The regulation of hypoxia-inducible factor (HIF-1) and the role of HIF-1 in *C. elegans* longevity

By

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CHAPTER 1: GENERAL INTRODUCTION

Oxygen is one of the most abundant elements on the earth. It is the final electron receptor of aerobic respiration, which is an essential energy generation process in living creatures. Oxygen homeostasis is crucial for the survival and function of cells (Semenza, 2000). In animals, the oxygen levels exposed to each individual cell are systemically controlled by the respiration system and circulatory system.

Hypoxia-inducible factor (HIF) transcription factors play key roles in oxygen homeostasis from invertebrate *C. elegans* to human beings. HIF is a heterodimeric transcription factor, consisting of an alpha subunit that is regulated by oxygen and a beta subunit (also termed ARNT) that can partner with related DNA-binding transcription factor proteins. Vascular endothelial growth factor, one of the earliest identified HIF-1 transcriptional targets, plays a central role in angiogenesis in vertebrate. In addition to promoting the formation of blood vessels for oxygen delivery, HIF also regulates erythropoietin that controls red blood cell production**.** Besides these genes, more than one hundred others have been identified to be regulated by mammalian HIF during oxygen deprivation (Elvidge et al., 2006; Manalo et al., 2005). Insufficient expression of HIF is associated with cardiovascular diseases (Semenza, 2000). On the other hand, overexpression of HIF also promotes metastasis of many cancers by providing oxygen and nutrients for fast dividing tumor cells (Semenza, 2009).

C. elegans has proven to be a good model system to study HIF (Epstein et al., 2001; Shao et al., 2009; Shen et al., 2005). *C. elegans* HIF-1 was first identified by Jiang et al. in our group in

2001 (Jiang et al., 2001b). It is regulated by evolutionarily conserved pathways (Epstein et al., 2001). Since then, we have focused on studying the regulation of HIF-1. In this thesis I describe collaborative research to understand the regulation and function of HIF-1 in *C. elegans*. First we found SKN-1/Nrf, the transcriptional regulatory phase II detoxification network regulator, negatively regulated HIF-1 protein stability. Later we demonstrated that SKN-1 activated *egl-9* transcription, thereby attenuating HIF-1 protein levels and HIF-1 activity. EGL-9 is HIF-1 prolyl hydroxylase and can hyproxylate HIF-1 at a conserved proline residue. The hydroxylation of HIF-1 by EGL-9 is essential for HIF-1 degradation in normoxia. In my second study, I found that HIF-1 mediated the life span extension associated with *clk-1* knockout. *clk-1* is a mitochondrial gene and encodes an enzyme which is responsible for the final step of CoQ9 synthesis.

THESIS ORGANIZATION

This thesis begins with a general introduction chapter which describes the background information related to the research for this dissertation. Chapter 2 is a literature review that contains 3 sections: HIF-1 and hypoxia signaling network; *C. elegans* transcription factors that mediate stress response and function in aging; and the role of mitochondria in the regulation of HIF. The chapter 3 contains a manuscript submitted to "*Developmental Biology*" entitled "Genome-wide RNA interference screen identifies SKN-1 / Nrf as a regulator of the HIF-1 hypoxia-inducible factor in *C. elegans*." In this manuscript, Zhiwei Zhai, Zhiyong Shao and Yi Zhang completed the genome-wide RNAi screen. Zhiwei Zhai and Zhiyong Shao further characterized 179 genes and generated Table 3_1. Zhiwei Zhai assayed the *Pnhr-57:GFP* expression and HIF-1 protein by RNAi in Figure 3_2 and Figure 3_3B. Dr. Jo Anne Powell-

Coffman discovered the conserved SKN-1 regulatory sequence on *egl-9* promoter. Dingxia Feng made *Pegl-9:gfp* constructs and Zhiyong Shao generated the transgenic animals with the constructs. Dingxia Feng did the experiments for Figure 3_3A and Figure 3_4 to Figure 3_8. Zhiwei Zhai prepared the whole manuscript for the publication. Dr. Jo Anne Powell-Coffman helped on the structure and revision of the manuscript. The chapter 4 is made up of a manuscript, entitled "The role of HIF-1 in longevity associated with mitochondria malfunction in *C. elegans*". Finally, chapter 5 contains a general conclusion.

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CHAPTER 2: LITERATURE REVIEW

HIF-1 AND HYPOXIA SIGNALING NETWORK

Molecular oxygen and hypoxia inducible factor (HIF)

In 1774 Joseph Priestley isolated an "air" that appeared to be completely new. He also proved the importance of this "new air" for animal life by placing a burning candle and a mouse together in a bell jar. The poor rodent showed deleterious effects when the candle consumed almost all the new "air" (Simon and Keith, 2008). Later this new air was shown to be oxygen, which is one of most abundant elements on the earth. 21% of room air is oxygen (O2), which serves as the terminal electron acceptor in the reactions that metabolize glucose to generate energy for life (Webster, 2007). The delivery of oxygen from the atmosphere to individual cells is a long journey. It involves in the respiration and circulatory systems' activities. The exposure level to oxygen is around 2-9% for individual cells in a mammal (Simon and Keith, 2008). For most cells, oxygen levels below 2% are hypoxic, meaning that the cells have to adapt to oxygen deprivation.

The cellular transcription profile is altered by hypoxia (Semenza, 1999; Shen et al., 2005). One of the major players in this transition is called hypoxia-inducible factor (HIF). HIF is a group of heterodimeric transcriptional factors (Jiang et al., 1996; Wang et al., 1995; Wang and Semenza, 1995). In mammals HIF consists of one of three subunits (HIF-1α, HIF-2α or HIF-3α) and one HIFβ subunit (ARNT or ARNT2). Each subunit belongs to the basic helix loop helix-

PAS (Per/Arnt/Sim) family. The HIFβ subunit is also known as aryl hydrocarbon receptor nuclear translocator (ARNT).

In 2001 Jiang et al. first identified HIF-1 in *C. elegans* (Jiang et al., 2001b). Nematodes *C. elegans* consists of 959 somatic cells in the adult hermaphrodite. There is no complex circulatory system in nematodes. Each cell is only a few cell widths from the gut or the cuticle, which presumably makes it easier for the oxygen to access each individual cell. *C. elegans* HIF-1 is a homolog of HIFα subunit in mammals. It can interact with AHA-1, a homolog of mammalian HIF-1β, in vitro (Jiang et al., 2001b; Powell-Coffman et al., 1998).

Post-translational regulation of HIFs

In mammals the stability and activity of HIFα are regulated by various post-translational modifications, such as phosphorylation and hydroxylation. HIFα subunits contain an oxygen dependent degradation (ODDD) domain (Huang et al., 1998). In normoxia, HIF is hydroxylated at two proline residues (Pro-564 and Pro-402) in the ODDD domain by a prolyl hydroxylase (PHD, also termed HPH or EGLN) (Huang et al., 2002; Ivan et al., 2001; Jaakkola et al., 2001). This prolyl hydroxylation allows HIFα to interact with pVHL (the Von Hippel-Lindau tumor suppressor protein). VHL is a component of an E3 ubiquitin ligase, and it targets HIFα for destruction in 26S proteasome (Huang et al., 2002). Other studies have also shown that in normoxia another important inhibitor FIH (Factor Inhibit HIF) could hydroxylate one asparagine residue in the C-terminal transcriptional activation domain of HIFα, and this modification prevents CBP/p300 coactivator from binding with HIF (Lando et al., 2002; Mahon et al., 2001). During oxygen deprivation, the enzymatic activity of PHD and FIH proteins are inhibited, and

HIF-1 is stabilized and able to bind CBP. It has also been reported that phosphorylation of HIFα at C-terminal transcriptional activation domain could enhance the CBP/p300 binding with HIF (Jiang et al., 2001a; Treins et al., 2002; Zhong et al., 2000).

The identity of the PHD / EGL-9 prolyl hydroxylases that target HIFα for degradation was first discovered in *C. elegans* (Epstein et al., 2001). *C. elegans* EGL-9 and VHL-1 are the orthologs of PHD and pVHL in mammals respectively. This is good proof that *C. elegans* is an excellent model organism to study HIF.

In addition to EGL-9′s role in oxygen dependent degradation of HIF-1, other students in our research group also found that EGL-9 could repress HIF-1 transcriptional activity through a *vhl-1*-independent mechanism in *C. elegans* (Shao et al., 2009; Shen et al., 2006). We were first attracted by the different expression pattern of *Pnhr-57:GFP* in *vhl-1* and *egl-9* mutant worms. *Pnhr-57:GFP* is HIF-1-dependent reporter and is rapidly up-regulated by hypoxia (Shen et al., 2006). Quantitative PCR experiments and protein blots showed the expression of HIF-1 target genes in *egl-9* mutants was much higher than those in *vhl-1* mutants. The HIF-1 protein expression in *egl-9* and *vhl-1* mutants was comparable as assayed by protein blots with a HIF-1 specific polyclonal antibody (Epstein et al., 2001; Shen et al., 2006). Collectively, these data suggested that EGL-9 may play a role in the regulation of HIF-1 targets independently of *vhl-1* function. By constructing and studying several *egl-9* and *hif-1* transgenic strains and mutants, Shao et al. proposed the dual functions of EGL-9 in the regulation of HIF-1 in *C. elegans*. First as HIF-1 prolyl hydroxylase, EGL-9 hydroxylates HIF-1 at the conserved proline residue. This is essential for HIF-1 to bind with VHL-l and for subsequent degradation. Second, EGL-9 suppresses HIF-1-mediated gene expression through an unknown mechanism, which is

independent of *vhl-1* function and has relatively little requirement for EGL-9 enzymatic activity (Shao et al., 2009).

In 2006, Shen et al. showed that *rhy-1 (Regulator of HYpoxia-inducible factor (hif-1))* negatively regulated HIF-1 activity. Loss-of-function mutations in *rhy-1* significantly increased HIF-1 targets expression. The induction of *Pnhr-57:GFP* was more than one hundred folds, whereas *hif-1* mRNA and protein levels only slightly increased in *rhy-1* mutant animals. Moreover the expression of HIF-1 targets further increased in *vhl-1; rhy*-1 double mutant compared to that in either *rhy-1* or *vhl-1* single mutant (Shen et al., 2006). The results suggested that RHY -1 inhibited the expression of HIF-1 targets through a *vhl-1*-independent pathway.

More interestingly, both *egl-9* and *rhy-1* mRNA were also up-regulated by hypoxia via a pathway that required HIF-1 function (Bishop et al., 2004; Shen et al., 2005). These creates negative feedback regulatory loops, which keep HIF-1 in check and prevents it from over-activation in wild type animal.

The study of HIF functions

In animals, angiogenesis and erythropoiesis are the two major mechanisms to increase oxygen delivery to different parts of the body. During oxygen deprivation, HIF-1 activates vascular endothelial growth factor (VEGF) and erythropoietin (EPO) expression to promote oxygen absorption and transportation. More than one hundred genes have been showed to be directly or indirectly regulated by HIF-1(Elvidge et al., 2006; Manalo et al., 2005). They are involved in many important physiological functions, such as angiogenesis/vascular remodeling which is essential for the growth, invasion, and metastasis of tumor cells. Insufficient expression

of HIFα causes ischemic stroke and coronary disease (Semenza, 2000). On the other hand, HIFs activity increases in many human cancers. The overexpression of HIFs and HIFs targets, particularly VEGF, promotes the formation of blood vessels; therefore helping to provide nutrients and oxygen for further proliferation and metastasis of the tumor. HIFs are considered to play an important role in tumor angiogenesis (Semenza, 2002; Semenza, 2009).

The mechanisms of oxygen homeostasis are evolutionarily conserved, and *C. elegans* is a good model to study HIF function. Since 2001, the study of *C. elegans* HIF-1, the homolog of HIF-1α in mammal, has provided important insights into hypoxia response networks. As mentioned before, the identity of the PHD / EGL-9 prolyl hydroxylases that target HIFα for degradation was first discovered in *C. elegans* (Epstein et al., 2001). HIF-1 is also required for the worm to survive and develop in hypoxia condition (Jiang et al., 2001b). In 2005 Shen et al. showed that 110 genes were induced at least 2-fold by hypoxia in *C. elegans* (Shen et al., 2005). Among these genes 63 of them were regulated by *hif-1*-dependent mechanisms.

C. elegans hif-1 has been shown to have important roles in stress response and aging. The heat acclimation (AC) caused the elevation of HIF-1 protein level, which is critical for AC-dependent tolerance to heat and heavy metals in *C. elegans*. This pathway has been proved to be evolutionarily conserved (Maloyan et al., 2005; Treinin et al., 2003). Recent studies have also discovered key roles of HIF-1 in the regulation of life span in *C.elegans* (Chen et al., 2009; Mehta et al., 2009; Zhang et al., 2009). Worms with stabilized HIF-1 live around 30% longer than wild type animals. *C. elegans* hypoxia signaling is also required for the resistance to pore-forming toxins (PFTs). *hif-1* mutant worms are more susceptible to PFT toxicity (Bellier et al., 2009).

HIF-1 also has roles in *C. elegans* neuronal pathfinding and aerotaxis behaviors. In 2006 Dr. Bargmann's group made a small chamber with a linear gradient from anoxia to atmospheric oxygen in the gas phase. With this chamber they showed that wild type nematodes preferred to stay at a median oxygen concentration of 10%, avoiding both high and low oxygen levels. The presence of food suppressed this oxygen aerotaxis behavior. Later in this study they found that a group of chemosensory neurons mediated the hypoxia avoidance in *C. elegans.* Recent study from same group showed that overexpression of *hif-1* by hypoxia or by loss-of-function mutation of *egl-9* shifted worm's preferred median oxygen concentration from 10% to 8% and also abolished the negative influence by food. The results suggested that *hif-1* function in a subset of chemsensory neurons and nonneuronal secretory cells mediated the shifting of oxygen preference. In 2008 Pocock et al. also showed that stabilized HIF-1 was responsible for the hypoxia induced neurodevelopmental defects (Pocock and Hobert, 2008). So HIF-1 function is also important in neuronal system in *C.elegans*.

C. ELEGANS TRANSCRIPTION FACTORS THAT MEDIATE STRESS RESPONSE AND FUNCTION IN AGING

Excess ROS generation by mitochondria can lead to macromolecular damage. The accumulation of these damaged nucleic acids, lipids, and proteins is thought to contribute significantly to aging. This is the central idea of the oxidative stress theory of aging (Beckman and Ames, 1998; Harman, 1956). Aging is an irreversible process, and it is one of the hottest topics in biomedical research. Aging has been studied in different model organisms (Guarente and Kenyon, 2000). Among these organisms, *C. elegans* may be one of the most favorable systems in recent decades.

Insulin/IGF-1 like signaling

Since Klass established the methods to isolate longevity mutants in *C. elegans* in 1983 (Klass, 1983), hundreds of mutants with increased life span have been identified. The most striking one is the *daf-2*, a homologue of both insulin receptors and IGF-1 receptors in mammals. The worms that carry loss-of-function *daf-2* mutation lived twice as long as wild type N2 worms did, which only live around 3 weeks at 20°C (Kenyon et al., 1993; Kimura et al., 1997). *daf-2* mutants increased the resistance to oxidative stress (Honda and Honda, 1999);(Lithgow et al., 1995), resistance to hypoxia (Scott et al., 2002), and also resistance to bacterial pathogens(Garsin et al., 2003; Troemel et al., 2006). DAF-2 has been shown to inhibit the function of multiple stress responsive transcription factors which include DAF-16, HSF-1 and SKN-1. *C. elegans* genome encodes multiple potential ligands for DAF-2, but their respective functions are not fully understood.

daf-16 encodes a FOXO family transcriptional factor and the major stress response factor that mediates DAF-2 function. Mutation of *daf-16* completely abolishes the life extension and pathogen-resistance phenotype of *daf-2* mutants. As expected *daf-16* mutants also reduced the resistance to other stresses (Barsyte et al., 2001; Honda and Honda, 1999; Larsen, 1993; Lin et al., 2001; Lithgow et al., 1994; Lithgow et al., 1995; Murakami and Johnson, 1996; Vanfleteren, 1993). Overexpression of *daf-16* promotes both longevity and stress resistance in *C. elegans*.

The studies in *C. elegans* made it clear that *daf-2*/insulin/IGF-1 receptors activate a conserved phosphatidylinositol-3-OH kinase (PI(3)K)/3-phosphoinositide-dependent kinase-1 (PDK1)/Akt signal transduction pathway which phosphorylates *daf-16*/FOXO and keeps it from

translocating to the nucleus. Full-genome microarrays, serial analysis of gene expression (SAGE) and chromatin immunoprecipitation revealed hundreds of genes that regulated by *daf-2/daf-16* pathway (Halaschek-Wiener et al., 2005; McElwee et al., 2003; Murphy et al., 2003; Oh et al., 2006). DAF-16 targets includes genes that mediate oxidative and heat stress response. DAF-16 induces downstream stress response genes expression and prevents the organism from aging. The insulin/IGF-1 signaling is evolutionarily conserved from *C. elegans* to human.

Heat shock factor 1 (HSF-1) and immunity

Heat shock factor 1 (HSF-1) is also part of *C. elegans* stress response network. *C. elegans* HSF-1 is orthologous to mammalian heat shock factor 1 (HSF1), which form homotrimeric complexes in heat stress conditions and then binds specific DNA sequences of downstream genes. As a heat shock transcription factor, HSF-1 regulates genes that are required for heat stress response such as heat shock proteins (HSPs). In 2006 Singh and Aballay found that heat shock treatment enhanced the resistance to *P. aeruginosa* (*PA14*) in *C. elegans*. HSF-1 function was critical in this process, and mechanism of the resistance to the bacteria pathogen was genetically distinguished from p38 MAPK/PMK-1 signaling pathway (Singh and Aballay, 2006a; Singh and Aballay, 2006b). HSF-1 also regulated longevity in *C. elegans*. RNAi knockdown of *hsf-1* shortens the adult life span (Garigan et al., 2002b), and overexpression of HSF-1 promotes longevity (Hsu et al., 2003).

