4 or young adult stage worms using Trizol reagent (Invitrogen). RNA samples were treated by RNase free DNase (Promega) before being reverse transcribed to complementary DNA using Oligo(dT)\textsubscript{18} primers and AffinityScript reverse transcriptase (Stratagene). Quantitative RT-PCR was performed using the iQ SYBR GREEN supermix (Bio-Rad) real-time PCR system, and each reaction included cDNA prepared from 50-100ng total RNA. The primers for K10H10.2 and inf-1 have been published previously (SHEN et al. 2006). The standard curve method was used to analyze the expression levels. inf-1 is not regulated by hypoxia and was used as an input control (SHEN et al. 2006). At least three biological replicates were analyzed for each experiment, and each PCR reaction was performed in duplicate. Student t-tests were used to assess statistical significance of differences.

**Yeast two-hybrid assay**

The coding region of the *C. elegans* egl-9 catalytic domain was generated by PCR and fused in frame to the GAL4 DNA-binding domain in the yeast expression vector pGBKT7 (bait). This construct did not result in autoactivation. *The swan-1* yeast two-hybrid construct (in the pACT2 prey vector) was described in a prior study and was kindly provided by E. Lundquist (YANG et al. 2006).

To assay for protein-protein interactions between the SWAN-1 and EGL-9 proteins, we used the Matchmaker\textsuperscript{TM} GAL4 Two-Hybrid systems (Clontech). In this system, the transcriptional activation domain (AD) and the DNA binding domain (BD) of the yeast GAL4 transcription activator are encoded by two different plasmids, termed pACT2 and pGBKT7, respectively. Each plasmid encodes a selectable marker. pACT2 uses leucine as selection marker, and pGBKT7 uses tryptophane as selection marker. The yeast strain used
in this system, *Saccharomyces cerevisiae* strain AH109, encodes four reporter genes, *HIS3* (imidazoleglycerol phosphate dehydratase, which catalyzes the sixth step in histidine biosynthesis), *ADE2* (phosphoribosylamino-imidazole-carboxylase in the biosynthetic pathway of adenine), *MEL1* (α-galactosidase) and *lacZ* (β-galactosidase), under the control of distinct GAL4 upstream activating sequences and TATA boxes. The yeast strain AH109 was cotransformed with two hybrid plasmids (pACT2 plasmid carrying *swan-1* sequence and pGKT7 carrying *egl-9* sequence) by the polyethylene glycol-lithium acetate method (Gietz et al. 1992; Schiestl and Gietz 1989). As controls, the *swan-1* plasmid was cotransformed with pGBK7, and the *egl-9* plasmid was cotransformed with pACT2. Both pGBK7 and pACT2 were also cotransformed into the yeast strain AH109. Yeast cells were grown in SD-agar plates lacking histidine, leucine and tryptophane (medium stringency selection) to screen for expression of *HIS3*. Twenty His+ colonies were grown in 2 ml of liquid SD-medium lacking histidine, leucine, and tryptophane shaken at 30°C for 24 h. Ten-fold serial dilutions of the cultures were made, and 5 μl of each dilution was plated onto SD-medium lacking histidine, adenine, leucine, tryptophane and containing X-α-galactosidase plates (high stringency selection). These plates were incubated at 30°C for 3 days to screen for expression of *ADE2* and *MEL1*.

**Pull-down assay**

The transgenic line expressing SWAN-1::GFP was kindly provided by Dr. Erik Lundquist (Yang et al. 2006). Pswan-1::swan-1::gfp transgenic worms were cultured on enriched peptone plates (1.2g NaCl, 20g peptone, 25g agar in 1L water. Autoclave. Cool to 55°C, then add 1ml of 5mg/ml cholesterol, 1ml of 1M MgSO4 and 25ml of 1M potassium phosphate, pH 6.0) spotted with NA22 bacteria. The collected worms were suspended in extraction buffer [100 mM PIPES, pH 6.0, 100 mM NaCl, 3 mM MgCl2, 1 mM EGTA, a mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% Triton X-100, Complete Protease Inhibitor Tablets (Roche), and phosphatase inhibitors (5 mM NaF and 1 mM
Na$_3$VO$_4$)]. The worm lysates were prepared by passing through a French press cell disrupter (Thermo Electron Corporation) at 8000 psi three times and cleared by centrifugation.

To perform the pull down assay, 20 ul protein G Sepharose beads were preloaded with anti-GFP monoclonal antibody (Roche). After 4 hrs, the beads were washed with extraction buffer for three times and incubated with worm lysates at 4°C for 4 hrs. The beads were washed three times in extraction buffer and incubated with 200 ul bacteria lysates expressing Maltose Binding Protein (MBP) fused EGL-9 at 4°C for 4 hrs. The beads were washed three times in extraction buffer and then boiled in 25 ul SDS loading buffer for 10 min. The supernatants were analyzed by SDS-PAGE and Western-blots using anti-MBP (NEB) antibody.

**Results**

**ia50 allele activates HIF-1 activity in vhl-1 mutants**

We designed an enhancer screen to search for the genes that inhibit HIF-1 activity. To facilitate the quick recovery of mutations in genes of interest, we have developed a visual system in which a GFP reporter was fused to the promoter of a HIF-regulated gene, nhr-57. This reporter has proven to be a reliable indicator of HIF function (SHEN et al. 2006). In animals expressing wild-type levels of HIF-1, the Pnhr-57::GFP signal was invisible in room air conditions under our fluorescent microscopy. The GFP signal increased in either vhl-1(ok161) or egl-9(sa307) mutants, consistent with the finding that HIF-1 was stabilized in both mutants (EPSTEIN et al. 2001; SHEN et al. 2006). In vhl-1(ok161) mutants, the GFP reporter was increased in the intestine but invisible in the hypodermis and pharynx (Figure 1E). However, the Pnhr-57::GFP signal increased dramatically in hypodermis, pharynx and intestine in egl-9(sa309) mutants (SHEN et al. 2006). Prior studies have established that
EGL-9 represses HIF-1 activity, in addition to its central role in VHL-1-dependent degradation of HIF-1. To identify the genes act in concert with EGL-9 to inhibit HIF-1 activity, we used EMS to mutagenize vhl-1(ok161) mutants and screened for F2 progeny with a Pnhr-57::GFP expression phenotype similar to that in egl-9 loss-of-function mutants.

We isolated several mutants that increased expression of the Pnhr57:GFP reporter in the vhl-1(ok161) background. Complementation tests revealed that 3 of the mutations were new alleles of rhy-1, a HIF-1 regulating gene identified previously in our lab (Table 1) (Shen et al. 2006). One of the mutant alleles, ia50, complemented both rhy-1 (ok1402) and egl-9(sa307), suggesting that it defined a new regulator of HIF. The enhanced GFP expression phenotype of ia50 was completely recessive and segregated as a single locus in a Medelian fashion. Interestingly, when we tried to outcross the vhl-1 mutant allele from ia50, all progeny (F2) that over-expressed the reporter were vhl-1 /- mutant. This suggests that the effects of the ia50 mutation are only visually detectable when HIF-1 protein is not destabilized by the VHL-1 pathway.

We also noticed egg-laying defects in ia50;vhl-1(ok161) mutant worms (Figure 1H, I), that were reminiscent of egl-9 mutants. Strong loss-of-function of egl-9 cause worms to hold their eggs in utero for longer than wild-type worms (Figure 1I), and this is suppressed by a loss-of-function mutation in hif-1, suggesting that the egg-laying defect requires enhanced HIF-1 activity (Shen et al. 2006). In addition, similar to egl-9 mutants, ia50;vhl-1(ok161) mutants had decreased brood size (Figure 1J). Taken together, these results indicated that the ia50 allele further increased HIF-1 activity in vhl-1 mutants to cause an phenotype similar to that seen in egl-9 strong loss-of-function mutants.
**ia50 is a loss-of-function mutation in swan-1(F53C11.8)**

We mapped \( \text{ia50} \) using a rapid single nucleotide polymorphism (SNP) method modified from Davis et al. (Davis et al. 2005). This method placed \( \text{ia50} \) close to + 6 mu on chromosome V (detailed in materials and methods). Three point mapping using two SNP markers near \( \text{ia50} \) [+5.83 and +6.42 mu (Table 2)] refined the location of \( \text{ia50} \) to + 5.67 mu on chromosome V (Figure 2A).