HSF-1 activity has been shown to mediate DAF-2 functions in *C. elegans*. First, the life span regulation mediated by *hsf-1* depends on insulin/IGF-1 like signaling pathway. Although HSF-1 overexpression could further extend the life span of *daf-2* mutant, *daf-16* RNAi

completely suppressed the increased longevity associated with HSF-1 overexpression. On the other hand, *hsf-1* RNAi also reduced the life span in *daf-2* mutants, but not in those with mutated *isp-1* or *eat-2*, which act independently of insulin/IGF-1 like signaling to promote the longevity in *C. elegans*. Second HSF-1 mediates the enhanced immunity against bacteria pathogens in *daf-2* mutants. Previous studies showed that *daf-2* mutants increased the resistance to *P. aeruginosa* (Garsin et al., 2003). RNAi inactivation of *hsf-1* reduced the pathogen resistance in *daf-2* mutants, but did not further compromise the defense of *daf-2*;*daf-16* double mutants (Singh and Aballay, 2006a; Singh and Aballay, 2006b).

The interaction between heat stress response and insulin/IGF-1 signaling pathway has been further addressed at the molecular level. In RT-PCR experiments, Hsu et al. also showed that the induction of a subset of DAF-16-dependent targets were also required HSF-1 function in *daf-2* mutant. DAF-16 function has also been shown to be critical for the expression of several heat-shock response genes in defense against heat stress. These data suggested that DAF-16 and HSF-1 may function together downstream of *daf-2* to promote longevity and enhance immunity in response to heat stress or reduction of insulin signaling in *C. elegans*.

Phase II detoxification network, SKN-1/Nrf

Like DAF-16 and HSF-1, SKN-1 is orthologous to Nrf in mammals and this class of DNA-binding transcription factors plays central roles in detoxification in living organisms.

The systemic detoxification is important for living creatures to defend themselves against endobiotic or xenobiotic toxins. It consists of three phases of detoxification. At phase I, the cell will solubilize the toxins through the modification of xenobiotics by phase I detoxification

enzyme such as cytochrome P450s (CYPs) and short chain dehydrogenases/reductases. This process is essential for the cell to excrete the toxins. Reactive oxygen species are produced as byproducts. Phase II detoxification involves scavenging the free radicals and repairing the damages caused by ROS. The glutathione-S-transferases (GSTs) and UDP—glucuronosyl/glucosyl transferases (UGTs) are the typical phase II detoxification enzymes. At the phase III, the conjugated toxins are moved by a series of transporters to kidney and intestine for the final excretion.

SKN-1 is a bZIP transcription factor and a regulator of phase II detoxification enzymes in *C. elegans*. At normal culture conditions, SKN-1 expressed at very low levels in the intestine and constitutively expressed in ASI neurons in young adult worms (An and Blackwell, 2003). In response to oxidative, xenobiotic or heat stress, SKN-1 accumulated in the intestinal nuclei. In 2009 a series of microarray experiments confirmed the central role of SKN-1 in the defense against oxidative and xenobiotic stresses in *C. elegans* (Oliveira et al., 2009). Different sets of genes with partial overlap are up-regulated or suppressed in a SKN-1 dependent mechanism in response to different xenobiotic stresses. The results suggested that SKN-1 also play a role in phase I detoxification as well as the phase II network. Earlier studies showed that moderate overexpression of SKN-1 extended worm life span and promoted resistance to oxidative stress/ROS, whereas *skn-1* mutants were highly sensitive to oxidative stress and had a short life span (An and Blackwell, 2003; Park et al., 2009). Thus the SKN-1/detoxification pathway also plays important roles in *C. elegans* aging.

The study of SKN-1 regulation suggests that SKN-1 can directly integrate multiple regulatory signals in *C. elegans*. Phosphorylation is the major means in the regulation of SKN-1 activity.

First the Ser-74 and Ser-340 residues of SKN-1 could be phosphorylated by active PMK-1, which resulted in the nuclear accumulation of SKN-1 protein (Inoue et al., 2005). Sodium arsenite treatments that activate PMK-1 promoted SKN-1 nuclear translocation. In *pmk-1* mutants, the nuclear localization of SKN-1 dramatically decreased in response to sodium arsenite. These data concluded that p38 MARK pathway promoted the nuclear localization of SKN-1 by phosphorylation modification of SKN-1 protein at Ser-74 and Ser-340 sites in response to oxidative stress.

Second, in 2005 An et al. reported that SKN-1 was shown to be phosphorylated at Ser-393 and Ser-397 sited by glycogen synthase kinase-3 (GSK-3), and this modification prevented SKN-1 from accumulating in the intestinal nuclei (An et al., 2005). A SKN-1::GFP (S393A) transgene was constitutively expressed in the nuclei at normal culture conditions, and it also enhanced the resistance to oxidative stress in *C. elegans*. In the absence of oxidative stress, GSK-3 inhibits SKN-1 nuclear translocation through the phosphorylation of SKN-1 at Ser-393 and Ser-397 sites.

Finally, in 2008 Tullet et al. found that reduction of insulin/IGF-1 like signaling also resulted in the accumulation of SKN-1 in the intestinal nuclei (Tullet et al., 2008). Further investigation showed that SKN-1 could be phosphorylated by AKT-1, AKT-2 and SGK-1, which worked downstream of DAF-2 and upstream of DAF-16. This is consistent with the finding that

DAF-2 signaling was *daf-16* independent. In this study they also found that expression of SKN-1 targets were regulated by DAF-2/DAF16 signaling pathway. The results revealed that the expression of a small subset of DAF-16 dependent genes was also required SKN-1 activity. Moreover the life span extension by constitutive activated SKN-1transgene was *daf-16-i*ndependent, which was consistent with the model. In conclusion, SKN-1 activity is tightly controlled by multiple signaling pathways in *C. elegans.*

Hypoxia signaling (HIF) and aging

The studies of DAF-2/DAF-16, HSF-1 and SKN-1 sketched out the stress response network in *C. elegans*. They work closely in the defense of heat, oxidative stress, xenobiotics, hormone reduction and other stressors. HIF-1 is a transcription factor that mediates response to oxygen deprivation (Jiang et al., 2001b). HIF-1 function is also essential for the tolerance to heat and heavy metals in *C. elegans* (Maloyan et al., 2005; Treinin et al., 2003). Mutation that resulted in HIF-1 hyperactivation has shown to promote *C. elegans* immunity against bacteria pathogens (Darby et al., 1999).

Recent studies have revealed an important role of HIF-1 in longevity in *C. elegans* (Chen et al., 2009; Mehta et al., 2009; Zhang et al., 2009). In 2009 we found that both overexpression of HIF-1 and a loss-of-function mutation of *hif-1* promoted the longevity and stress resistance in *C. elegans* (Zhang et al., 2009). In the study of HIF-1 overexpression we employed HIF-1 transgene in the longevity and stress resistance assay. The substitution of Proline-621 by glycine residue prevented EGL-9-mediated HIF-1 hydroxylation and degradation by EGL-9/VHL-1 pathway. The *hif-1 (P621G)* lines lived up to 34% longer than wild-type N2 worms. More

interestingly, the life span extension proportionately correlated with various HIF-1 expression levels in different *hif-1 (P621G)* transgenic lines. The mechanism by which *hif-1 (P621G)* extends lifespan in worms was genetically distinguished from the insulin signaling-like pathway (DAF-16) and SKN-1/Nrf pathway. In the presence of abundant O2 and nutrients, loss-of-function mutation of *hif-1* also promoted the longevity. And both overexpression of HIF-1 and loss-of-function mutation of *hif*-1 promoted the resistance against heat and t-butyl-peroxide stresses (Zhang et al., 2009).

An independent study from Dr. Kaeberlein's research group provides additional support for part of our conclusion. They showed that RNAi inactivation of *vhl-1* or *egl-9* extended the life span of *C. elegans* (Mehta et al., 2009). HIF-1 was stabilized in either *vhl-1* or *egl-9* mutant animals (Epstein et al., 2001; Shen et al., 2006). Loss-of-function mutation of *hif-1* completely abolished the life span extension associated with *vhl-1* knockdown. It suggested that overexpression of HIF-1 accounted for the life span extension in *egl-9* or *vhl-1* RNAi knockdown animals. However, they also reported that the life span of *hif-1* mutants was not significantly different from that of wild type N2 worms. In the study of Dr. Kaeberlein's group they fed the worms UV-killed bacteria on the NGM plates supplemented with 50 μm FUDR. Previously we also found that the *vhl-1* mutants lived longer than wild type N2 at 20 °C, but only on the plates that contained FUDR (unpublished data). FUDR (5-deoxyriboside) is an inhibitor of DNA synthesis (Taylor et al., 1962) and a drug broadly used in cancer treatment. In longevity assays, FUDR inhibits the reproduction of worms, so the worms do not have to be transferred to new plate frequently due to the deprivation of food by newly hatched worms. Many researchers include FUDR into the agar plates in the longevity assays. *vhl-1* is the homologue of mammalian

VHL (Von Hippel-Lindau Disease), which is a tumor suppressor. Interestingly, we did find that *vhl-1* mutant worms were sick and lived shorter than wild type N2 on the plates without FUDR (unpublished data), whereas they lived healthier and much longer on the FUDR plates. Since FUDR by itself could increase the life span of some mutant worms and interfere with the results, we decided not to include any antibiotics or drugs in the media in our longevity assay. The different longevity assay protocols used in these two studies may account for the different observations and conclusions.

In the meantime, a third study focused on the life span extension conferred by loss function mutation of *hif-1*. Chen et al showed that mutation of *hif-1* or RNAi knockdown *hif-1* promoted the longevity and mutation of *egl-9* had no effect on longevity in *C. elegans*. *hif-1* mutants could not further extend the life span caused by dietary restriction (DR). Their primary observation that *hif-1* mutants lived longer than wild type N2 worms is the same as our finding. However in our study we found that *hif-1* loss-of-function mutation extended the life span via pathway that required *daf-16* (insulin/IGF-1 signaling) signaling, whereas they showed that mutation in *daf-16* could not suppress the increased longevity associated with *hif-1* knockdown and *hif-1* RNAi further extended the life span of *daf-2*. They also showed that *ire-1*, which encodes an ER membrane protein that mediates the ER stress response, is required for the increased longevity associated with loss function mutation of *hif-1*. They performed all the longevity assays on plates supplemented with 50 μg/mL FUDR and at 25°C, which is different from the temperatures used in the other two studies. Again different longevity assay methods may lead to different observations and conclusions. But these three independent studies all demonstrated that HIF-1 played an important role in longevity and stress response in *C. elegans*.

In addition to the life span modulation, several studies also investigated the interaction between HIF-1 hypoxia signaling and other stress response pathways. First, as previously reviewed HIF-1 could be activated by heat acclimation (Treinin et al., 2003), like HSF-1 and SKN-1. Second, the microarray studies suggested that HIF-1 and DAF-16 may share some common targets genes in *C. elegans*.

Although these studies have pointed out that HIF-1 is a part of *C. elegans* stress response network, the molecular mechanism of interactions between hypoxia signaling (HIF-1) and other signaling pathways are still poorly understood. Among these stress response transcription factors, SKN-1 may be most closely related to HIF-1. From the studies of mitochondria activity in mammals, we knew that mitochondrial ETC complex III is the major site of reactive oxygen species (ROS) generation (Nohl et al., 2003; Raha and Robinson, 2000). Hypoxia also increased the production of mitochondrial ROS, which is essential for the HIF stabilization. Excess ROS production caused oxidative stress and activated the regulator of Phase II detoxification Nrf/SKN-1 network. Nrf/SKN-1 and hypoxia signaling (HIF) may cooperate in keeping the balance between oxygen and ROS levels.

THE ROLE OF MITOCHONDRIA IN THE REGULATION OF HIF

Mitochondria, the oxygen sensor

In 1998, Chandel et al. proposed that mitochondria had an important role in the regulation of HIF-1α protein stability in mammalian cells (Chandel et al., 1998). They showed that hypoxia (1.5% oxygen) failed to stabilize HIF-1α in rho zero Hep3B cells in which mitochondrial DNA were depleted. This result suggested that mitochondrial function was required for sensing or

responding to changing oxygen levels in cells. The mitochondrial electron transport chain (ETC) leakage is considered to be a major source of reactive oxygen species (ROS) (Chandel et al., 2000). In this study, Chandel and colleagues found that the ROS level increased in hypoxic conditions in human Hep3B cells. Treating wild-type Hep3B cells with antioxidants abolished hypoxia-induced increases in HIF-1α protein levels. This observation was confirmed by later studies which also showed that application of hydrogen peroxide (H_2O_2) scavengers such as N-acetylcysteine blocked hypoxic induction of HIF-1α (Kim et al., 2002). Several studies showed that exogenous H_2O_2 treatment could induce the accumulation of HIF-1α even at normoxia condition (Chandel et al., 1998; Chandel et al., 2000). Collectively, these studies supported a model in which ROS generated from mitochondria has a central role in the induction of HIF-1α protein accumulation (Chandel et al., 2000). However, other studies challenged this model. It has been reported that inhibition of mitochondria ETC function by some pharmacological inhibitors of ETC complex I and III had no effect on hypoxia-induced HIF-1α stability (Doege et al., 2005; Srinivas et al., 2001; Vaux et al., 2001). In 2005 three articles were published in "*Cell Metabolism*". These studies showed that siRNA knock down of Rieske iron-sulfur protein (Brunelle et al., 2005; Guzy et al., 2005) or targeted mutategenesis to the cytochrome C gene (Mansfield et al., 2005) of ETC complex III decreased the production of ROS and diminished the induction of HIF-1α in response to hypoxia. ROS generation was assessed by a more sensitive assay (fluorescence resonance energy transfer, FRET) in these studies. They provided further support for an important role of mitochondrial ROS in cellular oxygen sensing and regulation of HIF-1α.

In 2005, two groups reported that the accumulation of mitochondria TCA cycle intermediates caused by genetic mutations of some key enzymes promoted HIF-1 stabilization in tumor cell line, and this is thought to contribute to the highly vascularization in solid tumor (Pollard et al., 2005; Selak et al., 2005). Nuclear encoded mitochondrial proteins fumarate reductase (FH) and succinate dehydrogenase (SDH: B,C,D) are tumor suppressors. Genetic mutations of FH lead to leiomyomas and renal cell cancer, whereas mutations in SDH cause paragangliomas and phaeochromocytomas (HPGL). Mutation of these two genes caused accumulation of fumarate and succinate and the stabilization HIF-1α in these tumor cell lines (Pollard et al., 2005; Selak et al., 2005).

How do mitochondrial ROS or TCA cycle intermediates regulated HIF-1α stability? HIF-1α prolyl hydroxylation reaction may provide some insight into this question. PHD/EGL-9 is a dioxygenase and catalyzes two coupled reactions. PHD/EGL-9 requires ferrous iron (Fe^{+2}) and ascorbate as coenzymes. 2-oxoglutarate, HIF-1α and oxygen are substrates, which will be converted to succinate and hydroxylated HIF-1α.

Two models are illustrated as Figure 2_1. Current models concur that mitochondrial signals regulate HIF function, but the relative importance of various mitochondrial signals and metabolites is still unclear (Bell et al., 2005; Klimova and Chandel, 2008).

In the first model the increased production of ROS may drive the Fenton Reaction, which converts Fe^{+2} to Fe^{+3}. Fe^{+2} is a coenzyme of HIF prolyl hydroxylase (PHD) and is required for the enzyme to assemble to its active form (Counts et al., 1978). Increased generation of mitochondrial ROS may disrupt the assembly of PHD by oxidizing Fe^{+2} to Fe^{+3}.

In the second model the PHD prolyl hydroxylation reaction requires 2-oxoglutarate (also known as α -ketoglutarate) as a substrate, and it produces succinate. Both succinate and 2-oxoglutarate are TCA cycle intermediates. Thus, genetic, environmental, or pharmacological perturbations of mitochondrial function that affect the cellular levels of 2-oxoglutarate and succinate might alter the PHD enzymatic activity (Takahashi et al., 2000).

C. elegans mitochondria, aging and HIF-1

In *C. elegans* Sugimoto et al found that hypoxia signaling was regulated by mitochondrial function (Sugimoto et al., 2008). They showed that RNAi knockdown of nuclear encoded *mtssb-1* (mitochondrial single-stranded DNA-binding protein), a key component of the mtDNA replication machinery, resulted in decreased mitochondria DNA copy number and the up-regulation of many hypoxia response genes, which included both *hif-1*-dependent and *hif-1*-independent genes. Among these genes, *nhr-57, egl-9,* F22B5.4 and K10H10.2 have also been well studied as targets of HIF-1 (Budde and Roth, 2010; Shao et al., 2009; Shen et al., 2005; Shen et al., 2006; Zhang et al., 2009).

However the role of mitochondria in regulation of HIF-1 is still poor understood in *C.elegans*. The studies in worms that carry mitochondrial function deficiency may provide some insight in the regulatory role of mitochondria in HIF-1 activity.

The effects of decreased mitochondrial function are mixed. RNAi knockdown or mutation of nuclear encoded mitochondrial genes often lead to life span extension in *C. elegans* (Hamilton et al., 2005; Hansen et al., 2005; Lee et al., 2003), In humans, mutation of genes involved in mitochondria functions, particularly in ETC, results in many disorders including tumors

(Wallace, 2005). The well-known human mitochondria related disease, Leigh's syndrome, is caused by reduction of mitochondrial ETC complex I NDUFS3 subunit. RNAi inactivation of *nuo-2* which encodes complex I NDUFS3/30KD subunit has been shown to increase the life span in *C. elegans.* To solve this human-nematodes paradox, Ventura et al. proposed the "Mitochondrial Threshold Effect Theory" based on the studies of *C. elegans* mitochondrial mutants. In this theory, there are two bounded thresholds of mitochondria dysfunction. The first one is a moderate level of mitochondria deficiency, which often leads to slow postembryonic development, small adult size, decreased brood size, and life span extension. The second threshold associates with severe dysfunction of mitochondria. The animals are often larval-arrested and sterile (Ventura et al., 2006). It is consistent with observations in the studies of mitochondrial longevity genes in *C. elegans*. Previous studies have shown that RNAi knockdown some mitochondria ETC gene promoted longevity in *C. elegans*. However the worms that carry genetic mutation of same gene are not viable and arrest at larval stage. It is true for almost all the currently identified mitochondria longevity genes from genome RNAi screens. Subsequent studies provide support for this theory (Rea et al., 2007). It explains why in *C. elegans* RNAi mediated knockdown of mitochondria genes often leads to increased life span, while in human mutation of the correspondent gene resulted in diseased and sometimes short-lived. However the mechanism of life span extension mediated by mitochondrial gene knockdown is still unknown.

Although most mitochondrial gene mutations resulted in larval-arrest and sterility, there are a few viable mitochondrial mutants. *clk-1* mutant is the first identified mitochondrial longevity mutant(Wong et al., 1995). In *clk-1* mutant worms, embryonic and post embryonic development

were slower in comparison to those in wild type N2 worms. The timings of defecation, swimming and pumping cycles were also affected by mutation of *clk-1*. Most strikingly, *clk-1* mutants live much longer than the wild type N2 worms (Ewbank et al., 1997; Wong et al., 1995). The life span extension of *clk*-1 mutants is genetically distinguished from that caused by mutation in the insulin/IGF-1 signaling like pathway (Lakowski and Hekimi, 1996; Lakowski and Hekimi, 1998). As previous reviewed, overexpression of HIF-1 extended the life span in *C. elegans*, which was also independent of insulin/IGF-1 signaling pathway (Zhang et al., 2009). It has been shown that mutation of some mitochondria ETC genes lead to HIF-1 stabilization and activation in mammals (Koivunen et al., 2007; Pollard et al., 2005; Selak et al., 2005). If it is also true in *C. elegans*, HIF-1 may function in the life span extension in mitochondrial mutants.

Moreover, biochemical analysis in *C. elegans* metabolism also provides some insight into the role of mitochondria function in HIF-1 regulation in *C. elegans.* Mitochondria is major oxygen consumption organelle in the cell, and their function is essential for the energy generation as well as pyrimidine biosynthesis, fatty acid metabolism, steroid biogenesis. *C. elegans* may employ a compensatory metabolic mechanism when mitochondrial ETC function is impaired. The studies of metabolism processes indicated that *C. elegans* could employ several anaerobic pathways at same time in presence of low or no oxygen (Barrett, 1984). So in response to the reduction of mitochondria function associated with ETC gene knockdown, *C.elegans* could switch to an anaerobic pathway e.g. glycolytic cycle, to generate the ATP and remove buildup of upstream metabolites. The main end products of anaerobic pathways in *C.elegans* were lactate, acetate, succinate, and propionates (Foll et al., 1999). As reviewed before, in studies of tumor cell lines, it has been shown that accumulation of TCA cycle

intermediates, succinate or fumurate, induced HIF-1 α stabilization and activity (Koivunen et al., 2007; Selak et al., 2005). It hinted that HIF-1 function could be induced as a byproduct of anaerobic metabolism pathway employed by worms with mitochondria function deficiency.

The study of mitochondria ETC inhibitors also suggested a regulatory role of mitochondria in HIF-1. Hydrogen sulfide (H_2S) is an inhibitor of cytochrome *c* oxidase, one component of the ETC complex III (Beauchamp et al., 1984). Trace amounts of H_2S could reduce the oxygen consumption by ten-fold in mice. In *C. elegans*, treatment of H_2S reduced metabolic rate by 30% (Van Voorhies and Ward, 2000). In 2007 Miller and Roth found that worms exposed to H_2S were more thermotolerant and lived longer compared to worms cultured in the room air (Miller and Roth, 2007). In the later study the Roth lab also showed that HIF-1 protein was induced in response to H_2S in *C. elegans*. Overexpression of HIF-1 by hypoxia or in *vhl-1* or *egl-9* mutants increased worms' tolerance to H_2S (Budde and Roth, 2010). The remaining questions are: How could the exposure to H_2S induce HIF-1 function? Through the inhibition of mitochondria ETC? Does HIF-1 activation mediate the life span extension in worms exposed to H_2S?

Recent study of mouse *clk-1* also suggested the role of mitochondria in regulation of HIF-1 (Wang et al., 2010). They showed that HIF-1 protein level increased in liver nuclei of *Mclk1* heterozygous mutant mice as well as in *Mclk1* siRNA knockdown cells. Since the mRNA level of HIF-1 did not changed by siRNA knockdown Mclk1, the impact of *Mclk1* on HIF-1 may be post-translational. They also showed that mitochondrial ROS level in Mclk1 siRNA knockdown cells increased, which is consistent with previous finding in Mclk1 heterozygous mutant mice (Lapointe and Hekimi, 2008).

The studies of mitochondria function from both mammals and *C. elegans* suggested that HIF-1 function could also be regulated by mitochondria function in *C. elegans*. However there was no direct evidence that demonstrates the regulatory role of mitochondria in HIF-1 in *C. elegans*. In this thesis research I demonstrated that HIF-1 activity was induced and mediated the life span extension associated mitochondria function deficiency.

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FIGURE LEGENDS

Figure 2_1. Mitochondria influence on HIF-1. Hydroxylation of HIF-1α by PHD (EGLN) requires 2-oxoglutarate and O2 as co-substrates. This reaction produces succinate and carbon dioxide. Ferrous iron (Fe+2) is required for PHD (EGLN) activity. Hypoxia induced mitochondrial ROS and accumulation of TCA cycle intermediates are two proposed pathways of regulation of HIF by mitochondria function. This figure is partially adopted from Figure 3 by King et al. 2006.

Figure 2_2. Illustration of stress response network in *C. elegans.*

This figure is partially adopted from Figure 1 by Tullet et al. 2006

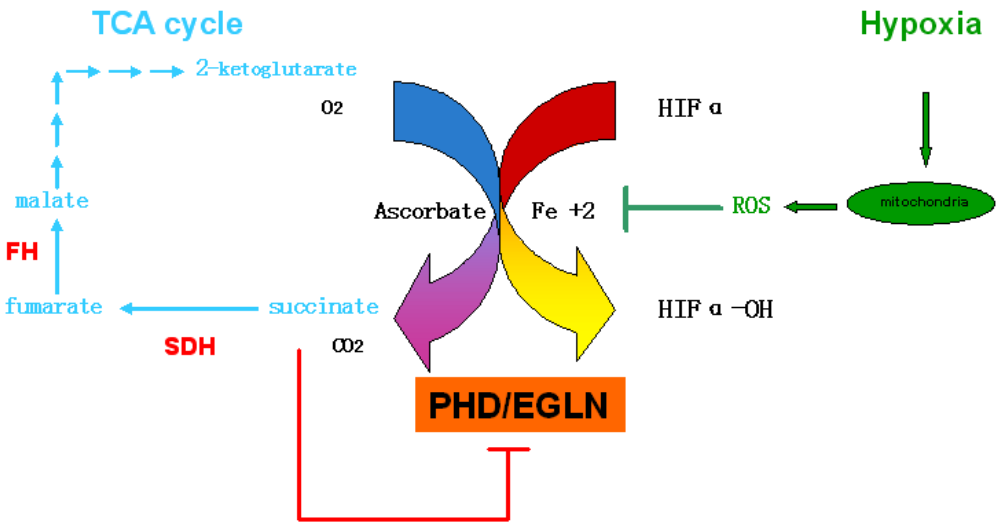

Figure 2_1. Mitochondria influence on HIF-1

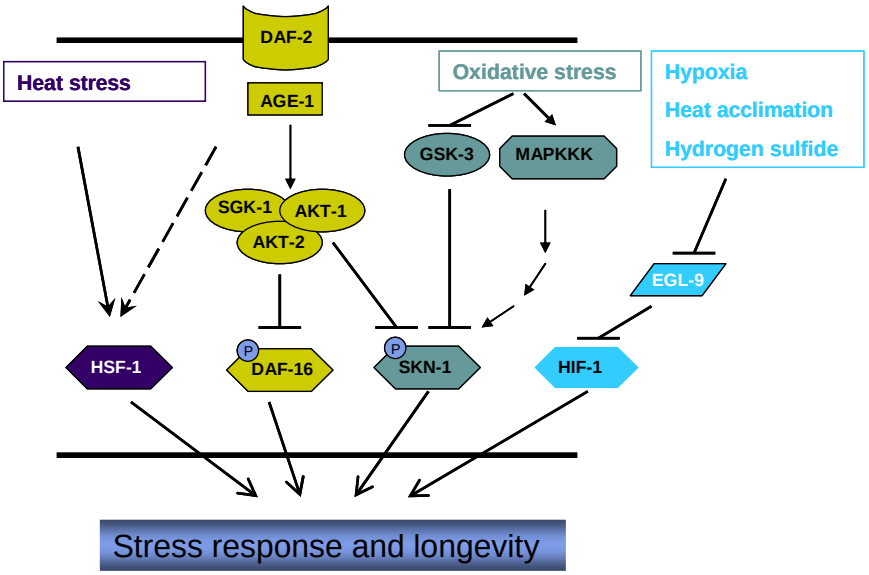

Figure 2_2. Illustration of stress response network in *C. elegans*

CHAPTER 3: GENOME-WIDE RNA INTERFERENCE SCREEN IDENTIFIES SKN-1/NRF AS A REGULATOR OF THE HIF-1 HYPOXIA-INDUCIBLE FACTOR IN C. ELEGANS.

ABSTRACT

During development, homeostasis, and disease, organisms must balance responses that allow adaptation to low oxygen (hypoxia) with those that protect cells from oxidative stress. The evolutionarily conserved hypoxia-inducible factor transcription factors (HIF) play central roles in oxygen homeostasis. To discover new genes and cellular signaling pathways that regulate HIF-1, we conducted a genome-wide RNAi screen in *C. elegans*. We found that RNAi-mediated mitochondrial or proteasomal dysfunction increased expression of a hypoxia-responsive reporter. Interestingly, many of these effects did not require *hif-1*. The RNAi screen led to the discovery that SKN-1/Nrf inhibits HIF-1. The SKN-1 transcription factor is responsive to oxidative stress, and SKN-1 is a key transcriptional regulator of phase II detoxification enzymes. Here, we show that SKN-1 acts through a putative SKN-1 binding site to activate *egl-9* expression. EGL-9 is the prolyl hydroxylase that targets HIF-1 for oxygen-dependent degradation and inhibits HIF-1 activity. Prior studies have shown that HIF-1 feeds back to activate *egl-9* expression. Thus, SKN-1 and HIF-1 both contribute to the regulation of *egl-9* expression, and we propose that this combinatorial control promotes adaptive changes in gene expression appropriate to different developmental contexts or environmental stresses.

INTRODUCTION

Oxygen homeostasis has profound effects on health and fitness. Oxygen serves as the terminal electron acceptor in the oxygen phosphorylation processes generate energy for life. When oxygen levels are low (hypoxia), cells and tissues must adapt quickly by increasing oxygen delivery, adjusting the levels of key metabolic enzymes, and limiting the accumulation of misfolded proteins. While oxygen is essential, it is also highly reactive. The reactive oxygen species (ROS) generated by cellular metabolism and signaling processes can damage macromolecules, and excess ROS are thought to contribute to cellular aging and deterioration (Finkel and Holbrook, 2000). The transcription factors and pathways that mediate hypoxia response and oxidative stress response have been studied intensively, but the mechanisms by which cells coordinate or fine-tune these networks to achieve oxygen homeostasis are not fully understood.

Hypoxia-inducible factor (HIF) transcription factors play key roles in oxygen homeostasis. HIF is a heterodimeric transcription factor, consisting of an alpha subunit that is regulated by oxygen and a beta subunit (also termed ARNT) that can partner with related DNA-binding transcription factor proteins. HIF was first discovered in mammals, and HIF transcription complexes have critical roles in both normal human development and in disease (Semenza, 2007; Weidemann and Johnson, 2008). Key elements of the hypoxia response networks are conserved in diverse animals, and HIF homologs have also been shown to have important functions in invertebrate development (Gorr et al., 2006; Jiang et al., 2001b; Lavista-Llanos et al., 2002).

The *C. elegans* hypoxia-inducible factor, *hif-1*, has important roles in stress response and in aging. Mutations or treatments that stabilize HIF-1 protein have been found to increase *C. elegans* adult lifespan, increase resistance to heat, and protect against certain bacterial pathogens (Bellier et al., 2009; Darby et al., 1999; Mehta et al., 2009; Treinin et al., 2003; Zhang et al., 2009). Wild-type *C. elegans* can survive and develop in moderately hypoxic conditions (0.5% oxygen at 20^{o}C), but animals that lack a functional *hif-1* gene cannot (Jiang et al., 2001b; Padilla et al., 2002; Van Voorhies and Ward, 2000). The ability to adapt to varying levels of oxygen may be essential in the soil microenvironments inhabited by *C. elegans*, especially in regions with plentiful microbial food. In laboratory conditions, on agar plates with abundant food and oxygen, *hif-1*-deficient animals are relatively heat resistant and live slightly longer than wild-type animals (Chen et al., 2009; Zhang et al., 2009). Misregulation of HIF-1 has been shown to cause egg laying defects, decreased fertility, and neuronal pathfinding aberrations (Pocock and Hobert, 2008; Shen et al., 2006; Trent et al., 1983; Zhang et al., 2009).

HIF protein levels and HIF activity are tightly regulated. When oxygen is abundant, the HIF alpha subunit is hydroxylated by the PHD/ EGL-9 enzymes. Once modified, HIFα protein interacts with the Von Hippel-Lindau tumor suppressor (VHL) and is targeted for ubiquitination and proteasomal degradation (Kaelin and Ratcliffe, 2008). This pathway for oxygen-dependent degradation of HIF protein is evolutionarily conserved. The *C. elegans hif-1, aha-1, egl-9,* and *vhl-1* genes are orthologous to mammalian HIFα, HIFβ, PHD, and VHL, respectively (Epstein et al., 2001; Jiang et al., 2001b; Powell-Coffman et al., 1998). Thus, in hypoxic conditions, HIF-1 protein is stable, and the transcription factor complex can activate the expression of a battery of genes that enable adaptation to low oxygen. The targets of *C. elegans* HIF-1 include *egl-9* and

rhy-1, genes that inhibit HIF-1 expression and activity (Bishop et al., 2004; Shao et al., 2009; Shen et al., 2005; Shen et al., 2006). In wild-type animals, these negative feedback loops keep HIF-1 activity in check and limit the potentially adverse effects of HIF-1 over-activation.

C. elegans responses to oxidative stress have also been studied intensively, and SKN-1, the ortholog of the mammalian Nrf2 transcription factor, plays a central role. SKN-1 activity is regulated by insulin / IGF-1-like signaling, the p38 map kinase pathway, and glycogen synthase kinase-3 (GSK-3) (An et al., 2005; Inoue et al., 2005). In optimal growth conditions, GSK-3 phosphorylates SKN-1, and SKN-1 is retained in the cytoplasm. Oxidative stress inhibits GSK-3 and induces nuclear accumulation of SKN-1. SKN-1 targets include phase II detoxification genes (An and Blackwell, 2003; Inoue et al., 2005; Oliveira et al., 2009; Tullet et al., 2008). Maternal *skn-1* is essential (Bowerman et al., 1992). Animals that receive SKN-1 from their mothers but do not carry a functional *skn-1* gene in their own genomes survive to adulthood, but are highly sensitive to oxidative stress and have a reduced adult lifespan (An and Blackwell, 2003; Park et al., 2009).

The mechanisms by which animals coordinate the activities of HIF-1 and oxidative stress responsive transcription factors such as SKN-1 are largely unknown. Here we describe a genome wide RNAi screen to identify regulators of *C. elegans* HIF-1. The data presented here support a model in which SKN-1 activates the expression of EGL-9, the oxygen-sensitive enzyme that targets HIF-1 protein for degradation and represses HIF-1-mediated gene expression.

MATERIAL AND METHODS

Strains.

The following strains were used in this study: wild-type N2 Bristol; ZG430: *Pnhr-57:gfp(iaIs07)IV; egl-9(sa307)V; hif-1(ia04)V; Phif-1:hif-1a:MYC:HA (iaIs28);* ZG120: *Pnhr-57:gfp(iaIs07)IV*; ZG509: *rrf-3(pk1426)II; Pnhr-57:gfp(iaIs07)IV;* ZG508: *rrf-3(pk1426)II; Pnhr-57:gfp(iaIs07)IV; hif-1(ia04)V;* ZG429: *hif-1(ia04)V; Phif-1:hif-1a:MYC:HA(iaIs28)*; ZG472: *hif-1(ia04)V; Pegl-9:gfp(iaEx84);* ZG487: *hif-1(ia04)V; P(m)egl-9:gfp(iaEx96);* ZG488: *skn-1(zu67)IV; hif-1(ia04)V; Pegl-9:gfp(iaEx84).* The transgenes expressing epitope-tagged HIF-1 protein were described and characterized previously (Zhang et al., 2009).

Genome wide RNAi Screen.

The RNAi screen was conducted as previously described (Simmer et al., 2003), with few modifications. Each bacterial clone (expressing double-stranded RNA for one gene) was cultured in L-broth with 50 ug/mL ampicillin and 12.5ug/mL tetracycline overnight at 37^{o}C. The following morning, the bacteria were inoculated into new L-broth with 100 ug/mL ampicillin for 6 hours at 37^{o}C before seeding on 24 well NGM agar plates with 25ug/mL carbenicillin and 2mM IPTG. Each RNAi clone was plated in duplicate. The following day, 15-25 L1 worms were added to each well. The plates were incubated at 15^{o}C for 5-6 days, and then the worms were screened for positive green fluorescence by stereomicroscopy. Bacterial RNAi clones that increased the reporter were rescreened in two independent replicates, and the plasmid inserts were validated by sequencing.

***Pegl-9:GFP* expression constructs**

To generate the *Pegl-9:*GFP construct, a fragment that contained 1.6kb of sequence upstream of the initiation ATG was amplified by PCR using the forward primer (*Sph*I) 5'-CGCGCATGCGTGTATGTGTGTGAAAGAG and the reverse primer (*Sal*I) 5'-GCGGTCGACGCAACTTTTTTCTGTCACATTCAG. The PCR product was cloned into the green fluorescence protein (GFP) vector pPD95.75 (gift from Andrew Fire). To create the point mutation construct (*P(m)egl-9:GFP*), predicted SKN-1 binding site TTTGTCAT was altered to CGACGGGC. Transgenic animals were generated by injection of DNA into the gonadal syncitium, using standard methods with *rol-6* (pRF4) as the co-injection marker (Mello et al., 1991). For each construct, two transgenic lines were generated and assayed.