We then screened cosmid DNA in that genetic interval to determine which genomic DNA fragment could rescue the \( \text{ia50} \) mutant phenotype and repress \( Pnhr-57::GFP \) expression. One cosmid clone, F53C11 suppressed \( Pnhr-57::GFP \) expression in the hypodermis and pharynx (data not shown). The genes on cosmid F53C11 included \( \text{swan-1} \) (F53C11.8). \( \text{swan-1} \) had previously been shown to encode a WD repeat protein and to be involved in regulation of Rac GTPases in neurons (Yang et al. 2006). Interestingly, SWAN-1::GFP fusion protein has been shown to be expressed in all cells except in intestine (Yang et al. 2006). This expression pattern is complementary to the \( Pnhr-57::GFP \) expression in \( vhl-1(ok161) \) mutants.

Sequencing the \( \text{swan-1} \) locus in \( \text{ia50} \) mutants revealed a G to A conversion at the splice acceptor site of intron 2 (Figure 2A). To investigate the consequences of this mutation, we sequenced cDNA from \( \text{ia50} \) animals, and we determined that of the \( \text{ia50} \) mutation resulted in a \( \text{swan-1} \) transcript that lacked intron 2. This missplicing caused a frameshift and introduced an early stop codon (Fig. 2A). The truncated SWAN-1 protein lacks 6 of its 8 WD repeat domains (Figure 2A). Furthermore, RNA-mediated interference (RNAi) of \( \text{swan-1} \) increased the expression of \( Pnhr-57::GFP \) in wild-type as well as in \( vhl-1(ok161) \) mutant worms (Figure 2B).

These data indicated that a loss-of-function mutation in \( \text{swan-1} \) could cause over-expression of HIF-1 target genes in conditions in which HIF-1 was stable. To further test this, we assayed \( Pnhr-57::GFP \) expression in \( \text{swan-1}(ok267); vhl-1(ok161) \) double
mutants. The swan-1(ok267) allele deletes sequences encoding 223 amino acid residues and is predicted to be a severe loss-of-function allele. These animals over-expressed the Pnhr-57::GFP HIF-1 reporter, as assayed by fluorescent microscopy (data not shown) and immunoblots (Figure 3A). Moreover, swan-1(ok267) failed to complement ia50 (data not shown), providing further evidence that the two mutations mapped to the same locus.

We noticed that immediately downstream of swan-1, there is another gene that encodes a WD repeat protein named SWAN-2 (F53C11.7). SWAN-2 is 45% identical at the amino acid level to SWAN-1 (Yang et al. 2006). To test the hypothesis that swan-2 also regulated expression of the HIF-1 reporter, we assayed expression of Pnhr-57::GFP in animals treated with swan-2 RNAi. As shown in Figure 2B, swan-2 RNAi had no effect. We conclude that swan-1, but not swan-2, regulates expression of HIF-1 target genes, as assayed by Pnhr-57::GFP expression and egg laying defects.

**SWAN-1 suppresses HIF-1 activity via a VHL-1-independent pathway.**

To more fully understand the swan-1 mutant phenotype, we quantitated the expression of HIF-1 target genes in wild-type animals and in animals that lacked oxygen-dependent degradation of HIF-1. We chose to assay three HIF-1 targets, Pnhr-57::GFP, F22B5.4 mRNA, and K10H10.2 mRNA, because each of these HIF-1 targets have been characterized in other studies (Shen et al. 2005; Shen et al. 2006) We used immunoblots to quantitate the expression of Pnhr-57::GFP. As shown in Figure 3A, swan-1(ok267) mutant worms expressed Pnhr-57::GFP at levels 5 times higher than in wild type. We further employed quantitative RT-PCR to assay the expression of K10H10.2 and F22B5.4 mRNAs. Previous studies had shown that the K10H10.2 and F22B5.4 genes were induced by hypoxia in a hif-1-dependent manner (Shen et al. 2005). As shown in Figure 3 B and C, the swan-1(ok267) mutation did not change the mRNA level of K10H10.2 and caused increase in F22B5.4
mRNA by 3.7 fold. The increase of F22B5.4 mRNA was not significant (p = 0.3) (Fig. 3B, C).

We further characterized the effects of swan-1 mutation on HIF-1 activity by analyzing double mutants lacking both swan-1 and vhl-1 functions. Characterization after the genetic screen had demonstrated that swan-1(ia50) mutation in vhl-1 mutants caused increased expression of Pnhr-57::GFP in the pharynx and hypodermis as detected by fluorescence (Fig. 1F). As shown in Figure 3D, in vhl-1(ok161) animals, the swan-1(ok267) allele increased Pnhr-57::GFP protein expression 2.4 fold. Analysis of K10H10.2 and F22G.5.4 mRNA levels by RT-PCR revealed a similar pattern. Expression of K10H10.2 and F22G5.4 mRNA increased 8 fold and 2 fold in swan-1(ok267); vhl-1(ok161), respectively, when comparing to vhl-1(ok161) mutants (Figure 3E, F).

To address whether SWAN-1 regulated HIF-1 protein expression, we assayed protein levels of HIF-1::tag minigene (Zhang et al. 2009). The epitope-tagged hif-1 encodes HIF-1a isoform and can rescue the HIF-1 function in hif-1 mutants (Zhang et al. 2009). As shown in the immunoblots in Figure 3G, the swan-1 mutation did not change the expression of HIF-1 protein. Similarly, swan-1(ok267); vhl-1(ok161) double mutants did not increase the HIF-1::tag protein level more than vhl-1-deficient worms (Fig. 3G). Collectively, these data show that loss-of-function mutations in swan-1 do not alter HIF-1 protein levels, but they do increase expression of HIF-1 targets.

**SWAN-1 interacts with EGL-9 to regulate HIF-1 activity**

In prior studies, we had discovered that EGL-9 has multiple functions, and it regulates HIF-1 activity via a novel mechanism that does not require EGL-9 hydroxylase activity (Shao et al. 2009). We hypothesized that SWAN-1 might interact with EGL-9 to repress HIF-1 function. To test this, we first tested whether SWAN-1 and EGL-9 acted in the same
pathway by measuring the expression of Pnhr-57::GFP in worms carrying loss-of-function mutations in swan-1 and egl-9. As shown in Figure 4A, swan-1(ok267);egl-9(sa307) mutants showed Pnhr-57::GFP expression levels comparable to those in egl-9(sa307) worms. This suggested that SWAN-1 regulates HIF-1 activity in the same pathway as EGL-9.

To test the hypothesis that SWAN-1 binds to EGL-9 directly, we employed yeast two-hybrid assays. The GAL-4 DNA binding domain was fused to SWAN-1, and the GAL-4 activation domain was fused to the EGL-9 catalytic domain. When these two fusion proteins were co-expressed, genes downstream of the GAL-4 enhancer sequences were expressed, as assayed by growth on auxotrophic media and by beta-galactosidase expression. As shown in Figure 4B, the presence of swan-1 and egl-9 catalytic domain fusion specifically activated both HIS5 expression and lacZ expression in the yeast two-hybrid system.

To further confirm the physical interaction of SWAN-1 and EGL-9, we purified either GFP (as control) or SWAN-1::GFP from transgenic worms and assayed their interaction with bacterially expressed EGL-9. As shown in Figure 4C, GFP tagged full length SWAN-1 specifically pulled down the MBP tagged EGL-9 protein.

**DISCUSSION**

Hypoxia-inducible factors play key roles in the regulation of oxygen homeostasis during development and pathological conditions (SEmenza 2007b; Shen et al. 2005). *C. elegans* hif-1, orthologous to mammalian HIF α genes, regulates most of the transcriptional changes in response to oxygen deprivation (Shen et al. 2005). HIF-1 protein is regulated by the evolutionarily conserved EGL-9/VHL-1 pathway, and in normoxia hydroxylated HIF-1 is targeted to proteasomal degradation (Epstein et al. 2001). Recent studies suggested that some regulatory networks modulate HIF-1 activity in parallel to the oxygen-dependent degradation pathway (Bishop et al. 2004; Shao et al. 2009; Shen et al. 2006). Employing
genetic approaches, we report here that SWAN-1 attenuates HIF-1 activity in mechanisms distinct from VHL-1-dependent degradation pathway.