Protein Blots

To assay relative expression levels of GFP reporters and HIF-1 protein, worm lysates were size fractionated on polyacrylamide gels and analyzed by Western blots. To assay *Pnhr-57:GFP* levels, 10-40 L4 or adult stage animals were assayed in each RNAi experiment, and 20 L4 animals were harvested in each experiment that assayed *Pegl-9:GFP* expression. Eighty animals were harvested for each experiment to assay HIF-1 levels. The antibodies dilutions were 1:500 for the GFP-specific monoclonal [from Roche] and 1:250 for the HA-specific mouse monoclonal [from Cell Signaling]. The secondary antibody (goat anti-mouse IgG+IgM from Biorad) was used at 1:2000 dilutions. The western blot images were analyzed by the Image J software. For each experiment, at least three independent biological replicates were included.

RNA extraction and quantitative RT-PCR

Total RNA was isolated from synchronized L4-stage animals using Trizol (Invitrogen). After being treated by RNase free DNase (Promega), total RNA was reverse transcribed to complementary DNA using Oligo dT_{18} primer and AffinityScript reverse transcriptase (Stratagene). PCR was performed using the iQ SYBR GREEN supermix (Bio-Rad) and Stratagene Mx4000 multiplex PCR system. In each experiment, three biological replicates were included. For each sample, three technical replicates were included. And each reaction included cDNA from 62.5ng total RNA. Relative mRNA quantification was performed using efficiency-corrected comparative quantification (Pfaffl, 2001). *inf-1*, a gene not regulated by hypoxia, was used as the reference gene (Shen et al., 2005; Shen et al., 2006). Primer sequences are available on request.

RESULTS

Genome wide RNAi screen

We conducted a screen to discover genes or cellular processes that inhibited HIF-1-mediated gene expression. Prior studies had shown that endogenous *nhr-57* mRNA and the *Pnhr-57:GFP* reporter were rapidly up-regulated by hypoxia treatment (Shao et al., 2009; Shen et al., 2005; Shen et al., 2006). To further test the hypothesis that *nhr-57* was a direct target of HIF-1, we performed chromatin immunoprecipitation experiments, and we found that the DNA associated with HIF-1 was enriched for *nhr-57* promoter sequences relative to controls (Figure 3_S1). We screened 16265 RNAi clones representing over 80% of the *C. elegans* genome (Kamath and Ahringer, 2003), and we identified 179 genes for which RNAi increased *Pnhr-*

57:GFP expression, as assayed by inspection under a fluorescent stereomicroscope (screen design illustrated in Fig 3_1A). These genes are listed in Table 3_S1 and are summarized in Figure 3_1B. Remarkably, 89 genes were predicted to have mitochondrial or metabolic functions, and the majority of these were electron transport chain components (42 genes) or subunits of mitochondrial ribosomes (24 genes). The second largest category of genes was protein folding or turnover (33 genes), and the majority of these were proteasomal components. The remaining major categories include vesicular transport, transporters and channels, signaling and cytoskeleton, transcription and DNA or RNA processing.

Recognizing that most eukaryotic genes are coordinately regulated by multiple transcription factors, we did a secondary screen to identify those RNAi treatments that clearly had a *hif-1*-dependent effect on *Pnhr-57:GFP* expression. Most of the 179 RNAi treatments increased expression of the reporter in *hif-1* deficient strains. Table 3_1 lists the 13 genes that had a much more pronounced effect on *Pnhr-57:GFP* expression in animals carrying wild-type *hif-1*. As expected, RNAi for *egl-9, rhy-1,* and *vhl-1,* three previously characterized negative regulators of *C. elegans* HIF-1, increased *Pnhr-57:GFP* expression in wild-type animals, but not in *hif-1* mutants.

We then focused on genes that had been shown to have evolutionarily conserved roles in stress response, as they might be informative to cross-talk between HIF-1 and other key pathways. The *sams-1* gene encodes S-adenosyl methionine synthetase, and this enzyme catalyzes the formation of a metabolically active form of methionine (SAMe) that can be used to methylate proteins, nucleic acids, and small metabolites (Finkelstein and Martin, 1986). Methionine metabolism is integrally linked to homocysteine levels and the folic acid cycle, and

clinical studies are ongoing to investigate the efficacy of SAMe dietary supplements to treat a range of ailments, including liver disease and depression (Lieber, 1999; Lieber, 2002a; Lieber, 2002b). Prior studies had shown that both *sams-1* and *hif-1* had roles in aging and polyglutamine-mediated proteotoxicity (Chen et al., 2009; Hansen et al., 2005; Mehta et al., 2009; Steinkraus et al., 2008; Zhang et al., 2009). In animals carrying a wild-type *hif-1* gene, *sams-1* RNAi increased *Pnhr-57:GFP* expression 10-fold, whereas in *hif-1* mutants *sams-1* RNAi increased expression of the reporter 3-fold (Figure 3_2A). *sams-1* RNAi did not alter HIF-1 protein levels (Figure 3_S2A). These data suggest a model in which *sams-1* regulates expression of *Pnhr-57:GFP* by both *hif-1*-dependent and *hif-1*-independent mechanisms.

sbp-1 encodes the *C. elegans* Sterol Regulatory Element Binding Protein (SREBP) basic helix-loop-helix zipper transcription factor. SBP-1 regulates lipid homeostasis, and SBP-1 has been shown to be activated in response to oxygen deprivation (McKay et al., 2003; Taghibiglou et al., 2009; Yang et al., 2006). To further understand the relationship between SBP-1 and HIF-1, we quantitated the effects of *sbp-1* RNAi on HIF-1 protein levels and on *Pnhr-57:GFP*. *sbp-1* RNAi did not cause a significant change in HIF-1 protein levels, as assayed by whole animal protein blots (Figure 3_S2B). In agreement with visual characterization of the *Pnhr-57:GFP* expression patterns, *sbp-1* RNAi caused a 4-fold increase in *Pnhr-57:GFP* expression, and this effect was dependent upon *hif-1* function (Figure 3_2B). These data indicate that *sbp-1* negatively regulates *Pnhr-57:GFP* via a pathway that requires *hif-1*, but SBP-1 does not control HIF-1 protein levels.

SKN-1 /NRF reduces HIF-1 protein levels and promotes *egl-9* expression

The effects of *skn-1* RNAi on expression of the *Pnhr-57:*GFP reporter were small but consistent. Depletion of *skn-1* by RNAi increased *Pnhr-57:GFP* levels 1.4-fold, relative to control bacterial food that carried an empty RNAi vector (Figure 3_3A; p-value< 0.01). *skn-1* RNAi had a more pronounced effect on HIF-1 protein levels. In animals fed bacteria expressing *skn-1* double-stranded RNA, HIF-1 protein levels increased 3.5-fold, relative to controls (Figure 3_3B). Reasoning that regulatory interactions between SKN-1 and HIF-1 might be informative to the mechanisms by which cells balance adaptation to hypoxia with oxidative stress response, we examined this interaction in greater depth.

In silico analyses identified a potential SKN-1 binding site in the *egl-9* promoter region (Figure 3_4A). This suggested a model in which SKN-1 promoted *egl-9* transcription, thereby inhibiting both HIF-1 protein stability and HIF-1 transcriptional activity. To test this, we compared *egl-9* mRNA levels in worms fed *skn-1* RNAi and control RNAi bacteria. In the room air, *skn-1* RNAi decreased *egl-9* mRNA levels by ~20% compared to control RNAi (p-value <0.01) (Figure 3_4B). HIF-1 has been shown to activate *egl-9* mRNA expression, creating a negative feedback loop (Bishop et al., 2004; Shen et al., 2005). In accordance with this, the effects of *skn-1* RNAi on *egl-9* mRNA levels were minimized by placing the animals in hypoxic conditions that induce HIF-1 (Figure 3_4B).

To more directly test the hypothesis that the putative SKN-1 binding site contributed to *egl-9* promoter activity, we generated a reporter construct in which 1.6 kb of *egl-9* regulatory sequences directed the expression of GFP (Figure 3_5A). We conducted these experiments in a

hif-1 mutant background to distinguish the effects of SKN-1 on *egl-9* expression from those of HIF-1. In transgenic animals in standard culture conditions (20^{o}C), *Pegl-9:GFP* was visible in several tissues, including the body muscle, vulva, pharynx, anterior intestine, rectal cells and additional cells in the tail (Figure 3_5B-C). When the animals were treated with heat shock conditions that had been shown to activate SKN-1 (29°C for 20h) (An and Blackwell, 2003), *Pegl-9:gfp* was expressed more strongly in the intestine (Figure 3_5D-E). Thus, *Pegl-9*:*GFP* responded to heat shock in a *hif-1*-independent manner.

We next asked whether heat shock induction of *Pegl-9:GFP* required *skn-1* function. Heat shock increased *Pegl-9:GFP* 2.5-fold in animals carrying the wild-type *skn-1* allele, but the reporter was not induced by heat shock in *skn-1(zu67)* mutants (Figure 3_6A). The effects of heat shock on the *Pegl-9:GFP* reporter in these two genetic backgrounds were significantly different (*p*-value <0.05), and analyses of an independent transgenic line yielded similar results (data not shown).

To test the hypothesis that the putative SKN-1 binding site in the *egl-9* promoter was required for heat shock induction of *Pegl-9:GFP*, we generated the *P(m)egl-9:GFP* construct, which contained mutations in the putative SKN-1 binding site (in red type in Figure 3_4A). In transgenic animals, *P(m)egl-9:GFP* was not induced by heat shock. There was a significant difference between relative induction of the *Pegl-9:GFP* and the *P(m)egl-9:GFP* constructs (*p*-value <0.01; Figure 3_6B). Experiments with a second *P(m)egl-9:GFP* transgenic line gave similar results (data not shown). Collectively, these data demonstrated that heat shock induction of *Pegl-9:GFP* required both *skn-1* function and the putative SKN-1 binding site in the *egl-9* promoter.

We employed *gsk-3* RNAi as an independent means of activating SKN-1. Prior studies had shown that SKN-1 was phosphorylated by glycogen synthase kinase-3 (GSK-3) and that this post-translational modification inhibited nuclear localization of SKN-1 and expression of the SKN-1 target gene *gcs-1* (An et al., 2005; Inoue et al., 2005). As shown in Figure 3_7, *gsk-3* RNAi increased expression of *Pegl-9:GFP*, and this effect was dependent upon the putative SKN-1 binding site. The difference in how the two reporters responded to *gsk-3* RNAi was statistically significant (*p*-value = 0.0013).

Collectively, these data supported a model in which SKN-1 activated *egl-9* transcription, thereby attenuating HIF-1 protein levels and HIF-1 activity. Prior studies had shown that *egl-9* expression levels were also controlled by the negative feedback loop in which HIF-1 activated *egl-9* expression (Bishop et al., 2004). Microarray analyses had identified genes that were induced by hypoxia via *hif-1*-dependent pathways and had discovered genes that were induced by SKN-1 in control conditions or in response to arsenite or peroxide (Shen et al., 2005; Oliveira et al., 2009). The sets of genes upregulated by SKN-1 or HIF-1 are largely non-overlapping, but a few genes are regulated by both transcription factors, including K10H10.2, F57B9.1, M05D6.5, and *rhy-1* (Figure 3_8A). The experiments in Figures 5 – 7 were conducted in *hif-1* loss-of-function strains, to isolate and examine the roles of *skn-1* and the putative SKN-1 binding site on *egl-9* expression. We conducted additional experiments to understand the net effects of the SKN-1 / EGL-9 regulatory interactions in animals carrying wild-type HIF-1. First, we examined the expression of K10H10.2. In agreement with prior studies (Oliveira et al., 2009), we found that *skn-1* RNAi reduced expression of K10H10.2 mRNA in normal culture conditions (*p*-value <0.0001; Figure 3_8B). For animals treated with control RNAi or *skn-1* RNAi, hypoxia

treatment increased K10H10.2 expression. Even in hypoxic conditions (2 hours at 0.5% oxygen), *skn-1* RNAi-treated animals expressed K10H10.2 mRNA at lower levels relative to controls (Figure 3_8B).

We then examined the effects of skn-1 RNAi on F22B5.4, a gene that had been established as a HIF-1 target (Bishop et al., 2004; Epstein et al., 2001; Shen et al., 2005), but had not been identified as responsive to SKN-1 in microarray analyses (Oliveira et al., 2009). Using quantitative RT-PCR experiments, we confirmed that skn-1 RNAi did not have a significant impact on F22B5.4 mRNA levels in normal culture conditions (Figure 3_8C). Further, skn-1 RNAi had little effect on F22B5.4 levels in hypoxic conditions (1 or 2 hours at 0.5% oxygen). Thus, while SKN-1 modulates egl-9 expression and HIF-1 levels, enabling cross-talk between the SKN-1 and HIF-1 pathways, the net effect is dependent upon the cell or promoter context.

DISCUSSION

These studies describe a regulatory link between SKN-1, a transcription factor activated by oxidative stress, and the HIF-1 hypoxia-inducible factor. SKN-1 acts through a putative binding site on the *egl-9* promoter to increase *egl-9* expression, thereby decreasing HIF-1 protein levels and repressing HIF-1-mediated gene expression. The enzymatic activity of EGL-9 is further modulated by mitochondrial function and by cellular oxygen levels (Kaelin and Ratcliffe, 2008; Klimova and Chandel, 2008). Thus, EGL-9 is well-positioned to integrate information about cellular oxygenation and oxidative stress and to affect adaptive changes in HIF-1 function.

RNAi screen identifies genes that regulate *nhr-57* expression

We identified 179 genes for which RNAi increased expression of the *Pnhr-57:GFP* reporter. Prior studies have demonstrated that *nhr-57* was induced by hypoxia in a *hif-1*-dependent manner and that over-expression of *Pnhr-57*:*GFP* in *egl-9* mutants required *hif-1* (Bishop et al., 2004; Budde and Roth, 2010; Shao et al., 2009; Shen et al., 2005; Shen et al., 2006). Moreover, Bellier et al (2009) found that HIF-1-mediated induction of *nhr-57* helped to protect *C. elegans* from the lethal effects of pore-forming toxins (Bellier et al., 2009). The studies presented here show that RNAi treatments can activate *Pnhr-57*:*GFP* through *hif-1*-independent pathways.

The RNAi screen revealed that *Pnhr-57*:*GFP* expression was influenced by the cellular processes that maintain metabolic function and protein homeostasis, and many of the genes integral to these processes have been shown to have roles in stress response and aging. RNAi for 41 different components of the mitochondrial electron transport chain increased *Pnhr-57*:*GFP*. In agreement with this, a recent study showed that inhibition of mitochondrial replication via *par-2.1 / mtssb-1* RNAi caused a strong induction of hypoxia-responsive genes, including *nhr-57* (Sugimoto et al., 2008). Most of the mitochondrial RNAi treatments that increase *Pnhr-57*:GFP have been shown to increase adult lifespan in *C. elegans* (Curran and Ruvkun, 2007; Hamilton et al., 2005; Hansen et al., 2005; Kim and Sun, 2007; Lee et al., 2003). The *Pnhr-57*:*GFP* reporter was also induced by RNAi-mediated depletion of many proteasomal components, and many of these treatments had been shown to affect polyglutamine toxicity and to induce expression of *Pgpdh-1*:*GFP*, a marker for osmotic stress and glycerol production (Lamitina et al., 2006; Nollen et al., 2004).

Further characterization of the 179 RNAi treatments that increased *Pnhr-57*:*GFP* identified 13 that had much stronger effects in animals carrying a wild-type *hif-1* gene. These genes included *vhl-1, egl-9,* and *rhy-1*. These three genes had all been identified in prior studies as negative regulators of HIF-1 (Epstein et al., 2001; Shen et al., 2006). The succinate dehydrogenase subunit *sdhb-1* was also found to have *hif-1*-dependent effects. This is in agreement with studies in cancer cell lines in which succinate inhibited the enzymatic activities of HIF prolyl hydroxylases (Koivunen et al., 2007; Selak et al., 2005). *sams-1* and *sbp-1* encode the *C. elegans* S-adenosyl methionine synthetase and the SREBP homologs, respectively. Analyses of HIF-1 protein levels and HIF-1-mediated gene expression suggest that *sams-1* and *sbp-1* limit HIF-1 transcriptional activity, directly or indirectly.

SKN-1 promotes *egl-9* expression, thereby inhibiting HIF-1

HIF-1 and SKN-1 are both key components of the *C. elegans* stress response network. HIF-1 is stabilized by hypoxia, and SKN-1 is activated by chemicals or treatments that generate reactive oxygen species (An and Blackwell, 2003; An et al., 2005; Inoue et al., 2005). Prior studies have shown that moderate over-expression of either SKN-1 or HIF-1 can extend the longevity of adult *C. elegans* (Mehta et al., 2009; Tullet et al., 2008; Zhang et al., 2009). Microarray analyses have shown that the genes activated by SKN-1 and HIF-1 are largely non-overlapping (Oliveira et al., 2009; Shen et al., 2005).

We report that *skn-1* RNAi decreases *egl-9* mRNA levels by ~20% in L4-stage animals. However, small changes in the level or activity of an enzyme can have dramatic effects, and *skn-1* RNAi results in a 3-fold increase in total HIF-1 protein levels. In L4 or young adult animals,

SKN-1 is expressed in the ASI neurons and in the intestine (Bishop and Guarente, 2007; Simmer et al., 2003). Hence, we would expect that the effects of SKN-1 on HIF-1 regulation could be greater in some tissues relative to others.

EGL-9 has been shown to have multiple functions. First, EGL-9 is the hydroxylase that targets HIF-1 protein for oxygen-dependent degradation. Second, EGL-9 inhibits HIF-1-mediated gene expression by a *vhl-1*-independent pathway. Mutations that impair HIF-1 hydroxylation or EGL-9 enzymatic activity do not prevent EGL-9 from inhibiting HIF-1 activity (Shao et al., 2009). SKN-1-mediated activation of *egl-9* expression would be expected to affect both HIF-1 levels and HIF-1 activity.

The *rhy-1* gene has also been identified as a potential transcriptional target of SKN-1. *rhy-1* mRNA levels were shown to be decreased by *skn-1* RNAi, and potential SKN-1 binding sites were identified in the *rhy-1* promoter region (Oliveira et al., 2009). *rhy-1* encodes a transmembrane protein that is predicted to acylate molecules other than proteins, and mutations in *rhy-1* result in dramatic up-regulation of HIF-1 target genes (Shen et al., 2006). Like *egl-9, rhy-1* is also positively regulated by HIF-1 (Shen et al., 2005). These data suggest a model in which the SKN-1 and HIF-1 transcription factors converge on the *egl-9* and *rhy-1* promoters to control HIF-1 stability and activity.

In some tissues and conditions, HIF-1 might have the greater impact on *egl-9* expression, whereas in other contexts, SKN-1 might play a more important role. To investigate this concept, we examined the effects of *skn-1* RNAi on the expression of two additional HIF-1 target genes. K10H10.2 encodes a predicted beta-synthase, and prior studies had shown that K10H10.2 was

induced by HIF-1 and that K10H10.2 mRNA expression was significantly reduced by *skn-1* RNAi (Shen et al., 2005; Shen et al., 2006; Shao et al., 2009; Oliveira et al., 2009). The data presented in Figure 3_8 of this study agree with these prior studies. Further, they show that *skn-1* RNAi does not preclude hypoxic induction of K10H10.2. The F22B5.4 gene has been shown to be strongly regulated by hypoxia and HIF-1 (Figure 3_8; Bishop et al., 2004; Shen et al., 2005), and *skn-1* RNAi has little effect on F22B5.4 expression. We conclude that the influence of SKN-1 on HIF-1-mediated gene expression is dependent upon developmental and environmental cues and upon promoter context.

Future studies will address the hypothesis that SKN-1 / Nrf homologs regulate the expression of HIF hydroxylases in other animals. This balance between surviving hypoxic stress and mitigating the potential damage caused by reactive oxygen species is especially important in cardiovascular development and disease. When ischemia blocks circulation to a tissue, oxygen levels drop, and cells induce HIF expression. Upon reperfusion and reoxygenation of the tissue, mammalian cells respond by rapidly degrading HIF and inducing Nrf2 (Kim et al., 2007; Leonard et al., 2006). Although mammalian HIF and Nrf2 share some common targets genes such as aldehyde dehydrogenase 1A1 or heme oxygenase HO-1, the batteries of genes induced by re-oxygenation are different from those upregulated by oxygen deprivation (Hough and Piatigorsky, 2004; Leonard et al., 2006). Induction of HIF hydroxylases by SKN-1 / Nrf2 homologs would help to facilitate the rapid changes in gene expression needed to limit repurfusion injury.

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Table 3_1: Genes for which RNAi caused *hif-1*-dependent changes in *Pnhr-57:GFP* expression.

	Gene	**Description**	***Pnhr-57:GFP* expression pattern caused by RNAi**	
			hif-1 (+)	***hif-1 (ia04)***
Mitochondrial/ Metabolism	T09B4.9	Mitochondrial import inner membrane translocase	Gut	Weak gut
	sdhb-1	Succinate dehydrogenase subunit	Weak gut	dim
	sams-1	S-adenosyl methionine synthetase	Gut and weak hypodermal	Gut
	sco-1	Putative cytochrome C oxidase assembly protein	Weak gut	dim
	W02F12.5	oxoglutarate dehydrogenase complex	Gut	Weak gut
Protein turnover	*rpn-11*	Proteasome regulatory particle	Gut	Weak gut
	rpn-12	Proteasome regulatory particle	Gut	Posterior gut
Transcription/ translation	*rrt-2*	Arginyl-tRNA synthetase	Gut	Weak gut
	sbp-1	Transcription factor: SREBP homolog	Gut	dim
	rrt-2	Arginyl-tRNA synthetase	Gut	Weak gut
	vhl-1	E3 ligase, Von Hipple Lindau	Gut	dim
Known regulators of HIF-1	*egl-9*	Prolyl hydroxylase	Gut*	dim
	rhy-1	Acyltransferase	Gut and hypodermis	dim

*Strong loss-of-function mutations in *egl-9* cause *Pnhr-57*:*GFP* expression in the gut and hypodermis. This *egl-9* RNAi clone caused a partial loss-of-function phenotype.

FIGURE LEGENDS

Figure 3_1. Genome-wide RNAi screen to identify negative regulators of HIF-1-mediated gene expression. **A.** Illustration of screen design. The screen identified RNAi treatments that

increased the expression of *Pnhr-57:GFP*, a reporter that has been shown to be induced by hypoxia and positively regulated by HIF-1. **B.** Summary of the 179 genes identified from the RNAi screen.

Figure 3_2. RNAi inactivation of *sams-1* or *sbp-1* increased *Pnhr-57:GFP* expression. **A.** RNAi for the *sams-1* S-adenosyl methionine synthetase gene increased *Pnhr-57*:*GFP* expression 10-fold in animals carrying the wild-type *hif-1* allele, and increased the reporter 3-fold in animals carrying the *hif-1(ia04)* deletion. **B.** RNAi for the *sbp-1* SREBP homolog increased expression of the reporter in animals carrying the wild-type *hif-1* allele, but had no effect on *Pnhr-57*:*GFP* expression in *hif-1(ia04)* mutants. GFP levels were determined by protein blots, and the control animals were fed on bacteria carrying the empty RNAi vector (L4440). The experiments were conducted in RNAi-sensitive strains [*rrf-3(pk1426*)]. The bars represent average differences between experimental and control RNAi treatments from at least five biological replicates. For both *sams-1* and *sbp-1*, the differences in RNAi effect between *hif-1(+)* and *hif-1(ia04)* strains are statistically significant (p-value <0.05, by student *t*-test).

Figure 3_3. Identification of SKN-1 as a regulator of HIF-1. **A.** *skn-1* RNAi increased expression of the *Pnhr-57:GFP* reporter. Reporter gene expression was quantitated in L4 stage animals in normal culture conditions or after 2 or 4 hours of hypoxia treatment (0.5% oxygen). The protein levels were calculated from three independent experiments and normalized to 0 hr control RNAi. Asterisks indicate significant differences between control RNAi and *skn-1* RNAi at any given time point. *: $p<0.05$; **: $p< 0.01$. **B.** *skn-1* RNAi increased HIF-1 protein levels.

These animals expressed an epitope-tagged HIF-1 protein (Zhang et al., 2009). Bar heights reflect the average effects of *skn-1* RNAi on HIF-1 protein levels, as determined by 3 biological replicates, and values were normalized to the control RNAi. Two different RNAi clones were assayed, and they are designated *skn-1(1)* and *skn-1(2)* here.

Figure 3_4. Identification of *egl-9* as potential transcriptional target of SKN-1. **A.** Sequence from the *egl-9* promoter aligned with established SKN-1 binding sites in *gcs-1, med-1,* and *med-2*. Asterisks identify sequence identities shared by all four promoter regions in this interval, and predicted SKN-1 binding sites are in red. **B.** *skn-1* RNAi decreased *egl-9* mRNA levels. *egl-9* mRNA levels were quantitated in 3 independent RT-PCR experiments in L4-stage animals in normal or hypoxic conditions (1 or 2 hours in 0.5% oxygen). In normal culture conditions, *skn-1* RNAi reduced *egl-9* mRNA levels in L4 stage animals 20%. Asterisks indicate significant differences between control RNAi and *skn-1* RNAi at any given time point. *: $p<0.05$; **: $p< 0.01$.

Figure 3_5. Heat shock alters *Pegl-9:GFP* expression. **A.** The *Pegl-9:GFP* construct includes 1.6 kb of sequence 5' to the *egl-9* translational start. GFP coding sequence is diagramed as a green box. The red oval indicates the position of the putative SKN-1 binding site. **B-E.** *Pegl-9:gfp* expression in L4-stage animals under normal culture conditions and heat shock. Animals are shown as DIC images (**B, D**) and corresponding images of GFP fluorescence (**C, E**). In all images, the head is to the right. **B, C.** Under normal conditions, *Pegl-9:GFP* was expressed in the body muscle, vulva, pharynx, anterior intestine, rectal cells and additional cells in the tail. **D, E.** After heat shock treatment (29°C for 20h), *Pegl-9:GFP* was expressed much

more strongly in the intestine. The *Pegl-9:GFP* expression patterns in the L1, L2, L3 and adults were similar to that in the L4 worms, under both normal and heat shock conditions.

Figure 3_6. SKN-1 acts through the putative SKN-1 binding site in the *egl-9* promoter to activate *egl-9* expression. **A.** Heat shock induced *Pegl-9:GFP* in animals carrying the wild-type s*kn-1* allele, but did not induce the reporter in animals carrying the *skn-1(zu67)* loss-of-function mutation. **B.** Heat shock increased total levels of *Pegl-9*:*GFP* expression, but did not increase the expression of the reporter in which the putative SKN-1 site was mutated [*P(m)egl-9:GFP*]. The vertical axes show the log2 fold change is plotted for each strain, as determined by five biological replicates. To distinguish the SKN-1-mediated changes in *Pegl-9:GFP* expression from those that were mediated by HIF-1, these experiments were conducted in animals that lacked *hif-1* function. The data is presented in tabular form in supplemental materials.

Figure 3_7. *gsk-3* RNAi induction of *Pegl-9:*GFP. *gsk-3* RNAi increased expression of *Pegl-9*:GFP, relative to control RNA (the L4440 empty vector). This effect was dependent upon the putative SKN-1 binding site in the reporter [mutated in *P(m)egl-9:GFP*]. The figure shows log2 fold change from four biological replicates, with standard errors.

Figure 3_8. Effects of *skn-1* on HIF-1-mediated gene expression. **A.** The data support a model in which SKN-1 and HIF-1 regulate *egl-9* expression. Prior studies have identified targets of the SKN-1 and HIF-1 transcription factors and described the negative feedback loop in which HIF-1 promotes the expression of EGL-9. Prior studies have identified K10H10.2, F57B9.1, M05D6.5, and *rhy-1* as targets of both SKN-1 and HIF-1 and have shown that HIF-1 promotes *egl-9* expression in a negative feedback loop (Shen et al., 2005; Oliveira et al., 2009). **B-C.** Effects of

skn-1 RNAi, relative to control RNAi, on the relative levels of K10H10.2 mRNA (**B**) or F22B5.4 mRNA (**C**). L4-stage animals were assayed in normal culture conditions or after 1 or 2 hours of hypoxia treatment (0.5% oxygen). Each point was calculated from three independent RT-PCR experiments. Data are presented in tabular format in supplemental materials. Asterisks indicate significant differences between control RNAi and *skn-1* RNAi at any given time point. *: $p<0.05$; **: $p< 0.01$; ****: $p<0.0001$

FIGURES

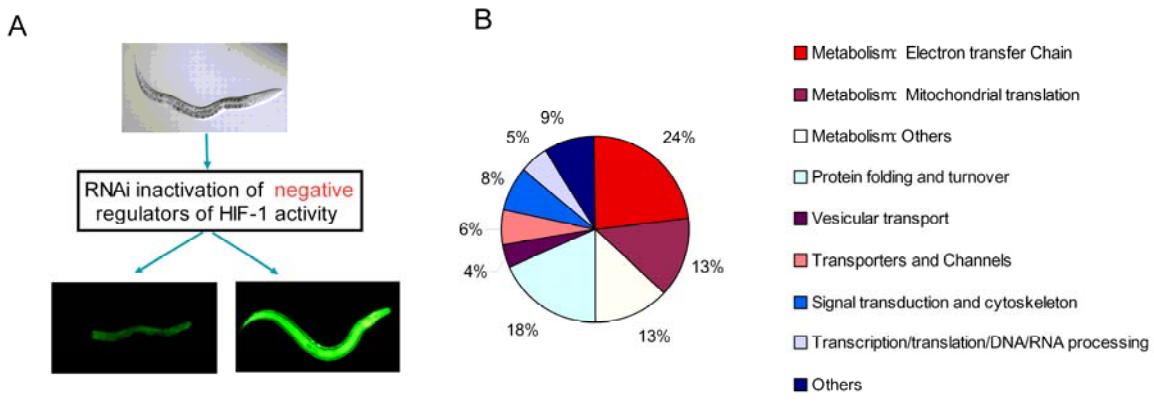

Figure 3_1. Genome-wide RNAi screen to identify negative regulators of HIF-1-mediated gene expression.

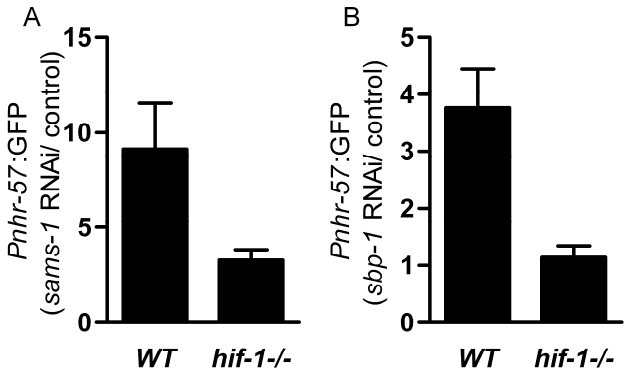

Figure 3_2. RNAi inactivation of *sams-1* or *sbp-1* increased *Pnhr-57:GFP* expression.

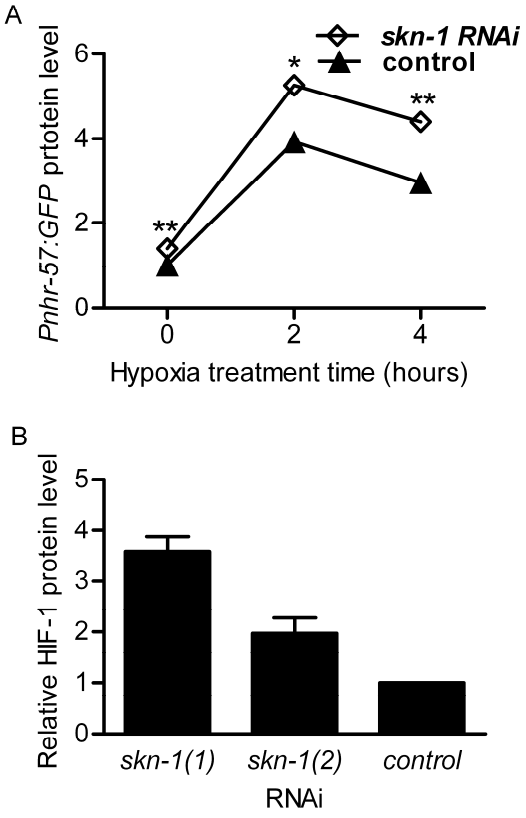

Figure 3_3. Identification of SKN-1 as a regulator of HIF-1.

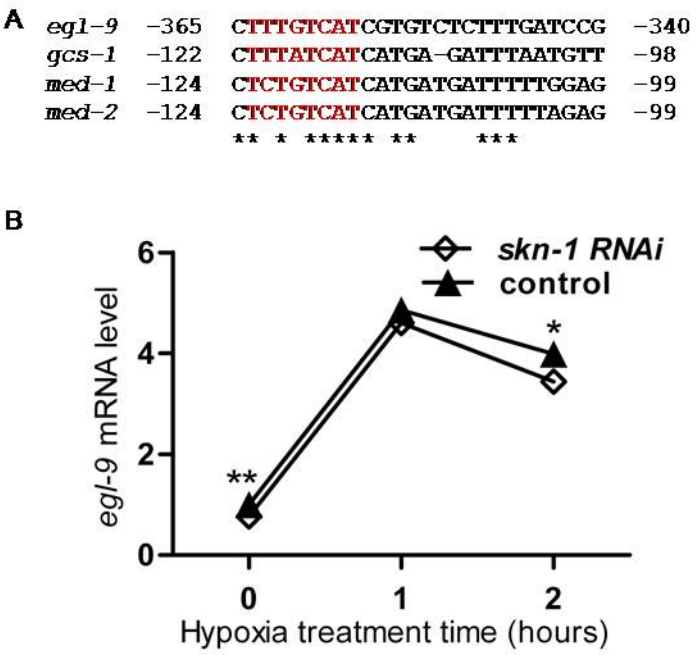

Figure 3_4. Identification of *egl-9* as potential transcriptional target of SKN-1.

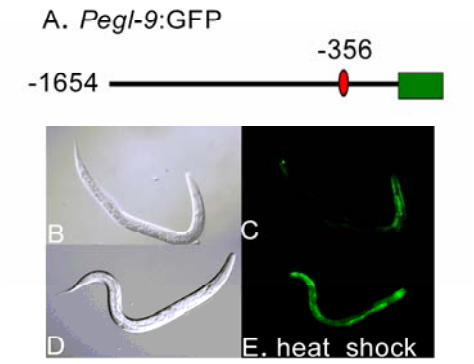

Figure 3_5. Heat shock alters *Pegl-9:GFP* expression.

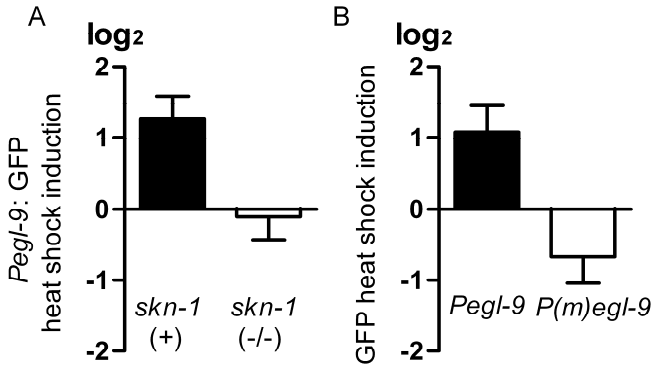

Figure 3_6. SKN-1 acts through the putative SKN-1 binding site in the *egl-9* promoter to activate *egl-9* expression.

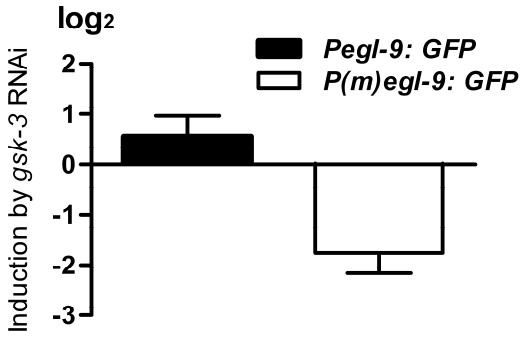

Figure 3_7. *gsk-3* RNAi induction of *Pegl-9:GFP*.