We first isolated an allele of swan-1 from a genetic screen designed to identify genes that inhibit HIF-1 activity. Subsequent mapping revealed a point mutation that disrupts the correct splicing of swan-1 mRNA. An existing deletion mutation of swan-1 largely recapitulates the phenotype of increased HIF-1 target reporter. The isolated swan-1 allele failed to complement the existing swan-1 deletion allele in inhibiting expression of HIF-1 target gene. Loss-of-function mutations of swan-1 increased expression of HIF-1 target genes but did not alter the HIF-1 protein level. Analysis of double mutants lacking both swan-1 and vhl-1 function demonstrated that loss-of-function mutation of swan-1 can further promote HIF-1 transcriptional activity in vhl-1 mutants without changing HIF-1 protein expression. We also found that SWAN-1 physically interacted with EGL-9 in yeast two-hybrid and pull-down assays. These results establish SWAN-1 as a novel HIF-1 regulator that normally inhibits HIF-1 transcriptional activity in parallel to VHL-1-mediated pathway that controls HIF-1 protein level.

swan-1 encodes a WD-repeat protein that has been reported to regulate Rac GTPase activity in C. elegans (Yang et al. 2006). SWAN-1 physically interacts with both Rac and UNC-115/abLIM, a Rac regulator. Over-expression of SWAN-1 suppresses Rac activity (Yang et al. 2006). Expression of Rac1 was shown to suppress HIF-1 protein expression in hypoxic Hep3B, HEK293, and NIH3T3 cells (Hirota and Semenza 2001). Our studies to date do not indicate that Rac family members control HIF-1 levels as assayed in whole animal lysates (data not shown). Thus, the interactions between SWAN-1 and Rac family members in migrating cells may not influence HIF-1 signaling as assayed in whole animals.

WD-repeat proteins have been found in all eukaryotes and they form a large family with diverse functions (Li and Roberts 2001; Smith et al. 1999). The WD-repeat domain folds into a beta propeller and often serves as a platform on which multiple protein complexes assemble reversibly (Li and Roberts 2001). Our data demonstrated that
SWAN-1 physically interacted with EGL-9. Intriguingly, Morg1, a WD-repeat protein in mammals has been found to form a complex with PHD2/EGLN3 to suppress HIF-1 activity (HOPFER et al. 2006). Amino acid sequence analysis demonstrated that SWAN-1 and Morg1 belong to different WD repeat protein families. It is interesting to see whether the functions of *C. elegans* SWAN-1 and mammalian Morg1 are conserved.

It waits to be determined how the interaction between SWAN-1 and EGL-9 contributes to suppressing HIF-1 activity. One possibility is that SWAN-1 forms a complex with HIF-1 and EGL-9, and may sequester HIF-1 from entering the nucleus. Another attractive model is that SWAN-1 may be involved in transcriptional repression. Recent studies have shown that WD-repeat proteins play important roles in chromatin remodeling and gene regulation (SUGANUMA et al. 2008b). Several WD-repeat proteins, including WDR5, RbBP5 and RbAp48, are components of histone modification complexes. WDR5 is associated with a SET-domain-containing histone methyltransferase as well as several histone acetyltransferases (DOU et al. 2006; MENDJAN et al. 2006; SUGANUMA et al. 2008a; WYSOCKA et al. 2005). RbAP48 is component of several chromatin-regulating complexes including histone deacetylase HDAC1, NuRD ATP-dependent chromatin remodeling complex and Polycomb group histone methyltransferase complex ESC-E(Z) (CZERMIN et al. 2002; HASSIG et al. 1997; KUZMICHEV et al. 2002; XUE et al. 1998; ZHANG et al. 1998). Future studies will test these hypotheses and help elucidate how sophisticated networks regulate HIF during development, homeostasis and diseases.

**FIGURE LEGENDS**

**Figure 1.** The *ia50;vhl-1(ok161)* animals have egg-laying defects similar to those in *egl-9* loss-of-function mutants. (A–F). Representative images of *Pnhr-57::GFP* expression in wild type and mutant animals. Fluorescent images are paired with Nomarski images of the same animal. (A,D) wild type; (B,E) *vhl-1(ok161)*; (C,F) *vhl-1(ok161), ia50*. (G, H) Representative
images of young adult wildtype (G) or vhl-1(ok161), ia50 animals (H) to illustrate the egg-laying defect. (I) Penetrance of egg-laying defect in vhl-1(ok161),ia50 mutants. (*** p<0.0001 compared to wild-type) (J) egl-9(sa307) and vhl-1(ok161),ia50 mutants have smaller brood sizes. (** p<0.01 compared to wild-type). The vhl-1(ok161) and egl-9(sa307) alleles are strong loss-of-function alleles.

**Figure 2. ia50 is an allele of swan-1.** (A) Genetic mapping of ia50. Three-point mapping with the indicated visible marker and single nucleotide polymorphisms placed ia50 to +5.67 mu on chromosome V. Cosmids injected for transformation rescue are shown. DNA sequencing revealed a point mutation at the splicing acceptor of exon 3 of swan-1. This results in failed splicing of intron 2, which introduces an early stop codon. swan-1 encodes a WD40 repeat family protein. A schematic of the SWAN-1 protein and the position of the stop codon introduced by ia50 are shown. The region deleted by the ok267 allele is also illustrated. (B) RNAi of swan-1 increased Pnhr-57::GFP protein level. RNAi of swan-2 had no effect on Pnhr-57::GFP expression.

**Figure 3. SWAN-1 regulates HIF-1 activity in animals that do not destabilize HIF-1.** (A-F) Expression of Pnhr-57::GFP and two HIF-1 target genes in wild-type and various mutants. Relative level of Pnhr-57::GFP was measured by immuno-blots. K10H10.2 and F22B5.4 mRNA levels were quantitated by real-time RT-PCR. Data were obtained from three independent experiments. * p<0.05 calculated by student t-test. (G) The level of transgenic HIF-1::HA protein were assayed in N2, swan-1(ok267), vhl-1(ok161), and swan-1(ok267);vhl-1(ok161) L4-stage worms.

**Figure 4. SWAN-1 interacts with EGL-9** (A) swan-1(ok267) did not change the Pnhr-57::GFP expression in egl-9(sa307) mutants. (B) Yeast harboring EGL-9 bait plasmids (across the top) without and with the SWAN-1 prey plasmid (see materials and methods). Yeast were grown on medium containing X-Gal to indicate lacZ activity and a positive
interaction (blue). Yeast harboring the bait plasmids or SWAN-1 alone were overgrown to ensure absence of lacZ activity. (C) GFP tagged SWAN-1 pulled down MBP tagged EGL-9. SWAN-1::GFP fusion protein or GFP protein were immuno-precipitated using worm lysate prepared from transgenic lines containing swan-1::gfp transgene or gfp transgene. The beads were then incubated with bacteria lysate expressing recombinant EGL-9-MBP protein. The input SWAN-1::GFP and GFP proteins were detected by anti-GFP antibody (right). Interacting proteins were fractionated by SDS-PAGE, western-blotted and probed with an anti-MBP antibody. Un incubated bacteria lysate was included as a control.

References


WYSOCKA, J., T. SWIGUT, T. A. MILNE, Y. DOU, X. ZHANG et al., 2005 WDR5 associates with histone H3 methylated at K4 and is essential for H3 K4 methylation and vertebrate development. Cell 121: 859-872.


Table 1: Analysis of double heterozygotes reveals a novel mutant allele \textit{ia50} that increases \textit{nhr-57}:GFP expression

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<th>phenotype in trans to \textit{egl-9(sa307)}</th>
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Table 2: SNP primers, locations and band sizes. In each pair of primers, the left primer is listed first; all primer sequences are given 5' to 3'. The Table is modified from Davis et al. (Davis et al. 2005)

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| X, 2            | 10,637,922        | F11A1 | 409, 133  | 542          | AGCAACAAAACATGCAACTATGG
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| X, 11           | 13,339,566        | F46G10 | 318, 191, 37 | 509, 37     | ACTGTTACCAGCCTCTCTGC
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| X, 17           | 14,547,382        | T24C2 | 409, 34  | 302, 107, 34 | GCTGGGATTTTGAAGAGTTGTT
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| X, 23           | 15,500,013        | H13N06 | 358, 134 | 492          | CAAATACCAAAGTTGATCGTG
|                 |                   |       |           |              | TTGTTGCAATTAATCAACGG | uCE6-1554 |
Figure 1. The *ia50;vhl-1(ok161)* animals have egg-laying defects similar to those in *egl-9* loss-of-function mutants.
Figure 2. *ia50* is an allele of *swan-1*. 
Figure 3. SWAN-1 regulates HIF-1 activity in animals that do not destabilize HIF-1.
Figure 4. SWAN-1 interacts with EGL-9
CHAPTER 6. GENERAL CONCLUSION

SUMMARY

Hypoxia-inducible factor (HIF) is the master regulator of transcriptional changes during hypoxia adaptation in *C. elegans* as well as in humans. In this dissertation, I used the nematode *C. elegans* as a model to study the role of HIF-1 in organismal aging. Major findings include: 1) HIF-1 over-expression through extra copies of a *hif-1* transgene can extend lifespan. This prolongevity effect is independent of *daf-16/FOXO* and *skn-1/NRF* transcription factors and may work downstream of the *daf-2* insulin like growth factor receptor. 2) the *hif-1* loss-of-function mutation also confers increased longevity at standard culture conditions. This longevity phenotype, unlike that of HIF-1 over-expression, is dependent on *daf-16/FOXO* and *skn-1/NRF*. 3) Both HIF-1 over-expression and *hif-1* loss-of-function mutations increase resistance to thermal stress and oxidative stress in *C. elegans*. 4) Hypoxia treatment extends lifespan in *C. elegans*. This longevity effect does not require functional HIF-1 signaling. Instead, IRE-1-mediated unfolded protein response pathway is required for hypoxia to extend lifespan.

The second part of the thesis is devoted to understand the regulatory networks that control the HIF-1 activity. Using forward genetic approaches, I identified *swan-1* as a novel negative regulator of HIF-1. Loss-of-function mutations in *swan-1* increase HIF-1 transcriptional activity in wildtype as well as *vhl-1* mutant background. However, loss-of-function mutations in *swan-1* do not enhance the egl-9 mutant phenotype, as assayed by increased expression of HIF-1 target genes. The data further demonstrated that SWAN-1 can physically interact with EGL-9. These results suggest a model in which SWAN-1 regulates HIF-1 activity via an egl-9-mediated mechanism that functions in parallel to the oxygen-dependent degradation pathway of HIF-1.
GENERAL DISCUSSION

HIF-1 modulates aging in C.elegans

Aging is a basic biological process and perhaps one of the most important factors associated with the incidence of many age-related diseases, such as cancer, diabetes and neurodegenerative diseases. Understanding the mechanisms of aging will not only shed light on the fundamental questions in biology, but also provide insights to the treatment of age-related diseases.

C. elegans, because of its powerful genetics and relatively short lifespan, has become an important model organism for the study of aging. Since the first discovery of the long-lived mutant worm in 1980s, hundreds of genes have been found to modulate aging in C. elegans (JOHNSON 2008). Among these genes, a handful of stress responsive transcription factors, including daf-16, skn-1, hsf-1 and pha-4, have been shown to be the critical regulators in the aging-regulation networks (HENDERSON and JOHNSON 2001; HSU et al. 2003; LIN et al. 2001; MORLEY and MORIMOTO 2004; PANOWSKI et al. 2007; TULLET et al. 2008).

In Chapter 2, I showed evidence that hif-1 is another stress response transcription factor that play important roles in modulating aging in C.elegans. Over-expressing HIF-1 through extra-copies of hif-1 transgene could extend life span to as much as 34% (Chapter 3 Figure 2, Table 1). HIF-1 over-expression in daf-16 or skn-1 mutation background extended the life span of mutant worms to a similar degree (Chapter 2 Figure 3, Table 2), suggesting that the prolongevity effect of HIF-1 over-expression is independent of DAF-16 and SKN-1. Interestingly, I found hif-1 loss-of-function mutations could also increase longevity at least 10% (Chapter 2 Figure 4). This longevity effect was completely abolished in daf-16 or skn-1 mutants, suggesting that HIF-1 over-expression and hif-1 loss-of-function may extend life span through distinct mechanisms.
Does HIF-1 over-expression modulate aging cell autonomously or systematically?

The genetic data in Chapter 2 leave open the possibility that hif-1 is in the downstream of daf-2 to regulate aging. However, the daf-2(1370) mutation did not cause a significant change in HIF-1 protein levels, or in the expression of HIF-1 target genes, as assayed by western-blot or qRT-PCR (Chapter 2, Figure 5). One explanation for this observation is that DAF-2 may regulate HIF-1 in specific cells or tissues to modulate aging. Indeed, prior studies have demonstrated that stress-responsive transcription factors can have non-cell-autonomous roles in organismal aging. For example, intestine-specific expression of DAF-16 completely restored the longevity of daf-16 deficient animals (LIBINA et al. 2003). Over-expressing SKN-1 in two neurons was shown to be sufficient to extend lifespan in C. elegans (BISHOP and GUARENTE 2007). Studies from Pocock and Hobert (POCOCK and HOBERT 2008) and Hoogewijs et al (HOOGEWIJS et al. 2007) suggested that DAF-16 modulates HIF-1 levels in some conditions or cell types. To define the sites where HIF-1 regulates lifespan extension in C. elegans, we can generate transgenic animals that express the hif-1 transgene in specific tissues. The lifespan and stress resistance assay of these transgenic animals will tell us whether HIF-1 controls downstream genes in specific responding cells to modulate aging.

A recent study from Chen et al showed that HIF-1 functions in specific neurons and muscles to modulate Dietary Restriction (DR) dependent lifespan extension (CHEN et al. 2009). In their study, loss-of-function mutation of hif-1 was found to extend lifespan in a nutrient-dependent manner. hif-1 loss-of-function mutant lived longer in rich nutrient condition but failed to extend lifespan under DR. Loss-of-function mutation of egl-9, which increased HIF-1 expression, abolished longevity effect of DR. Using transgenic animals that express egl-9 in various tissues under egl-9 mutant background, these authors found that
eglu-9 expression in body wall muscle and serotonergic neurons was important for HIF-1-mediated lifespan extension via DR. In addition, a recent study from Chang et al (CHANG and BARGMANN 2008) demonstrated that HIF-1 functions both in neurons and gonad endocrine cells to regulate oxygen preference in C. elegans. We predict that over-expression of HIF-1 acts in multiple tissues to modulate lifespan.

Which genes regulated by HIF-1 are important for life span extension?

HIF-1 regulates the transcriptional changes of a wide range of genes for organisms to adapt to hypoxic conditions. Prior studies in our lab have identified 63 genes that are regulated by hypoxia in a hif-1 dependent manner (SHEN et al. 2005). It is conceivable that some of them may play important roles in regulating aging. Of particular interest are those genes involved in signal transduction, metabolism, transcription and stress responses. In future studies, it would be interesting to use RNAi to knockdown these genes to test whether they are required for HIF-1 over-expression mediated life span extension.

There are several potential caveats, however, in referencing the previous genome-wide microarray study. First, as discussed above, HIF-1 may regulate gene expression in specific cells or tissues to extend life span. Some hif-1 regulated genes that are important for aging-regulation may not be reflected in samples prepared from whole animals. Second, my studies in Chapter 3 indicated that HIF-1 over-expression induced by hypoxia during larval development is insufficient to extend life span (Chapter 3 Figure 1C). It is worth noting that the samples used in previous microarray study were prepared from L3 larvae, which may not correctly reflect the transcriptional changes associated with adult life span extension. Towards the goal of identifying HIF-1 targets that modulate adult longevity, future studies could analyze the gene expression patterns of long-lived animals that over-express HIF-1. The result may be more informative in evaluating which genes might be involved in the prolongevity effects of HIF-1 over-expression.
Hypoxia modulates aging in *C. elegans*

After establishing HIF-1 as an important transcription factor that modulates aging in *C. elegans*, one intriguing question to ask is whether hypoxia influences longevity. In my PhD study, I found that hypoxia extended the adult life span of *C. elegans* by around 20% (Chapter 3 Figure 1, Table1). I also showed that hypoxia treatment during the adult stage conferred longevity in *hif-1* loss-of function mutants as well as HIF-1 over-expression transgenic worms (Chapter 3 Figure 2, Table1). These results suggested that *hif-1* is not the only signaling pathway that regulates hypoxia-mediated longevity in *C. elegans*. I further demonstrated that hypoxia can extend life span via mechanisms independent of the *daf-2* insulin like growth factor pathway (Chapter 3 Figure 3, Table 1). I presented evidence that IRE-1 mediated ER stress response is induced by hypoxia and is required for hypoxia mediated longevity effect (Chapter 3 Figure 4 and 5).

The biological significance of hypoxia

Hypoxia is important to many biological processes, including development, regeneration and animal hibernation, and is associated with a number of diseases, such as stroke, ischemia and cancer. Hypoxia tolerance varies between species and cell types, and is a major determinant in many pathological conditions. For example, hypoxia in stroke and ischemia can cause neuronal dysfunction and cell death, while tumor hypoxia is relevant to resistance to chemotherapy and radio-therapy (BERTOUT et al. 2008). In my study, hypoxia treatment delays aging and increases thermotolerance and oxidative stress resistance. On the other hand, severe hypoxia (<0.3%) at high temperatures (26°C) can result in hypoxic injury and worm death (SCOTT et al. 2002). Understanding the roles of hypoxic response signaling in different hypoxia conditions may help us to develop novel therapies for hypoxia-related diseases.
Multiple signaling pathways regulate hypoxia mediated longevity

HIF-1 regulates most of the transcriptional changes that enable organisms to adapt to hypoxia. A previous microarray study in our lab has revealed that transcriptional expression of some genes, including daf-16, is regulated by hypoxia in a hif-1-independent manner (Shen et al. 2005). The data that hypoxia can extend life span of hif-1 and daf-16 deficient animals suggest that metabolic changes responding to hypoxia are mediated by multiple signaling pathways. In my study, ER stress response pathway is clearly involved in hypoxia mediated longevity. However, whether induced ER stress response is sufficient to extend life span or just required for hypoxia tolerance still remains unclear.