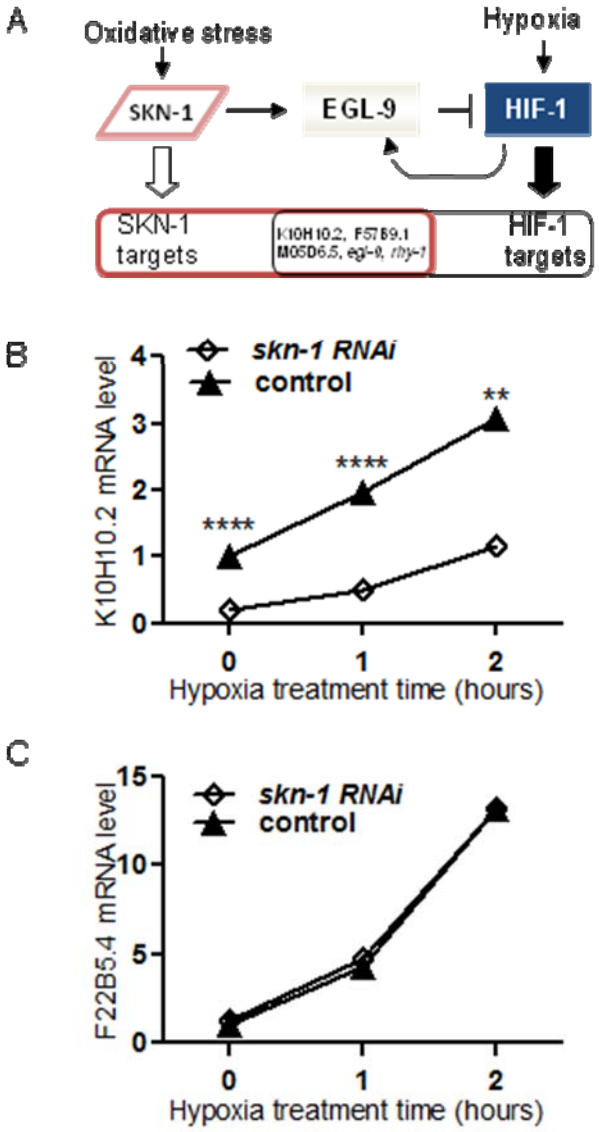

Figure 3_8. Effects of *skn-1* on HIF-1-mediated gene expression.

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURES

Figure 3_S1

A. *nhr-57* promoter sequence and primers used from chromatin immunoprecipitation experiments

```
                        1F →
ttgaataatttgattagctgattggcttatgacatcagctgcgggaagtcattatgcaaacgtg
aataatttggtagtacgtgtgttttattgttttctatttttgaattcaataacttgaataaga
aaaatataattgaaaactaacaatgatgcaaacaaacaaaata
                      ←1R               2F →
aacttacatcatatctttcgcttcttcaactcaaagagtgtttattgtactttacttttctctc
tctccctctcactctccatacctttttcatttcccaagtgtctatcaacaaaacatacacaaaa
agaggcgtaactctcactcaaaagatcagctggagaactatgcgtacgtgattagtggaacgtc
taacctcccgcgtctccacattcaatcgatcactat
                                ←2R               3F→
ccgaacaaattgtcatcgttttcggaaaacccagacctcgctgtaatttttgcatgatttcca
tctttgagacaccattttggtttgcatttctcacgtagacttcctggaggtcaccggtctttta
tcttatcaatcgtaattgttagactatataaaacgtgcggtgt
                                              ← 3R
tcaactacatgcttttgtatttttaaagagtgaatcaagagatgttggtggctcgcg
```

B. Primers sequences

nhr-57 1 F 5'---attggcttatgacatcagctgc---3’

nhr-57 1 R 5'---cactctttgagttgaagaagcg---3’

nhr-57 2 F 5'---cgcttcttcaactcaaagagtg---3’

nhr-57 2 R 5'---ttacagcgaggtctgggttttc---3’

nhr-57 3 F 5'---gaaaacccagacctcgctgtaa---3’

nhr-57 3 R 5'---ccaacatctcttgattcactct---3’

inf-1 F 5'---ttccgttcaggatcttcccgtgtt---3’

inf-1 R 5'---tctcggtgacgaagttgatggcaa---3’

C.

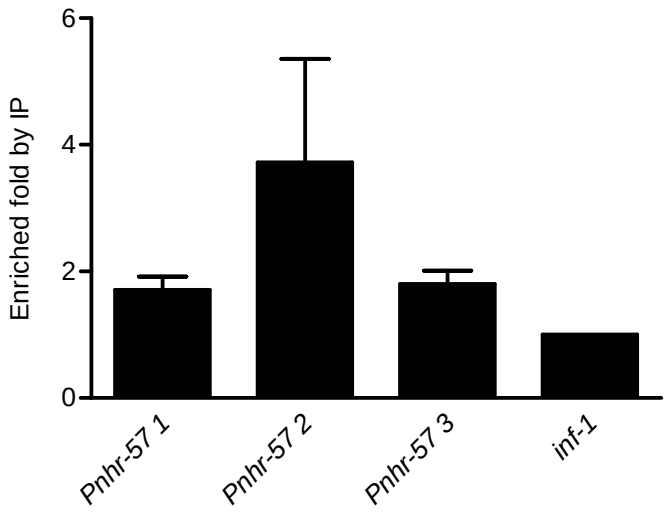

Figure 3_S1C: Chromatin co-immunoprecipitation data.

In these experiments the endogenous *hif-1* locus was disrupted by the *ia04* deletion and HIF-1 function was restored by the *Phif-1:hif-1a:MYC:HA* transgene (Zhang et al., 2009). The relative amounts of genomic sequences that co-immunoprecipitated with *HIF-1:MYC:HA* was determined by quantitative PCR. The bars show the average enriched fold from at least 3 independent replicates.

Data for Figure 3_S1C in tabular form

	Relative enriched fold			
Experiments	*Pnhr-57 1*	*Pnhr-57 2*	*Pnhr-57 3*	*inf-1*
1	2.106	10.196		1.
2	1.257	1.310		1.
3	2.158	2.621	2.000	1.
4	1.853	2.550	2.028	1.
5	1.173	1.931	1.376	1.
Average	1.709	3.721	1.801	1

Figure 3_S2

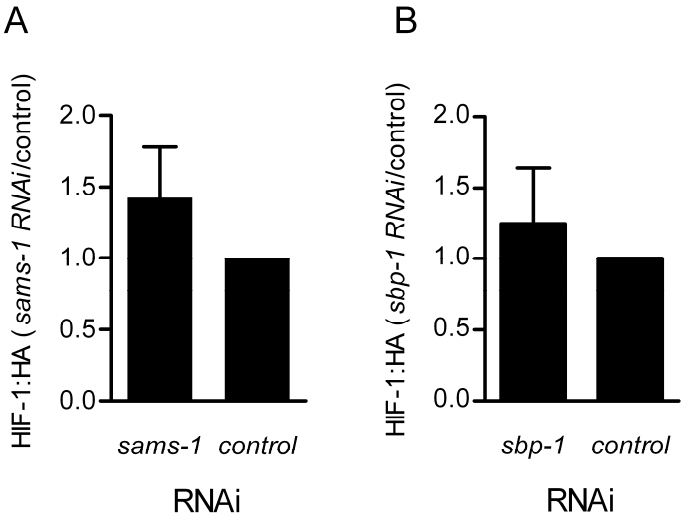

Figure 3_S2: *sams-1* ans *sbp-1* RNAi did not change HIF-1 protein levels.

Expression levels of *Phif-1:hif-1a:HA* transgene were assayed in adults treated with control RNAi (empty vector L4440), *sams-1* RNAi (A) or *sbp-1* RNAi (B) (Zhang Y et al., 2009). The

bars show the averages from at least 3 independent replicates and the error bar represented the standard error.

Data for Figure 3_S2A in tabular form

Experiments	1	2	3	4	Average
Phif-1:hif-1a:MYC:HA (*sams-1* RNAi/control RNAi)	1.08	1.9	2.13	0.6	1.42

Data for Figure 3_S2B

Experiments	1	2	3	Average
Phif-1:hif-1a:MYC:HA (*sbp-1* RNAi/control RNAi)	1.445	1.805	0.500	1.25

Data from Figure 3_2A: The repression of *Pnhr-57:GFP* by *sams-1* is partially dependent on HIF-1 activity

Pnhr-57: GFP on RNA*i*	WT（ *sams-1*/L4440）	*hif-1 -/-* (*ia04*)（ *sams-1*/L4440）
Exp1	13.5	4
Exp2	4.5	4.0
Exp3	3.7	3.9
Exp4	15	3.6
Exp5	15	3.7
Exp6	3.0	0.6
Mean	**9.12**	**3.3**
s.e.	**2.42**	**0.54**
p-value (paired t-test)	**0.048**	

Data from Figure 3_2B: *sbp-1* negatively regulates *Pnhr-57:GFP* expression through *hif-1* dependent mechanism

Pnhr-57:GFP on RNA*i*	WT（ *sbp-1*/L4440）	*hif-1 -/-* (ia04)（ *sbp-1*/L4440）
Exp1	4.357021	0.732547
Exp2	2.605624	0.918757
Exp3	1.734806	0.843413
Exp4	5.103881	1.45511
Exp5	5.047583	1.776325
Mean	**3.77**	**1.14**
s.e.	**1.52**	**0.45**
p-value (paired t-test)	**0.009606**	

Data from Figure 3_3A: Effect of *skn-1* RNAi on hypoxia induction of *Pnhr-57:GFP* protein levels

Time in hypoxia	Genotype	Relative blot intensity	*p*-value
0 hr	control RNAi	1.0	0.0086
	skn-1 RNAi	1.4	
2 hr	control RNAi	3.9	0.0165
	skn-1 RNAi	5.3	
4 hr	control RNAi	3.0	0.00305
	skn-1 RNAi	4.4	

Pnhr-57:GFP levles were calculated from three independent western blotting experiments. Values were normalized to 0 hr control RNAi. *P*-values were used to determine whether the induction differences between control RNAi and. *skn-1* RNAi were significant at each hypoxia treatment time point.

Data from Figure 3_3B: Effect of *skn-1* RNAi on *Phif-1:HIF-1a:HA expressions*

Exp	1	2	3	4	Average
skn-1(1)/L4440	*3.18*	*4.44*	*3.49*	*3.24*	3.59
skn-1(2)/L4440	*N/A*	*1.46*	*2.49*	*1.97*	1.97

Data from Figure 3_4B: Effect of *skn-1* RNAi on hypoxia induction of *egl-9* mRNA levels

Time in hypoxia	Genotype	Relative expression level	*p*-value
0 hr	control RNAi	1.0	0.002
	skn-1 RNAi	0.8	
1 hr	control RNAi	4.9	0.41
	skn-1 RNAi	4.6	
2 hr	control RNAi	4.0	0.045
	skn-1 RNAi	3.4	

egl-9 mRNA levels were calculated from three independent RT-PCR experiments using efficiency-corrected comparative quantification (Pfaffl, 2001). *inf-1* was used as the reference gene. *P*-values were used to determine whether the expression level differences between control RNAi and *skn-1* RNAi were significant at each hypoxia treatment time point.Data from Figure

Data from Figure 3_6A: Heat shock induces *Pegl-9:GFP* in a *skn-1* dependent manner.

Heat shock duration	Genotype	Log2 fold change Ls mean	p-value
12 hr	*skn-1 (+)*	1.3	0.015
	skn-1 (zu67)	-0.1	
14 hr	*skn-1 (+)*	1.1	0.223
	skn-1 (zu67)	0.5	

Log2 fold change least square means were calculated from four independent western blotting experiments. The fold change was determined by dividing the *Pegl-9:GFP* level in heat shock treatment by the *Pegl-9:GFP* level of corresponding non-heat shock control. *P*-value was used to determine whether the *Pegl-9::GFP* induction difference between *skn-1 (+) and skn-1 (zu67)* was significant.

Data from Figure 3_6B: Mutation of the putative SKN-1 binding site reduced the activation of *Pegl-9:GFP* by heat shock.

Heat shock duration	Genotype	Log2 fold change Ls mean	*p*-value
12 hr	*Pegl-9:GFP*	1.1	0.0036
	P(m)egl-9:GFP	-0.7	
14 hr	*Pegl-9:GFP*	0.5	0.0850
	P(m)egl-9:GFP	-0.4	
16 hr	*Pegl-9:GFP*	0.5	0.6605
	P(m)egl-9:GFP	0.2	

Log2 fold change least square means were calculated from five independent western blotting experiments. The fold changes were determined by dividing the GFP expression levels in the heat shock treatment by the GFP levels of corresponding non-heat shock control. *P*-value were used to determine whether the induction differences between *Pegl-9:GFP* and *Pegl-9(m):GFP* were significant at each heat shock time point.

The induction of *Pegl-9:GFP* was greatest at 12 hours so this condition was used for subsequent experiments and the data from the 12 hours treatment is represented in Figure 6B.

Data from Figure 3_7: *gsk-3 RNAi induction of Pegl-9:GFP* and *P(m)egl-9:GFP*.

Duration of RNAi treatment	Genotype	Log2 fold change Ls mean	*p*-value
96 hr	*Pegl-9:GFP*	0.3	0.137
	P(m)egl-9:GFP	-0.7	
102 hr	*Pegl-9:GFP*	0.6	0.0013
	P(m)egl-9:GFP	-1.8	
120 hr	*Pegl-9:GFP*	0.1	0.3
	P(m)egl-9:GFP	-0.6	

Log2 fold change least square means were calculated from four independent western blotting experiments. The fold change was determined by dividing the GFP expression levels in the *gsk-3* RNAi treatment by the GFP levels in corresponding control RNAi treatment. *P*-values were used

to determine whether the *GFP* induction differences between *Pegl-9:GFP* and *Pegl-9(m):GFP* were significant at each RNAi treatment time point.

These studies identified the treatment condition in which *gsk-3* RNAi had the greatest effect (102 hours RNAi treatment) and the data from this time point are shown in Figure 7.

Data from Figure 3_8B: Effect of *skn-1* RNAi on hypoxia induction of K10H10.2 mRNA levels

Time in hypoxia	Genotype	Relative expression level	*p*-value
0 hr	control RNAi	1.0	<0.0001
	skn-1 RNAi	0.2	
1 hr	control RNAi	2.0	<0.0001
	skn-1 RNAi	0.5	
2 hr	control RNAi	3.1	0.0019
	skn-1 RNAi	1.2	

K10H10.2 mRNA levels were calculated from three independent RT-PCR experiments using efficiency-corrected comparative quantification (Pfaffl, 2001). *inf-1* was used as the reference gene. *P*-value were used to determine whether the expression level differences between control RNAi and *skn-1* RNAi were significant at each hypoxia treatment time point.

Data from Figure 8C: Effect of *skn-1* RNAi on hypoxia induction of F22B5.4 mRNA levels

Time in hypoxia	Genotype	Relative expression level	*p*-value
0 hr	control RNAi	1.0	0.13
	skn-1 RNAi	1.3	
1 hr	control RNAi	4.3	0.46
	skn-1 RNAi	4.8	
2 hr	control RNAi	13.2	1.00
	skn-1 RNAi	13.2	

F22B5.4 mRNA levels were calculated from three independent RT-PCR experiments using efficiency-corrected comparative quantification (Pfaffl, 2001). *inf-1* was used as the reference gene. *P*-value were used to determine whether the expression level differences between control RNAi and *skn-1* RNAi were significant at each hypoxia treatment time point.

Supplemental material and methods

Chromatin Immunoprecipitation and CHIP PCR.

Worms containing strong loss-of-function mutations in *hif-1* and *egl-9* and carrying the rescuing transgene *Phif-1:hif-1a:MYC:HA* (Zhang et al., 2009) were grown on enriched NGM plates at 20^{o}C. The animals were pelleted by low-speed centrifugation to ~ 1 ml pellets for each strain. The animals were washed 3 times in cold M9 buffer. The DNA was crosslinked to proteins by incubation in 2% formaldehyde for 30 minutes at room temperature in PBS. Chromatin immunoprecipitation was carried out as previous described (Chu et al., 2002) with some modifications. The samples were sonicated six times for 15-second at 30% power using an Amtek sonic dismembrator (model 150). The HIF-1 complexes were immunoprecipitated with monoclonal antibodies specific to the myc epitope. After the reverse crosslinking reactions and purification, the DNA fragments were analyzed by quantitative PCR. We designed 3 pairs of primers to amplify different regions on the promoters of *nhr-57*. The primer sequences used for CHIP PCR are in Supplemental Figure 1B. The *inf-1* gene was used a negative control to monitor non-specific binding to protein G beads or the anti-myc antibody. The relative enrichment of each 3 *nhr-57* promoter region was calculated and normalized to that of *inf-1*.

Statistical analyses

To compare *Pnhr-57:GFP* expression levels in animals treated with *skn-1* RNAi and control (L4440 empty vector) RNAi at each time point, the Log2 of each value was calculated and analyzed using an ANOVA model to generate the *P*-values. In these analyses, time point (time in hypoxia), RNAi treatment (*skn-1* RNAi or control RNAi), hypoxia time by RNAi treatment interaction, and the three independent replicates were treated as fixed effect factors.

egl-9, K10H10.2 or F22B5.4 mRNA levels were assayed by RT-PCR and were compared to the *inf-1* control. To compare the effects of *skn-1* RNAi control (L4440 empty vector) RNAi on the expression of each mRNA, the Log2 relative enrichment was analyzed using an ANOVA model. In these analyses, the time in hypoxia, RNAi treatment (*skn-1* RNAi or control RNAi), time point by RNAi treatment interaction, and the three independent replicates were each treated as fixed effect factors.

To compare the effects of heat shock on *Pegl-9:GFP* expression in *hif-1 (ia04)* versus *hif-1 (ia04);skn-1(zu67)* animals at each time point, Log2 fold changes were analyzed using an ANOVA model to generate the *P*-values. The fold change was determined by dividing the western blot intensity of the heat shock sample by the intensity of corresponding non-heat shock sample. Heat shock time (12h or 14h), genotype [*hif-1 (ia04)* or *hif-1 (ia04);skn-1(zu67)*], time point by genotype interaction, and the four independent replicates were each treated as fixed effect factors.