There are at least two models to explain the requirement of ire-1 in hypoxia-induced lifespan extension. First, UPR components might be induced by hypoxia and act in parallel to HIF-1 to extend adult longevity. Alternatively, animals that are defective for UPR components may suffer more from hypoxic stress, offsetting the benefits of hypoxia. To help distinguish between these possibilities, future studies could test the hypothesis that over-expression of UPR components have prolongevity effects.

When considering alternative models, it is important to recall that C. elegans prefer oxygen levels in the range of 5 – 14% (Chang and Bargmann 2008). Room air is, therefore, hyperoxic. It is possible that hyperoxia suppresses longevity and that removing worms from hyperoxia (to 5% or 1% oxygen) results in less proteotoxicity and longer life, provided the UPR is intact. To test this hypothesis, we may assay lifespan of worms under 5% and 10% oxygen, and determine whether the change of lifespan, if any, depends on functional UPR.

A recent study from Chen et al (Chen et al. 2009) showed that ire-1 mutation significantly reduces the longevity effect of DR. ire-1 mutation completely suppressed the lifespan extension by hif-1 loss-of-function mutation in rich nutrient condition. These results
and ours suggested that functional UPR is required for multiple signaling pathways in modulating lifespan.

My work opens the door to new questions about hypoxia and aging. It will be important to identify the genes and pathways that mediate the hif-1-independent effects of hypoxia. It would be informative to compare the gene expression patterns of hypoxia-treated wild-type or hif-1-deficient adults with those of adult animals over-expressing HIF-1. To study the hif-1-independent signaling pathways involved in hypoxia mediated longevity, we can use gene expression analysis to identify the changes of gene expression after hypoxia treatment in adult stage. The results can then be compared with those genes regulated by HIF-1 over-expression in hif-1 transgenic worms (as discussed above) to measure which genes may modulate longevity in parallel with hif-1.

The transcriptional induction of gene expression is a major mechanism in hypoxia adaptation (Wenger 2000). However, transcriptional changes are not the only responses that help organisms to adapt to hypoxia. In mammalian cells, hypoxia can suppress mRNA translation by activating UPR (Wouters and Koritsinsky 2008). Abundant results have shown that protein homeostasis plays important roles in modulating aging (Hansen et al. 2007; Pan et al. 2007a; Syntichaki et al. 2007). Recently, Anderson et al. showed that depletion of an arginyl-transfer RNA (tRNA) synthetase results in increased resistance to high-temperature hypoxia treatment. This hypoxia resistance phenotype required functional UPR, suggesting that the role of UPR in suppressing translation may be conserved in C.elegans (Anderson et al. 2009). Therefore, an intriguing model is that hypoxia may extend lifespan in C.elegans by decreasing translation rate through UPR.

Oxygen deprivation of mammalian cells also represses mRNA translation by inhibiting the TOR (Target of Rapamycin) pathway (Wouters and Koritsinsky 2008). In C. elegans, inhibition of the TOR pathway extends lifespan and produces autophagy (Hansen et al. 2008; Jia et al. 2004; Vellai et al. 2003). Recent studies suggested that TOR signaling pathway plays important role in DR-dependent lifespan extension (Chen et al.
Hence, it is worthwhile to test whether hypoxia can also inhibit TOR pathway in *C. elegans*, and whether the inhibited TOR activity accounts for the life span extension induced by hypoxia.

**SWAN-1 regulates HIF-1 in *C. elegans***

Using a visual assay based genetic screen, prior lab mates have isolated an array of mutant alleles that increase the expression of a HIF-1 target gene (Shen et al. 2006). In Chapter 5, I showed that some of these mutations mapped to *rhy-1*, and one identified a novel regulator of HIF-1. The *ia50* mutant allele was mapped to *swan-1(F53C11.8)*, which encodes a WD repeat protein. The *ia50* allele harbors a G to A conversion at the splicing acceptor site of intron 2. This results in a mis-spliced mRNA that includes an early stop codon and may encode a truncated protein with only 2 of the 8 WD motifs. This molecular characterization and additional genetic analyses show that *is50* is likely a loss-of-function mutation of *swan-1*. My genetic data suggest that SWAN-1 regulates HIF-1 via a mechanism distinct from the VHL-1 mediated degradation pathway: 1) the HIF-1 dependent *Pnhr-57::GFP* reporter is expressed in distinct patterns and different levels in *swan-1* mutants and *vhl-1* deficient animals (Chapter 4 Figure 1); 2) HIF-1 protein levels do not change in *swan-1* deficient worms, while the *vhl-1* mutation causes around 3-5 fold increase in HIF-1 protein levels (Chapter 4 Figure 3) (Shao et al. 2009; Shen et al. 2006); 3) Worms that carry loss-of-function mutations in both *swan-1* and *vhl-1* express significantly higher levels of HIF-1 target genes compared with each single loss-of-function mutant (Chapter 4 Figure 3).

**The dual functions of EGL-9***

EGL-9 is the enzyme that catalyzes the hydroxylation of HIF-1, which allows its degradation in normoxia (Epstein et al. 2001). Hence, EGL-9 clearly serves as an oxygen sensor in
regulating HIF-1 stability. Prior studies indicate that in addition to its role in controlling HIF-1 protein level, EGL-9 also inhibits HIF-1 transcriptional activity (Shen et al. 2006). Other members in our group has tested this hypothesis rigorously and shown that the function of EGL-9 in attenuating HIF-1 transcriptional activity did not require its enzymatic activity of hydroxylation (Shao et al. 2009). However, the molecular basis of this inhibition is still unclear. My findings that swan-1 mutation failed to increase HIF-1 activity in egl-9 mutant background and that SWAN-1 interacted with EGL-9 physically both support a model in which EGL-9 inhibits HIF-1 activity at least in part through interacting with SWAN-1.

**How does swan-1 regulate HIF-1 activity?**

WD-repeat proteins form a large family with diverse functions. Many WD-repeat proteins have been found to serve as a platform to facilitate formation of large protein complexes (Li and Roberts 2001). SWAN-1 was first identified in a yeast two-hybrid screen searching for the proteins interact with the Rac GTPase effector UNC-115/abLIM (Yang et al. 2006). Further studies indicated that SWAN-1 also interacted with Rac GTPase (Rac-2) and inhibited Rac GTPase function in cell morphogenesis and cytoskeletal organization (Yang et al. 2006). Our data that SWAN-1 physically interacted with EGL-9 indicated that SWAN-1 may participate in multiple pathways through binding with its partners.

Previous experiments using the SWAN-1::GFP transgene suggested that SWAN-1 is expressed in most of the cells except the intestine and that it predominantly locates in the cytoplasm. Intriguingly, in vhl-1 loss-of-function mutant, HIF-1 reporter Pnhr-57::GFP express predominantly in intestine. swan-1, vhl-1 double mutant has a broader expression pattern of Pnhr-57::GFP. It is enticing to speculate that SWAN-1 might form a complex with HIF-1 and sequester HIF-1 from localizing to nucleus. To this end, co-immuno precipitation (co-IP) experiment using SWAN-1::GFP transgene can not detect the interaction between
SWAN-1 and HIF-1. However, it is possible that their interaction is limited in specific tissue or not strong enough to be measured by our assay.

Another attractive model of how SWAN-1 regulates HIF-1 is that SWAN-1 may repress the expression of HIF-1 target genes. Many WD-repeat proteins have been found to involve in chromatin remodeling and gene regulation (Suganuma et al. 2008b). One approach to test this hypothesis is to use chromatin immunoprecipitation (CHIP) assay to measure whether SWAN-1 resides in the vicinity of HIF-1 target genes on chromosomes.

Further studies of SWAN-1 may shed light on some of the key questions in HIF-1 signaling. How does EGL-9 repress HIF-1 activity? Is HIF-1 differentially regulated by VHL-1, EGL-9, or SWAN-1 in different tissues? Do SWAN-1, EGL-9, and RHY-1 act through a common pathway to repress the expression of HIF-1 target genes, or do they act in parallel? Which of these regulatory mechanisms are evolutionarily conserved? Screens for SWAN-1-interacting proteins and novel screens for genes mutable to the vhl-1, swan-1 double mutant phenotype are promising approaches to these important questions.

REFERENCES


SHEN, C., Z. SHAO and J. A. POWELL-COFFMAN, 2006 The Caenorhabditis elegans rhy-1 gene inhibits HIF-1 hypoxia-inducible factor activity in a negative feedback loop that does not include vhl-1. Genetics 174: 1205-1214.


Appendix I. Co-immunoprecipitation of HIF-1 interacting proteins from *Caenorhabditis elegans*

Hypoxia inducible factor (HIF) transcription factor is the master regulator that mediates most of the transcriptional changes that enable adaptation to oxygen deprivation. The heterodimeric HIF complex consists of two subunits, both of which can interact with DNA and contain bHLH and PAS domains. The complex binds DNA in a sequence-specific manner [Note: the monomers do not]. While HIFβ interacts with other bHLH-PAS proteins, HIFα subunit is acutely regulated by cellular oxygen levels and is dedicated to oxygen homeostasis. The stability of the HIF α subunit is regulated by the EGLN/pVHL pathway: when cellular oxygen is high enough, HIF-α is hydroxylated by EGLN enzymes, which are 2-oxoglutarate dioxygenases. This modification in turn allows HIF-α to be recognized by pVHL, an ubiquitin E3 ligase, which poly ubiquitinates HIF α and targets it for proteosomal degradation. This oxygen-dependent degradation pathway is conserved in humans and *C. elegans*. The orthologs of HIF-α, HIF-β, EGLN and VHL in *C. elegans* are *hif-1, aha-1, egl-9* and *vhl-1*, respectively (Epstein et al. 2001; Jiang et al. 2001; Powell-Coffman et al. 1998).

Our prior studies support a model in which EGL-9 has two functions (Shao et al. 2009; Shen et al. 2006). First, EGL-9 hydroxylates HIF-1 in an oxygen-dependent manner which enables HIF-1 to bind VHL-1 and be degraded in normoxia. Second, EGL-9 inhibits the expression of HIF-1 target genes by an unknown mechanism that does not require EGL-9 hydroxylase activity and does not involve VHL-1.

How might EGL-9 repress expression of HIF-1 target genes? One possibility is that EGL-9 is present in a complex of HIF together with some transcription repressors. Indeed, it was proposed that in mammalian cells EGLN1 could recruit the ING4 transcription repressor
to the promoter of HIF target genes (Ozer and Bruick 2005). Alternatively, EGL-9 might be involved in some other modifications of HIF-1 that regulate its localization or activity. It is also possible that EGL-9 might have other substrate(s) that in turn attenuates HIF-1 activity. In order to gain insight to HIF-1 regulation and function, I attempted to define HIF-1 complexes through co-immunoprecipitation (co-IP).

In order to identify the HIF-1 associating proteins with co-immunoprecipitation (co-IP), I first generated a construct in which an epitope tag was fused to hif-1 (Figure 1 A and also described in Chapter 2). Additional constructs and strains are listed in Table 1. The original design of this epitope enabled the tandem affinity purification of HIF complexes through myc, strep and HA tags. However, pilot experiments demonstrated that the binding efficacy of HA or strep tags were too low to be used in purification (data not shown). Therefore, I opted for one step purification in which I used the myc tag to immunoprecipitate transgenic HIF-1 protein, followed by tobacco etch virus (TEV) protease digestion to release the HIF-1 complex from the beads.

The epitope tagged hif-1 construct was inserted into the genome of the worms by gold-particle bombardment (Praitsis et al. 2001). Stable transgenic lines carrying epitope-tagged hif-1 were crossed to vhl-1(ok161) or egl-9(sa307) mutant backgrounds and the HIF-1 protein levels were assayed by western-blot. In agreement with the result of endogenous HIF-1, vhl-1(ok161) or egl-9(sa307) mutations increased epitope-tagged HIF-1 levels by about 4-fold (Epstein et al. 2001; Shen et al. 2006) (Figure 1 B). The activity of HIF-1 was also measured by detecting the expression of a HIF-1-regulated GFP reporter gene (Shen et al. 2006). Epitope-tagged HIF-1 rescued the expression of the Pnhr-57:GFP reporter in a strain carrying a deletion in the endogenous hif-1 gene (Figure 2). Moreover, we found that the egl-9(sa307) mutation had a much greater effect on the expression of Pnhr-57:GFP than did the vhl-1(ok161) mutation. This confirmed that HIF-1:myc was regulated by both the VHL-1/EGL-9 pathway for degradation and the VHL-1-independent pathway by which EGL-9 represses HIF-1 activity.
Using this transgenic strain, I did some pilot co-immunoprecipitation experiments to test whether the HIF complex could bind to the myc antibody coupled sepharose beads and then be released by protease digestion. Indeed, endogenous AHA-1 was co-immunoprecipitated and also released after protease digestion together with epitope-tagged HIF-1, which indicated that at least the prototypic HIF complex was preserved in the assay (Figure 1 C).

To generate sufficient starting materials for co-IP, I optimized large-scale production of nematode transgenic lines using enriched peptone plates (1.2g NaCl, 20g peptone, 25g agar in 1L water. Autoclave. Cool to 55ºC, then add 1ml of 5mg/ml cholesterol, 1ml of 1M MgSO_4 and 25ml of 1M potassium phosphate, pH 6.0) spotted with NA22 bacteria lawn. The transgenic line was grown at 20ºC on 4 100mm enriched peptone plates until most of the worms were starved L1 larvae. The worms were then washed off the plates using cold M9 buffer and aliquotted onto 40 100mm enriched peptone plates. Two days later, the worms were harvested using cold M9 buffer and centrifuged at 2000× g. Typically, 10 grams of worms (around 10 ml worm pellet) could be collected from 40 100mm plates. The worm pellet was washed twice in cold M9 buffer and then resuspended in 40 ml lysis buffer [100 mM PIPES, pH 6.0, 100 mM NaCl, 3 mM MgCl_2, 1 mM EGTA, a mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% Triton X-100, Complete Protease Inhibitor Tablets (Roche), and phosphatase inhibitors (5 mM NaF and 1 mM Na_3VO_4)]. The mixture could then be frozen down in 10 ml aliquots in liquid nitrogen and stored at -80ºC.

To generate whole worm extracts, a single freeze/thaw cycle was performed before the suspension was lysed by passing through a French Press cell disrupter (Thermo Electron Corporation) at 8000 psi three times (Albertha et al., 2006). The mixture was clarified by centrifugation at 16000× g for 20 minutes at 4ºC and the soluble supernatant fraction was collected.

To prepare Mab9E10 (myc)-coupled protein G beads, 1 mL of sepharose Protein G Beads (ZYMED laboratories) (sepharose beads show less non-specific binding compared to
agarose beads) was incubated at 20°C with mouse ascite (2 mg of protein, from Developmental Studies Hybridoma Bank) for 2 hours, and washed with 10 volumes of 0.2 M sodium borate, pH 9.0. Antibody bead coupling was performed by adding 2mM dimethylpimelimidate dihydrochloride at 20°C for 30 min (POLANOWSKA et al. 2004). The coupling reaction was stopped by washing with 5 volumes of 0.1 M Tris-HCl, pH 8.0, and 10 volumes of phosphate buffered saline (PBS). The coupled beads were stored at 4°C in two volumes of PBS with 0.5% sodium azide. Prior to co-IP, the beads were washed with Binding buffer (50 mM Tris-HCl, pH 7.3, 0.1 M KCl, 1mM EDTA and 10% glycerol).

To perform co-IP, worm lysate prepared from each transgenic line was incubated with c-myc antibody coupled sepharose beads at 50 uL of beads per mL extract. ProteinG-sepharose beads without coupling of myc antibody, where most of the none-specific binding came from, were used as controls. The absorption was done at 4°C for 6 h. The beads were then washed with binding buffer 5 times before they were aliquotted to separate tubes at 100 uL slurry (about 50 uL beads) per tube. The cleavage reaction was performed by adding 2 uL 0.1 M DTT and 2 uL AcTEV (10U/uL) (Invitrogen) to each tube and incubated at 4°C overnight with gentle shaking (for some unknown reason, I found digestion result was much better in small reaction volume). The slurry was then transferred to a Micro bio-spin column (Bio-rad). The elution was collected by low speed centrifugation.

The proteins eluted from protease digestion were concentrated by Trichloroacetic Acid (TCA) precipitation. One fifth volume of 100% TCA and 10 ug insulin (Sigma, 5 ug/uL in H2O) (to help precipitate low amount of proteins, optional) was added to elution and incubated on ice for 30 minutes. The precipitation was harvested by centrifugation at 16000× g and washed with 300 uL cold acetone. The precipitation was then vacuumed dry and dissolved in 10 uL 1M Tris-HCL (pH8.0) and 10 uL 2× SDS-PAGE buffer [100mM Tris-HCl pH6.8, 20%(v/v) glycerol, 4% (w/v) SDS, 200mM DTT, 0.2% (w/v) bromphenol blue]. The sample was resolved on 10% SDS-PAGE gel and stained with Novex Colloidal Blue Staining Kit (Invitrogen). Bands were then excised from the gel and subjected to
LS-MS/MS Mass spectrometry analysis by TSQ Vantage Triple Quardrupole mass spectrometer (Thermo Scientific) at UC Davis Proteomic Core Facility.