To assess the differences in heat shock induction of the *Pegl-9:GFP* and *P(m)egl-9:GFP* reporters, Log2 fold changes were analyzed using an ANOVA model to generate the *P*-values. The fold change was determined by dividing the western blot intensity of the heat shock sample by the intensity of corresponding non-heat shock sample. Heat shock treatment, genotype, time by genotype interaction, and the five independent replicates were each treated as fixed effect factors.

To assess whether the *Pegl-9:GFP* and *P(m)egl-9:GFP* reporters responded to *gsk-3* RNAi differentially, Log2 fold changes were analyzed using an ANOVA model. The fold change was determined by dividing the western blot intensity of the *gsk-3* RNAi sample by the intensity of corresponding control RNAi samples. RNAi treatment, time by RNAi treatment interaction, and the four independent replicates were each treated as fixed effect factors.

Reference

Chu, D. S., Dawes, H. E., Lieb, J. D., Chan, R. C., Kuo, A. F., Meyer, B. J., 2002. A molecular link between gene-specific and chromosome-wide transcriptional repression. Genes Dev. 16**,** 796-805.

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Zhang, Y., Shao, Z., Zhai, Z., Shen, C., Powell-Coffman, J. A., 2009. The HIF-1 hypoxia-inducible factor modulates lifespan in *C. elegans*. PLoS One. 4**,** e6348.

CHAPTER 4: THE ROLE OF HIF-1 IN LONGEVITY ASSOCIATED WITH MITOCHONDRIA MALFUNCTION IN *C. ELEGANS*

ABSTRACT

Mitochondrion is a key organelle in the cell. It plays important role in the energy production and regulation of metabolism. Mitochondria consumed most of the molecular oxygen in the cell. Previous studies have shown the regulation role of mitochondria on the HIF-1 stability through the increased production of mitochondrial ROS or accumulation of TCA cycle intermediates in tumor cell lines. As a transcription factor, HIF-1 also regulated mitochondrial function through its target genes.

C. elegans has been established to be excellent model to study post mitotic aging. In *C.elegans* mitochondrial genes has also play an important role in longevity. Previous RNAi genomic screens found that RNAi inactivation of mitochondrial genes extended C. elegans life span. Genetic mutants in mitochondrial genes promoted the longevity of C. elegans. *clk-1* is one of the few viable mitochondrial mutants that extended the life span. In *C.elegans*, both *hif-1* mutant and HIF-1 overexpressed worms lived longer than wild type animals.

Here we tested the hypothesis that HIF-1 function is regulated by mitochondrial gene *clk-1* and is essential for life span extension associated with the mutation in clk-1 in C.elegans. We found that loss-of-function mutation of mitochondrial gene *clk-1* induced one HIF-1 target gene's

expression without affecting HIF-1 protein stability. The life span extension of *clk-1* was partially suppressed in *hif-1* genetic mutant. Overexpression of HIF-1 further extended the life span of *clk-1* mutant. The data presented here support a model that moderate dysfunction of mitochondria induces HIF-1 taget, thereby promoting longevity in *C. elegans*.

INTRODUCTION

Hypoxia inducible factor (HIF) plays an important role in oxygen homeostasis in all metazoans. *C. elegans* HIF-1 is mammalian HIF-1α (Jiang et al., 2001). It is regulated by evolutionarily conserved Prolyl hydrodroxylase PHD/EGLN pathway (Epstein et al., 2001).

Last year our group identified a key role of HIF-1 in *C. elegans* longevity (Zhang et al., 2009). We employed HIF-1 transgene in the life span and stress resistance assays. The substitution of proline-621 by glycine residue prevents HIF-1 from being hydroxylated and degraded by the EGL-9/VHL-1 pathway. The *hif-1 (P621G)* transgenic lines live up to 34% longer than wild-type N2 worms. The life span extension proportionately correlated with the various HIF-1 protein levels in different *hif-1 (P621G)* transgenic lines. The mechanism by which *hif-1 (P621G)* extends lifespan in worms is genetically distinguished from the insulin/IGF-1 (DAF-2/DAF-16) signaling-like pathway and SKN-1/Nrf phase II detoxification pathway. An independent study from *Dr. Kaeberlein's* research group provided additional support for our conclusion (Mehta et al., 2009). They showed that RNAi inactivation of *vhl-1* or *egl-9* extended the life span and enhanced the resistance to polyglutamine and β-amyloid toxicity in *C. elegans*. The increased longevity associated with *vhl-1* RNAi knockdown was completely abolished by loss-of-function mutation of *hif-1*. Together with our finding, it demonstrated that

HIF-1 played an important role in longevity in *C. elegans*. However the mechanism behind this longevity effect is largely unknown.

In recent decades, *C. elegans* researchers have also revealed an important role of mitochondria in longevity. The first identified mitochondrial longevity gene is *clk-1* (clock-1) (Wong et al., 1995). *clk-1* mutants phenotype has been well characterized in several studies (Ewbank et al., 1997; Wong et al., 1995). The average life span of *clk-1* mutants is around 40% longer compared to that of wild type N2 animals and the life span extension in *clk-1* mutants is independent of insulin/IGF-1 signaling pathway. *clk-1* mutant strain has lower embryonic and postembryonic development rates.

Later studies found that CLK-1 encodes an enzyme which is responsible for the final step of ubiquinone (CoQ9) synthesis (Ewbank et al., 1997; Stenmark et al., 2001). CoQ is an important component of mitochondria electron transfer chain (ETC). It receives electrons from ETC complex I and complex II, then shuttles them to the ETC complex III. Mitochondria electron transfer chain is partially compromised in *clk-1* mutant animals. Biochemical study showed that *clk-1* mutants had lower reactive oxygen species concentration and higher AMP:ATP ratio compared to wild type N2 worms (Kayser et al., 2004).

Recent study of mouse *clk-1* showed that HIF-1 protein level increased in liver nuclei of *Mclk1* heterozygous mutant mice as well as in *Mclk1* siRNA knockdown cells (Wang et al., 2010). It suggested that HIF activity could be induced by the mutation of mitochondrial genes.

Our finding from genome-wide RNAi screen also provided an important piece of evidence. In the screen we found almost all the current known mitochondrial longevity genes of

which RNAi inactivation induced *Pnhr-57:GFP*, a *hif-1*-dependent reporter (In chapter 3, Table 3_S1). It suggested that there may be an interaction between mitochondria function and HIF-1 activity in *C. elegans.*

The findings from our genetic screen and the study of mouse *clk-1* both indicated that HIF-1 activity may be induced in mitochondrial longevity mutant. Here we tested the hypothesis that HIF-1 can be activated by mutation of mitochondrial ETC genes, therefore mediate the life span extension associated with the mutation of mitochondria ETC gene. *clk-1* mutant has been well characterized phenotypically and molecularly, whereas most of the other mitochondrial mutants are lethal or sterile. So we employed *clk-1* mutant in our study and also examined other mitochondria ETC genes by RNAi.

MATERIAL AND METHODS

C. elegans strains

MQ130: *clk-1(qm30)III*

RB1234: *clk-1(ok1247)III*

ZG503: *clk-1 (qm30)III; Pnhr-57:gfp(iaIs07)IV*

ZG506: *clk-1 (qm30)III; Pnhr-57:gfp(iaIs07)IV; hif-1(ia04)V*

ZG513: *clk-1 (qm30)III; hif-1(ia04)V*

ZG514: *clk-1 (ok1247)III; hif-1(ia04)V*

ZG517: *clk-1 (qm30)III; Phif-1:hif-1(P621G)a:MYC (iaIs34)*

ZG518: *clk-1 (ok1247)III; Phif-1:hif-1(P621G)a:MYC (iaIs34)*

ZG519: *clk-1 (qm30)III; hif-1(ia04)V; Phif-1:hif-1a:MYC:HA(iaIs28)*

ZG520: *clk-1 (ok1247)III; hif-1(ia04)V; Phif-1:hif-1a:MYC:HA(iaIs28)*

The transgenes *Phif-1:hif-1(P621G)a:MYC (iaIs34) and Phif-1:hif-1a:MYC:HA(iaIs28)* were previously described (Shao et al., 2009; Zhang et al., 2009).

Bacteria strains

Q-less: GD1, ubiG::Kan, zei::Tn10dTet, contained a deletion of the ubiG gene required for Q biosynthesis (Hsu et al., 1996).

Q-replete: GD1:pAHG, a Q8-replete strain of E. coli

Protein blot

Prior to feed assays, all strains were maintained at 20 °C for at least two generations with abundant food. To assay *Pnhr-57:gfp* expression, the synchronized worms were cultured until they reached L4 stage. L4 worms were transferred to plates with Q-less or Q-replete bacteria. After 48 hours, 20 adult worms from each plate were boiled in 2XSDS loading buffer. And worm lysates were size fractioned on polyacrylamides gels and analyzed by western blots. The primary antibody dilution was 1:500 for GFP specific mouse monoclonal [from Roche). To assay HIF-1 protein level, 80 synchronized young adult worms fed on OP50 were boiled in 2XSDS loading buffer. And worm lysates were size fractioned on polyacrylamides gels and

analyzed by western blots. The primary antibody dilution was 1:250 for HA specific mouse monoclonal [from Cell Signaling] .

Longevity assay

The longevity assay methods were previously described (Zhang et al., 2009). Prior to the assays, all strains were maintained at 20 ℃ for at least two generations with abundant food.

In non RNAi longevity assay, at day 0, the synchronized L4 worms were transferred to NGM plates with newly spotted OP50. Then the worms were transferred to newly spotted plates every other day until day 10. From day 10 the worms number on each plate were recorded every other day and transferred to newly spotted NGM plate every six days until all of them were dead. The non RNAi longevity assay NGM plates did not contain Fudr or any antibiotic.

In mitochondria gene RNAi longevity assay, each bacteria clone was grown in 2mL LB medium with 50ug/mL ampicillin at 37 ℃ for 6 hours, then spotted 200mL on each RNAi feeding plate. The RNAi feeding plate is the NGM plate with 5mM IPTG and 25ug/mL carbenicillin. Let the plate dry for overnight at room temperature. At the next day 6 adult worms were allowed to lay eggs on RNAi feeding plate for 6 hours before removal. Kept the plates in 20℃ for 2 or 3 days, transferred the synchronized L4 to RNAi feeding plate with newly induced bacteria. We put 20-25 worms on each plate. Then the worms were transferred to RNAi feeding plates with newly induced bacteria every other day until day 10. From day 10 the worms number on each plate were recorded every other day and transferred to RNAi feeding plate with newly induced bacteria every six days until all of them were dead. The RNAi longevity assay NGM plates did not contain Fudr or any antibiotic except carbenicillin.

The life span data were analyzed by JMP7. And p-values were determined by log-rank test.

RESULTS

Pnhr-57:GFP expression level increases in *clk-1* mutant worms feeding on Q-less or Q-replete bacteria.

Pnhr-57:GFP reporter has been previously described and widely used as a monitor of HIF-1 activity (Budde and Roth, 2010; Shao et al., 2009; Shen et al., 2005; Shen et al., 2006; Zhang et al., 2009). Our recent study further demonstrated that *nhr-57* is a direct target of HIF-1 transcription factor in *C. elegans* (unpublished data). Q-less and Q-replete bacteria were also previously described (Jonassen et al., 2002; Larsen and Clarke, 2002). Q-less bacteria (GD1) contain a deletion mutation of the ubiG gene that is required for CoQ biosynthesis, so Q-less bacteria cannot generate CoQ8. However *clk-1* mutant worms require bacteria-derived dietary CoQ8 for development and reproduction, so when fed on the Q-less bacteria, *clk-1* mutants arrest at larval stage. Q-replete bacteria (GD1:pAHG) contain ubiG transgene which can rescue the synthesis of CoQ8.

When fed on Q-less bacteria or Q-replete bacteria, both *clk-1* mutant and *clk-1; hif-1* double mutant showed the induction of *Pnhr-57:GFP* compared to wild type animals and *hif-1* single mutant respectively. But the expression pattern and level of GFP are different between *-1* mutant and *clk-1; hif-1* double mutant. On Q-less bacteria, *Pnhr-57:GFP* was highly expressed through the whole body in *clk-1* mutants (Figure 4_1A), whereas in *clk-1;hif-1* double mutants, GFP is strongly induced in the intestine (Figure 4_1C). The different GFP expression patterns in *clk-1* mutants and *clk-1; hif-1* double mutants were not obvious when feeding worms Q-

replete bacteria (Figure 4_1B and 4_1D). The GFP expression levels in *clk-1* mutant and *clk-1; hif-1* double mutant worms were also assayed by protein blot on both bacteria sources. On Q-less bacteria, GFP expression in *clk-1* single mutant is 2-fold higher than that in *clk-1; hif-1* double mutant (Figure 4_2A). Similar result was obtained in worms fed on Q-replete bacteria (Figure 4_2B). Although *Pnhr-57:GFP* was induced, HIF-1 protein level did not change in *clk-1* mutant animals (Figure 4_2C). The results suggested that CLK-1 regulated *hif-1*-dependent reporter's expression without affecting HIF-1 protein stability.

Loss-of-function mutation of *hif-1* suppresses longevity of *clk-1* mutant worms

clk-1 is also one of the earliest identified longevity genes in *C. elegans*. At 20 °C the mean life span of wild type nematode is around 3 weeks. *clk-1* loss-of- function mutant strains live 30-40% longer than wild type N2 animals (Ewbank et al., 1997; Wong et al., 1995). Overexpression of HIF-1 also promotes the longevity in *C. elegans* (Mehta et al., 2009; Zhang et al., 2009). Here we found that the average life span of *clk-1*(ok1247) mutants was around 33 days, whereas the mean life span of the worms carrying deletion mutations in both *clk-1* (ok1247) and *hif-1* locus was only 22 days. Loss-of-function mutation of *hif-1* completely abolished the life span extension of *clk-1* (ok1247) mutants (Figure 4_3A). For another clk-1 mutant strain, *clk-1*(qm30), their average life span is around 29 days and that of *clk-1*(qm30); *hif-1*(ia04) double mutants is about 27 days (Figure 4_3B). From the study of two different alleles of *clk-1* mutant, it suggested that *clk-1* mutants require HIF-1 function to promote the longevity in *C. elegans*.

HIF-1 transgene fully restores the life span extension in *clk-1* mutants.

To confirm that activation of HIF-1 function accounts for the increased longevity associated with *clk-1* knockout, we preformed a rescue experiment with *Phif-1:hif-1a:MYC:HA(iaIs28)* transgene. *Phif-1:hif-1a:MYC:HA* transgene was previously described (Zhang et al., 2009). This *Phif-1:hif-1a:MYC:HA* transgene has been shown to partially restore endogenous HIF-1 function (Shao et al., 2009; Zhang et al., 2009). The mean life span of worms carrying both *Phif-1:hif-1a:MYC:HA* transgene and *hif-1* deletion mutation was around 22 days, about 2 days longer than that of wild type N2 worms (Zhang et al., 2009). So here we introduced *Phif-1:hif-1a:MYC:HA* transgene into *hif-1;clk-1* double mutant animals and assayed their life spans. By average, the worms carrying *Phif-1:hif-1a:MYC:HA* and *hif-1; clk-1*(ok1247) double mutations lived 3 days longer than *clk-1*(ok1247) single mutant (Figure 4_3C). Similar result was found in the study of *clk-1*(qm30) strain (Figure 4_3D). It confirmed that HIF-1 function is essential for the life span extension of *clk-1* mutants.

Overexpression of HIF-1 further extends the life span of *clk-1* mutant animals.

It has been shown that *hif-1(P621G)* gain-of-function transgenic animals stabilized HIF-1 protein and promoted the longevity in *C. elegans* (Zhang et al., 2009). Here we also tested the influence of *hif-1(P621G)* on the increased longevity associated with *clk-1* knockout by crossing *hif-1*(P621G) transgene to *clk-1* loss-of-function mutant. These animals live longer than the worms carrying *clk-1* loss-of-function mutation alone (Figure 4_3 E-F). It suggests that HIF-1 overexpression and *clk-1* mutation have additive effect on the life span extension in *C. elegans*.

HIF-1 overexpression does not further extend the life span in mitochondria gene knockdown worms.

Previous RNAi screens discovered *C. elegans* longevity (Hamilton et al., 2005; Hansen et al., 2005; Lee et al., 2003). RNAi knockdown each of these mitochondria genes promoted the longevity in *C. elegans*. We have shown that HIF-1 modulated the life span extension of *clk-1* mutant. Here we tested whether the HIF-1 function also accounted for the increased longevity associated with other mitochondria ETC gene knockdown by RNAi. Two genes were chosen for the longevity assay according to their significant effect on life span extension demonstrated by RNAi knockdown in previous studies (Lee et al., 2003). T02H6.11 encodes QCR7 subunit of ubiquinol cytochrome c reductase which is part of mitochondria ETC complex III and D2030.4 encodes NDUFB7/B18 subunit of mitochondria ETC complex I NADH:ubiquinone oxidoreductase. RNAi inactivation of either T02H6.11 or D2030.4 extends the life span in a *daf-16* independent manner (Hansen et al., 2005; Lee et al., 2003). Here we showed that *hif-1(P621G)* gain-of-function transgene increased the longevity of worms fed on RNAi control (L4440) bacteria (Figure 4_4A), which was consistent with our previous finding of the worms fed on OP50 (Zhang et al., 2009). However the worms carrying *hif-1(P621G)* transgene did not live longer than wild type N2 worms when both of them were cultured on T02H6.11 RNAi bacteria or D2030.4 RNAi bacteria (Figure 4_4B-C). Overexpression of HIF-1 cannot further extend the life span associated with T02H6.11 or D2030.4 gene knockdown. We also assayed the life span of *hif-1* loss-of-function mutants in this RNAi longevity study. However, most of worms carrying *hif-1* loss-of-function mutation arrested at larvae stage and survived for 5-6 weeks when they were fed on T02H6.11 RNAi bacteria or D2030.4 RNAi bacteria (data not

shown). This also suggests that HIF-1 function is essential for the worms with mitochondria function deficiency.