To this end, several candidate proteins have been identified from one experiment (Table 2). The results from the proteomic analysis need to be confirmed by genetic and biochemical experiments to reveal the biological significance of the interaction.

Collaborating with Mark Roth’s research group, we have generated a monoclonal antibody against HIF-1. This antibody provides opportunity to immunoprecipitate the endogenous HIF-1 complex. One potential caveat of this approach is that monoclonal antibody may recognize the epitope that interacts with HIF-1 associated proteins and thereby fail to immunoprecipitate the HIF-1 complex. Another concern is that IgG protein will impose a heavy background due to the lack of method to release HIF-1 complex off the beads. Nevertheless, HIF-1 monoclonal antibody can be used in co-IP experiments to confirm the results from our proteomic analysis. Similarly, the AHA monoclonal antibody could be used to IP the HIF-1 transcription complex, and interacting proteins in a wild-type animal could be compared to those in a hif-1 mutant.

**FIGURE LEGENDS**

**Figure 1. Identify HIF-1 interacting proteins through co-immunoprecipitation.** (A) Diagram of the epitope-tagged *hif-1*. Detailed diagram of epitope tag is also illustrated. (B) epitope-tagged HIF-1 transgene is functional in vivo. Western-blot comparison of the *vhl-1(ok161)* and *egl-9(sa307)* phenotypes in *hif-1(ia04)* mutant background indicates that HIF-1::myc is appropriately regulated by these genes: both mutations increase HIF-1::myc protein to similar levels but the *egl-9(sa307)* mutation derepresses expression of the Pnhr-57::GFP reporter to a much higher level. (C) Western-blot shows a result of a co-IP experiment of HIF-1 associating proteins with c-myc antibody coupled sepharose beads.
Lysate prepared from *vh1-1(ok161)* worms carrying the *Phif-1::hif-1::tag* transgene was loaded on c-myc antibody coupled sepharose beads. The HIF complex binding to the beads was released using AcTEV protease digestion. AHA-1, the partner of HIF-1, was present in the elution while TUBULIN was absent. AHA-1 was detected by mouse monoclonal antibody 10H8 (JIANG et al. 2001); α-TUBULIN was detected by mouse monoclonal antibody clone DM1A (Sigma). (D) Elutions from co-IP experiments was concentrated by TCA and resolved on 10% PAGE gel. After coomassie-blue staining, bands specifically appeared on transgenic line were excised and subject to MALDI-Mass Spectrometry analysis. One band was identified as RFP-1.

**REFERENCES**


SHEN, C., Z. SHAO and J. A. POWELL-COFFMAN, 2006 The Caenorhabditis elegans rhy-1 gene inhibits HIF-1 hypoxia-inducible factor activity in a negative feedback loop that does not include vhl-1. Genetics **174**: 1205-1214.
### Table 1: Epitope-tagged HIF-1 transgenes.

<table>
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<th>Plasmid name</th>
<th>Description</th>
<th>Epitope tag</th>
<th>Original strains</th>
<th>Reference</th>
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<tr>
<td>pFOX1</td>
<td>Phif-1::hif-1a</td>
<td>6× myc</td>
<td>ZG417</td>
<td>Zhang et al., 2009</td>
</tr>
<tr>
<td>pFOX4</td>
<td>Phif-1::hif-1a (P621G)</td>
<td>6× myc</td>
<td>ZG550, ZG552, ZG554, ZG556</td>
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<tr>
<td>pFOX5</td>
<td>Phif-1::hif-1a (ΔODD)</td>
<td>6× myc</td>
<td>ZG436, ZG440, ZG441</td>
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<td>HA+6× myc+strep</td>
<td>ZG429</td>
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<tr>
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<td>HA+6× myc+strep</td>
<td>ZG584, ZG590</td>
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<tr>
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<td>HA+6× myc+strep</td>
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### Table 2. Mass-spec results.

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<th>Probability</th>
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<tr>
<td>cyp-13A7 (cytochrome P450 homolog)</td>
<td>T10B9.10</td>
<td>59</td>
<td>98%</td>
</tr>
<tr>
<td>hh-11</td>
<td>F58A4.7a</td>
<td>46</td>
<td>90%</td>
</tr>
<tr>
<td>teg-1 (tumorous Enhancer of glp-1, uncharacterized, protein-protein interaction)</td>
<td>Y47D3A.27</td>
<td>42</td>
<td>85%</td>
</tr>
<tr>
<td>hgo-1 (Homogentisate Oxygenase)</td>
<td>W06D4.1</td>
<td>49</td>
<td>75%</td>
</tr>
<tr>
<td>acs-18 (fatty acid CoA synthetase family)</td>
<td>R09E10.3</td>
<td>79</td>
<td>72%</td>
</tr>
<tr>
<td>crn-3 (cell death related nuclease)</td>
<td>C14A.4</td>
<td>68</td>
<td>71%</td>
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APPENDIX II. ADDITIONAL RESULTS

Does HIF-1 over-expression regulate mitochondria to extend lifespan?

In Chapter 3, I demonstrated that over-expression of HIF-1 protein can extend lifespan in *C. elegans*. However, the mechanisms behind this longevity effect remained unknown. Given that hypoxia-inducible transcription factors mediate a critical cellular metabolic adaptation to hypoxia, it is conceivable that a similar metabolic change activated by HIF-1 may contribute to the lifespan extension.

Studies from Dang and Denko groups demonstrated that in mammalian cells, HIF1 actively suppresses aerobic metabolism through the tricarboxylic acid cycle (TCA) by directly trans-activating the gene pyruvate dehydrogenase kinase 1 (PDK1) (Kim et al. 2006; Papandreou et al. 2006). PDK1 phosphorylates and inhibits pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl-CoA and fuel the mitochondrial TCA cycle. The inhibition of TCA cycle and sequentially oxidative phosphorylation results in attenuation of reactive oxygen species (ROS) generation (Kim et al. 2006). If this pathway is conserved in *C. elegans*, it may account for the lifespan extension mediated by HIF-1 over-expression by decreasing ROS production.

Indeed, one homolog of PDK, *pdhk-2 (ZK370.5)*, is identified in *C. elegans*. Down-regulating *pdhk-2* by RNAi abrogated the longevity effect of HIF-1 over-expression (Figure 1, Table 1). To test the hypothesis that HIF-1 over-expression suppressed PDK phosphorylation, I employed an antibody (Novus Biological) that recognizes phosphorylated PDH to measure the phosphorylation level of PDH. My results showed that phosphorylation of PDH actually decreased in HIF-1 over-expressing worms (Figure 1 B). These results indicate that HIF-1 over-expression may alter the mitochondrial metabolism in *C. elegans*.
However, relationship between the mitochondrial metabolic change and lifespan extension is complicated.

**hif-1 is involved in SIR2 pathway to modulate aging in C. elegans.**

SIR2 was first identified in yeast as a heterochromatin component that is associated with gene silencing at mating loci and telomeres (IVY et al. 1986). The proteins encoded by yeast SIR2 and its orthologs form a NAD-dependent protein deacetylase family that mediates the chromatin silencing (BLANDER and GUARENTE 2004; BRACHMANN et al. 1995; IMAI et al. 2000). SIR2 genes have been shown to modulate aging in *Saccharomyces cerevisiae*, *C.elegans* and *Drosophila melanogaster* (KAEBERLEIN et al. 1999; ROGINA and HELFAND 2004; TISSENBAUM and GUARENTE 2001). Transgenic worms carrying extra copies of sir-2.1 live longer. This longevity effect of SIR2 over-expression is daf-16 dependent (TISSENBAUM and GUARENTE 2001). It has been proposed that SIR-2.1 interacts with 14-3-3 protein to regulate the nuclear localization of DAF-16 upon stress (BERDICEVSKY et al. 2006; WANG et al. 2006).

To test whether *hif-1* is involved in life span extension mediated by SIR2 over-expression, I assayed the life span of *hif-1(ia04);sir-2.1(geIn3)* and *vhl-1(ok161);sir-2.1(geIn3)* worms (geIn3 represents the chromosome integration of multiple copies of sir-2.1 transgene). I found that *hif-1(ia04)* suppressed the pro-longevity effect of *sir-2.1(geIn3)* while *vhl-1(ok161)* mutation further extended the life-span of long-lived *sir-2.1(geIn3)* worms (Figure 2, Table 2). These results suggest that *hif-1* is required for *sir-2.1*-mediated life span extension.
A role of autophagy in lifespan extension mediated by HIF-1 over-expression and hypoxia.

Autophagy is a process in which a cell degrades and recycles macro-molecules and organelles for energy and building blocks. During autophagy, portions of the cytoplasm are wrapped in double-membrane vesicles, called autophagosomes, which then fuse with lysosomes to degrade their contents. The degraded products are subsequently recycled to the cytoplasm (LEVINE and KLIONSKY 2004).

Recent studies have demonstrated that autophagy plays important roles in life span regulation (TOTH et al. 2008). Inhibiting autophagy by RNA interference (RNAi) of bec-1, which is critical for initiation of autophagosome, suppressed the longevity phenotype of daf-2 mutant worms (MELENDEZ et al. 2003). Dietary restriction also required functional autophagy to extend lifespan in C.elegans (HANSEN et al. 2008; JIA and LEVINE 2007).

Hypoxia has been shown to be one of the strongest signals to trigger autophagy. In mammalian cells, increased HIF-1 induces autophagy through . Recent study

We tested the hypothesis that autophagy is involved in the lifespan extension mediated by HIF-1 over-expression and hypoxia. As shown in Figure 3B and Table 3, down-regulating bec-1 by RNAi suppressed life span extension mediated by HIF-1 over-expression. Similarly, inhibiting autophagy by bec-1 RNAi abrogates the pro-longevity effect of hypoxia (Figure 3D, Table 4). These results demonstrated that autophagy is required for HIF-1 over-expression as well as hypoxia mediated life span extension.

Figure Legends

Figure 1. pdhk-2 is involved in lifespan extension mediated by HIF-1 over-expression. (A)RNA interference of pdhk-2, a homolog of pyruvate dehydrogenase kinase (PDK),
suppresses longevity effect of HIF-1 over-expression. (B) The level of phosphorylated pyruvate dehydrogenase (PDH) decreases in HIF-1 over-expression worms.

**Figure 2. hif-1 is involved in SIR2 pathway to extend the lifespan in *C.elegans*.** hif-1(ia04) suppresses the long-lived phenotype of sir-2.1 over-expression worms while vhl-1(ok161) mutation further extends the life span of sir-2.1 over-expression worms. The proportion of animals alive is plotted against adult age. Lifespan values are given in Table1; assays were carried out at 20ºC.

**Figure 3. Autophagy is required for lifespan extension mediated by HIF-1 over-expression and hypoxia.** (A, B) bec-1 RNAi suppresses long-lived phenotype of HIF-1 over-expression worms. (C, D) bec-1 RNAi suppresses pro-longevity effect of hypoxia. The proportion of animals alive is plotted against adult age. Lifespan values are given in Table1; lifespan assays of HIF-1 over-expression were carried out at 20ºC. Hypoxia lifespan assays were carried out at 25ºC.

**References**


Table 1: Effects of pdhk-1 RNAi on the longevity of wildtype and HIF-1 over-expression worms.

<table>
<thead>
<tr>
<th>strain</th>
<th>Adult life span mean ±S.E.</th>
<th>Maximum lifespan</th>
<th>Percentage v. control</th>
<th>n</th>
<th>p† value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 (L4440 RNAi)</td>
<td>17.1 ± 0.6</td>
<td>24</td>
<td></td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>IaIs32 (L4440 RNAi)</td>
<td>23.3 ± 0.5</td>
<td>32</td>
<td>36</td>
<td>48</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>N2 (pdhk-2 RNAi)</td>
<td>20.4 ± 0.6</td>
<td>26</td>
<td></td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>IaIs32 (pdhk-2 RNAi)</td>
<td>19.3 ± 0.7</td>
<td>26</td>
<td>-4</td>
<td>40</td>
<td>0.40</td>
</tr>
<tr>
<td>N2 (L4440 RNAi)</td>
<td>16.8 ± 0.8</td>
<td>28</td>
<td></td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>IaIs32 (L4440 RNAi)</td>
<td>22.8 ± 0.8</td>
<td>30</td>
<td>36</td>
<td>44</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>N2 (pdhk-2 RNAi)</td>
<td>20.1 ± 0.5</td>
<td>26</td>
<td></td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>IaIs32 (pdhk-2 RNAi)</td>
<td>21.9 ± 0.4</td>
<td>26</td>
<td>9</td>
<td>38</td>
<td>0.54</td>
</tr>
<tr>
<td>N2 (L4440 RNAi)</td>
<td>19.4 ± 0.8</td>
<td>28</td>
<td></td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>IaIs32 (L4440 RNAi)</td>
<td>23.1 ± 0.5</td>
<td>28</td>
<td>19</td>
<td>42</td>
<td>0.015</td>
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<tr>
<td>N2 (pdhk-2 RNAi)</td>
<td>20.7 ± 0.6</td>
<td>28</td>
<td></td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>IaIs32 (pdhk-2 RNAi)</td>
<td>19.5 ± 0.8</td>
<td>28</td>
<td>-6</td>
<td>43</td>
<td>0.29</td>
</tr>
</tbody>
</table>

These lifespan assays were performed at 20°C.

† The p value was calculated by log-rank test as a comparison to N2 worms treated with control RNAi (L4440).

Table 2: hif-1 is involved in the longevity of SIR2 over-expression worms.

<table>
<thead>
<tr>
<th>strain</th>
<th>Adult life span mean ±S.E.</th>
<th>Maximum lifespan</th>
<th>Percentage v. control</th>
<th>n</th>
<th>p† value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIR2.1 O/E</td>
<td>23.9 ± 1.0</td>
<td>38</td>
<td></td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>SIR2.1O/E;hif-1(ia04)</td>
<td>19.6 ± 1.1</td>
<td>32</td>
<td>-18</td>
<td>34</td>
<td>0.008</td>
</tr>
<tr>
<td>SIR2.1O/E;vhl-1(ok161)</td>
<td>28.0 ± 1.8</td>
<td>48</td>
<td>17</td>
<td>44</td>
<td>0.005</td>
</tr>
<tr>
<td>SIR2.1 O/E</td>
<td>22.0 ± 0.8</td>
<td>36</td>
<td></td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>SIR2.1O/E;hif-1(ia04)</td>
<td>19.5 ± 0.7</td>
<td>30</td>
<td>-11</td>
<td>35</td>
<td>0.027</td>
</tr>
</tbody>
</table>
**Table 3: Effects of bec-1 RNAi on the longevity of HIF-1 over-expression worms.**

<table>
<thead>
<tr>
<th>strain</th>
<th>Adult life span mean ±S.E.</th>
<th>Maximum lifespan</th>
<th>Percentage v. control</th>
<th>n</th>
<th>p† value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 (L4440 RNAi)</td>
<td>18.4 ± 0.7</td>
<td>26</td>
<td></td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>lal32 (L4440 RNAi)</td>
<td>23.3 ± 0.6</td>
<td>30</td>
<td>27</td>
<td>46</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>N2 (bec-1 RNAi)</td>
<td>17.1 ± 0.5</td>
<td>22</td>
<td></td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>lal32 (bec-1 RNAi)</td>
<td>17.5 ± 0.5</td>
<td>24</td>
<td>2</td>
<td>46</td>
<td>0.51</td>
</tr>
</tbody>
</table>

† The p value was calculated by log-rank test as a comparison to N2 worms treated with RNAi.

**Table 4: Effects of bec-1 RNAi on the longevity of hypoxia treatment.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hypoxia life span mean ±S.E. (n)</th>
<th>Nomoxia life span mean±S.E. (n)</th>
<th>Percentage v. normoxia</th>
<th>p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 (control RNAi)</td>
<td>18.0 ± 0.3 (43)</td>
<td>15.5 ± 0.4 (33)</td>
<td>16</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>N2 (control RNAi)</td>
<td>18.9 ± 0.4 (48)</td>
<td>16.5 ± 0.3 (47)</td>
<td>15</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>bec-1(RNAi)</td>
<td>14.5± 0.4 (38)</td>
<td>14.6 ± 0.4 (41)</td>
<td>0</td>
<td>0.96</td>
</tr>
<tr>
<td>bec-1(RNAi)</td>
<td>15.5± 0.3 (48)</td>
<td>14.9 ± 0.3 (51)</td>
<td>4</td>
<td>0.10</td>
</tr>
</tbody>
</table>

† The p value was calculated by log-rank test as a comparison between worms cultured in hypoxia and those cultured in normoxia.
Figure 1. A role of pdhk-2 in lifespan extension mediated by HIF-1 over-expression.

Figure 2. *hif-1* interacts with *sir2.1* in modulating aging.

Figure 3. Autophagy is required for lifespan extension induced by HIF-1 over-expression.
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