DISCUSSION

These studies describe a role of HIF-1 in aging associated with mitochondria function deficiency in *C. elegans*. Mitochondrial gene *clk-1* regulates the expression of HIF-1 target without affecting HIF-1 protein levels. HIF-1 function is essential for the life span extension associated with *clk-1* knockout. The results confirm that mitochondria play an important role in the regulation of hypoxia signaling in *C. elegans*.

clk-1 modulates the expression of *hif-1*-dependent reporter on different bacteria dietaries

clk-1 encodes a key enzyme which is responsible for the synthesis of functional CoQ in respiration chain. *clk-1* mutant worms require bacteria-derived dietary CoQ8 for development and reproduction. Fed on Q-less bacteria or Q-replete, *Pnhr-57:GFP* expression increased in *clk-1* mutants. Previous studies have demonstrated that *nhr-57* was one of HIF-1 targets and induced by hypoxia in a *hif-1*-dependent manner (Shen et al., 2005; Shen et al., 2006). However in the previous RNAi screen we showed that *Pnhr-57:GFP* was activated by some RNAi treatment though *hif-1*-independent pathway in chapter 3. Here we also found that worms carrying loss-of-function mutation of both *clk-1* and *hif-1* genes induced *Pnhr-57:GFP* expression. But compared to that in *clk-1* single mutants, the induction in *clk-1;hif-1* double mutants was weaker (Figure 4_2). The regulation of *Pnhr-57:GFP* reporter by CLK-1 is partially *hif-1*-dependent. Moreover we also showed that HIF-1 protein levels do not change in

clk-1 mutants. Together we concluded that CLK-1 regulated the expression of this *hif-1*-dependent reporter without affecting HIF-1 protein stability in *C. elegans*.

The life span extension in *clk-1* mutants is partially mediated by HIF-1 function

After showing that the *hif-1*-dependent reporter is regulated by CLK-1, we investigated whether HIF-1 activation mediates the phenotypes associated with *clk-1* knockout. *clk-1* is one of the earliest identified longevity genes in *C. elegans* (Wong et al., 1995). Here we also found that worms carrying loss-of-function mutation of *clk-1* (both *qm30* and *ok1247* alleles) lived around 30 days by average, whereas the mean life span of wild type N2 worms was around 20 days. Previously we also identified an important role of HIF-1 in the regulation of *C. elegans* longevity (Zhang et al., 2009). In the life span assay of worms carrying both loss-of-function mutation of *clk-1* and *hif-1*, we found that loss-of-function mutation of *hif-1* partially suppressed life span extension in *clk-1* mutants (Figure 4_3 A-B). Additionally *hif-1(iaIs28)*, a transgene including 5.2 kb of *hif*-1 5′ regulatory sequence and *hif-1* full cDNA sequence completely restored *clk-1* mutants′ life span suppressed by loss-of-function mutation of *hif-1* (Figure 4_3 C-D). The results confirmed that HIF-1 function was at least partially required for the increased longevity associated with *clk-1* knockdown.

The additive effect on longevity of *hif-1(P621G)* and mutation of *clk-1*

Overexpression of HIF-1 further extended the life span in *clk-1* mutants (Figure 4_3 E-F). It seems to be conflicting with our previous conclusion that HIF-1 and CLK-1 act in the same pathway to promote the longevity. It can be explained by additive effect on *hif-1* targets expression by mutation of *clk-1* and *hif-1(P621G)* transgenes. From the study of *egl-9* and *rhy-1*,

we know that *hif-1* activity can be regulated at different levels in *C. elegans*. First EGL-9 regulates HIF-1 protein stability in the same pathway as *vhl-1* does. In *egl-9* or *vhl-1* mutants HIF-1 is stabilized (Epstein et al., 2001; Shen et al., 2006). EGL-9 also inhibits HIF-1 transcriptional activity which does require VHL-1 function or EGL-9 enzymatic activity as HIF-1 prolyl hydroxylase (Shao et al., 2009). RHY-1 mainly suppressed the expression of *hif-1* targets without affecting HIF-1 protein levels (Shen et al., 2006). The expression of *hif-1* targets was higher in *vhl-1*;*rhy-1* double mutants than that in either *vhl-1* or *rhy-1* single mutant. Here we showed that *Pnhr-57:GFP* reporter was induced in worms that carry loss-of-function of *clk-1* and HIF-1 protein did not change in *clk-1* mutants . It suggests that CLK-1 regulates HIF-1 transcriptional activity. Like in *rhy-1;vhl-1* double mutants, we may expect greater expression of *hif-1* targets in *clk-1; hif-1(P621G)* than those in worms that carry either *clk-1* mutation or *hif-1(P621G)* transgene alone. It has been shown that overexpression of HIF-1 extended life span in a dosage-dependent manner. It indicated that the longevity may be determined by varying expression level of HIF-1 or HIF-1 targets. Loss-of-function mutation of *clk-1* and *hif-1(P621G)* transgene should have additive effect on the regulation of *C. elegan* life span, if they regulate the expression of HIF-1 targets at different levels.

Other mitochondria longevity gene and *hif-1*

Both T02H6.11 and D2030.4 encode mitochondrial ETC components. RNAi knockdown of either gene causes life span extension in *C. elegans* (Hansen et al., 2005; Lee et al., 2003). Here we showed that *hif-1(P621G)* failed to extend the life span in T02H6.11 or D2030.4 RNAi knockdown worms. It suggests that HIF-1 function may also be involved in the life span extension associated with mitochondrial ETC genes knockdown. Moreover, not like *clk-1;hif-1*

double mutants which are viable and fertile, most of the worms carrying *hif-1* loss-of-function mutation arrested at larvae stage when they were fed on T02H6.11 or D2030.4 RNAi bacteria (data not shown). This is not informative on whether HIF-1 also mediated the life span extension in these mitochondrial ETC genes knockdown animals. But from another aspect it suggested that HIF-1 function may be essential for the viability of worms with mitochondria ETC function deficiency.

In this study only one *hif-1*-dependent reporter was tested in *clk-1* mutant. Although this reporter was induced in clk-1 mutant in a partially *hif-1*-dependent manner, we can't safely draw the conclusion on HIF-1 activity only based this information. From another aspect, the longevity study suggested that HIF-1 activity was induced in *clk-1* mutant.

Two *clk-1* mutants were employed in this study. In the *clk-1(ok1257)* strain, mutation of *hif-1* completely suppressed its life span extension. However *clk-1(qm30);hif-1* double mutant only 2 days shorter than *clk-1(qm30)* single mutant. Although statistics suggested rejecting the null hypothesis that *hif-1* didn't affect *clk-1* longevity in both cases, we still don't know why *hif-1* preformed differently in these two different *clk-1* strains. More strains should be tested before we can draw any strong conclusion.

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TABLES

Table 4_1. Quantification of *Pnhr-57:GFP expression* and HIF-1 protein level in *clk-1* mutants

	Exp1	Exp2	Exp3	Average
Pnhr-57:GFP [*clk-1(qm30)/clk-1(qm30);hif-1(ia04)*]	3.7	1.8	4.6	**2.98**

	Exp1	Exp2	Exp3	Exp4	Average
Pnhr-57:GFP [*clk-1(qm30)/clk-1(qm30);hif-1(ia04)*]	1.7	3.1	2.8	2.4	**2.5**

	Exp1	Exp2	Exp3	Average
Phif-1:hif-1:MYC:HA(iaIs28) [*clk-1(ok1247)/clk-1(ok1247);hif-1(ia04)*]	0.92	1.86	0.80	**1.19**

Table 4_2. Effect of HIF-1 function on the longevity of *clk-1* mutants

A.

Strain	**Mean**	**Std err**	Extension	**n**	***p*† value**
clk-1(qm30)	31.9	0.7		41	
clk-1(qm30);hif-1(ia04)	27.2	1.3	-14.7%	26	0.0066
clk-1(qm30)	28.2	1.0		50	
clk-1(qm30);hif-1(ia04)	25.3	0.8	-10.3%	48	0.0028
clk-1(qm30)	28.8	0.9		40	
clk-1(qm30);hif-1(ia04)	25.1	0.6	-12.8%	39	0.0003
clk-1(qm30)	26.2	1.5		22	
clk-1(qm30);hif-1(ia04)	24.9	0.7	-5.0%	48	0.15
clk-1(qm30)	33.1	0.8		39	
clk-1(qm30);hif-1(ia04)	29.6	0.8	-10.6%	68	0.020
clk-1(qm30) ;hif-1(iaIs34)	34.3	1.4	3.5%	44	0.0064
clk-1(qm30)	29.8	0.7		83	
clk-1(qm30);hif-1(ia04)	29.8	0.7	0%	101	0.56
clk-1(qm30) ;hif-1(iaIs34)	34.4	0.8	15.4%	87	<0.0001
clk-1(qm30) ; hif-1(ia04);hif-1(iaIs28)	33.4	0.8	12.1%	62	0.0025
clk-1(qm30)	26.0	0.5		106	
clk-1(qm30);hif-1(ia04)	27.3	0.8	5%	60	0.18
clk-1(qm30) ; hif-1(iaIs34)	31.4	0.7	20.8%	91	<0.0001
clk-1(qm30) ; hif-1(ia04);hif-1(iaIs28)	37.2	0.7	43.1%	35	<0.0001

† p-value was calculated by log-rank test as a comparison to the survival function of *clk-1(ok1247)* in same experiment.

B.

Strain	**Mean**	**Std err**	Extension	**n**	***p*† value**
clk-1(ok1247)	34.2	1.5		31	
clk-1(ok1247);hif-1(ia04)	23.9	1.2	-30%	18	<0.0001
clk-1(ok1247)	32.2	1.1		45	
clk-1(ok1247);hif-1(ia04)	24.0	0.9	-25%	42	<0.0001
clk-1(ok1247)	31.6	0.9		41	
clk-1(ok1247);hif-1(ia04)	22.0	0.7	-30%	27	<0.0001
clk-1(ok1247)	30.0	1.6		40	
clk-1(ok1247);hif-1(ia04)	23.1	0.8	-23%	29	<0.0001
clk-1(ok1247)	31.8	1.0		68	
clk-1(ok1247);hif-1(ia04)	22.0	0.5	-31%	36	<0.0001
clk-1(ok1247) ; hif-1(iaIs34)	38.5	1.3	21%	43	<0.0001
clk-1(ok1247)	36.0	0.7		91	
clk-1(ok1247);hif-1(ia04)	22.6	0.5	-37%	75	<0.0001
clk-1(ok1247) ;hif-1(iaIs34)	37.8	0.8	5%	75	0.07
clk-1(ok1247) ; hif-1(ia04);hif-1(iaIs28)	36.5	0.7	1%	66	0.67
clk-1(ok1247)	35.7	1.1		49	
clk-1(ok1247);hif-1(ia04)	22.2	0.4	-38%	97	<0.0001
clk-1(ok1247) ; hif-1(iaIs34)	36.5	1.0	1%	82	0.22
clk-1(ok1247) ; hif-1(ia04);hif-1(iaIs28)	35.5	1.2	0%	31	0.63

† p-value was calculated by log-rank test as a comparison to the survival function of *clk-1(ok1247)* in same experiment.

Table 4_3. Effect of HIF-1 function on the longevity of worms with mitochondria function deficiency

Strain	RNAi	Mean	Std err	Extension	n	*p*† value
N2	L4440	19.9	0.6		84	
hif-1(ia04)	L4440	22.3	0.8	12%	64	<0.0001
hif-1(iaIs34)	L4440	23.8	0.6	20%	43	<0.0001
N2	T02H6.11	31.5	1.4		40	
hif-1(iaIs34)	T02H6.11	32.4	1.8	2%	17	0.94
N2	D2030.4	25.7	1.0		52	
hif-1(iaIs34)	D2030.4	24.7	0.5	-4%	44	0.004
N2	L4440	18.4	0.7		49	
hif-1(ia04)	L4440	22.7	0.7	23%	35	<0.0001
hif-1(iaIs34)	L4440	22.4	0.8	22%	22	<0.0001
N2	T02H6.11	32.5	1.3		50	
hif-1(iaIs34)	T02H6.11	27.3	0.8	-16%	35	0.0002
N2	D2030.4	25.6	1.3		42	
hif-1(iaIs34)	D2030.4	27.9	0.7	-9%	43	0.94
N2	L4440	17.1	0.5		49	
hif-1(ia04)	L4440	22.3	0.9	30%	19	<0.0001
hif-1(iaIs34)	L4440	21.2	0.1	24%	55	<0.0001
N2	T02H6.11	31.3	1.1		44	
hif-1(iaIs34)	T02H6.11	30.1	0.8	-4%	55	0.17
N2	D2030.4	28.1	1.1		46	
hif-1(iaIs34)	D2030.4	30.4	1.2	8%	44	0.09
N2	L4440	18.5	0.8		46	
hif-1(ia04)	L4440	23.9	0.6	29%	85	<0.0001
hif-1(iaIs34)	L4440	25.8	0.5	39%	83	<0.0001
N2	T02H6.11	30.1	1.4		35	
hif-1(iaIs34)	T02H6.11	30.3	0.9	1%	50	0.60
N2	D2030.4	27.2	1.3		37	
hif-1(iaIs34)	D2030.4	30.4	0.9	12%	35	0.51

† p-value was calculated by log-rank test as a comparison to the survival function of N2 fed on same RNAi bacteria and in same experiment.

FIGURE LEGENDS

Figure 4_1. *Pnhr-57:GFP expression* in *clk-1* mutants and *clk-1;hif-1* double mutants on different bacteria source

A-B. *Pnhr-57*:*GFP* expression pattern in worms that carry loss function mutation of *clk-1(qm30)* on Q less bacteria (A) and Q-replete bacteria (B)

C-D. *Pnhr-57:GFP* expression pattern *clk-1 (qm30);hif-1(ia04)* double mutant worms on Q less bacteria (C) and Q-replete bacteria (D)

Figure 4_2. Quantification of *Pnhr-57:GFP expression* and HIF-1 protein level in *clk-1* mutants

A The vertical bar represented the relative GFP expression levels of *clk-1(qm30)* mutants to those in *clk-1(qm30);hif-1(ia04)* double mutants fed Q-less bacteria. In each experiment, the synchronized worms were cultured until they reached L4 stage. L4 worms were transferred to plates with Q-less bacteria. After 48 hours, 20 adult worms from each plate were boiled in 2XSDS loading buffer. And worm lysates were size fractioned on polyacrylamides gels and analyzed by western blots. The relative expression of GFP in *clk-1(qm30)* mutants to those in *clk-1(qm30);hif-1(ia04)* was calculated. The experiment was repeated for 3 times and error bar represent the standard error of ratios.

B The vertical bar represented the relative GFP expression levels of *clk-1(qm30)* mutants to those in *clk-1(qm30);hif-1(ia04)* when fed the worm Q-replete bacteria. In each experiment, the synchronized worms were cultured until they reached L4 stage. L4 worms were transferred to

plates with Q-replete bacteria. After 48 hours, 20 adult worms from each strain were boiled in 2XSDS loading buffer. And worm lysates were size fractioned on polyacrylamides gels and analyzed by western blots. The relative expression of GFP in *clk-1(qm30)* mutants to those in *clk-1(qm30);hif-1(ia04)* was calculated. The experiment was repeated for 4 times and error bar represent the standard error of ratios.

C The vertical bar represented the relative *Phif-1:hif-1a:MYC:HA(iaIs28)* expression levels of *clk-1(ok1247)* to those in *clk-1(ok1247);hif-1(ia04)* when fed the worm OP50. For each strain, 80 synchronized young adult worms fed on OP50 were boiled in 2XSDS loading buffer. And worm lysates were size fractioned on polyacrylamides gels and analyzed by western blots. The relative *Phif-1:hif-1a:MYC:HA(iaIs28)* expression levels of *clk-1(ok1247)* to those in *clk-1(ok1247);hif-1(ia04)* was calculated. The experiment was repeated for 3 times and error bar represent the standard error of ratios.

Figure 4_3. Effect of HIF-1 function on the longevity of *clk-1* mutants

A-B. The Kaplan-meier survival curves of *clk-1* mutants and *clk-1;hif-1(ia04)* double mutants at 20 ℃. Loss-of-function of *hif-1* shortened the life span of *clk-1* mutant worms. p-value was calculated by log-rank test and less than 0.0001.

C-D. The Kaplan-meier survival curves of *clk-1* mutants and *clk-1;hif-1(ia04); Phif-1:HIF-1a:MYC:HA(iaIs28)* mutants at 20 ℃. HIF-1 wild type transgene fully restored the longevity of *clk-1;hif-1(ia04)* double mutant worms. p-value was calculated by log-rank test and less than 0.0001.

E-F. The Kaplan-meier survival curves of *clk-1* mutants and *clk-1;hif-1(ia04); Phif-1:HIF-1(P621G)a:MYC:HA(iaIs34)* mutants at 20 ℃. Overexpression of HIF-1 further extended the life span of *clk-1* mutant worms. *p-value* was calculated by log-rank test and less than 0.0001.

Figure 4_4. Effect of HIF-1 function on the longevity of worms with mitochondria function deficiency

A. The Kaplan-meier survival curves of wild type N2 worms, *hif-1*(ia04) mutant worms and worms carrying *hif-1(iaIs34)* gain-of-function mutation on control RNAi bacteria (L4440). Overexpression of HIF-1 extended the life span when worms were fed on control RNAi bacteria (L4440). Loss-of-function mutation of *hif-1* also slightly increased the longevity.

B. The Kaplan-meier survival curves of wild type N2 strain and *hif-1(iaIs34)* strain on T02H6.11 RNAi bacteria. In T02H6.11 RNAi knockdown worms, *hif-1(iaIs34)* failed to further extend the life span.

C. The Kaplan-meier survival curves of wild type N2 strain and *hif-1(iaIs34)* strain on D2030.4 RNAi bacteria. In D2030.4 RNAi knockdown worms, *hif-1(iaIs34)* failed to further extend the life span.

FIGURES

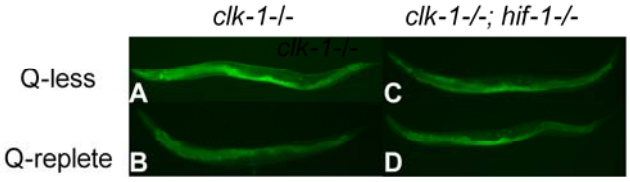

Figure 4_1. *Pnhr-57:GFP expression* in *clk-1* mutants and *clk-1;hif-1* double mutants on different bacteria source

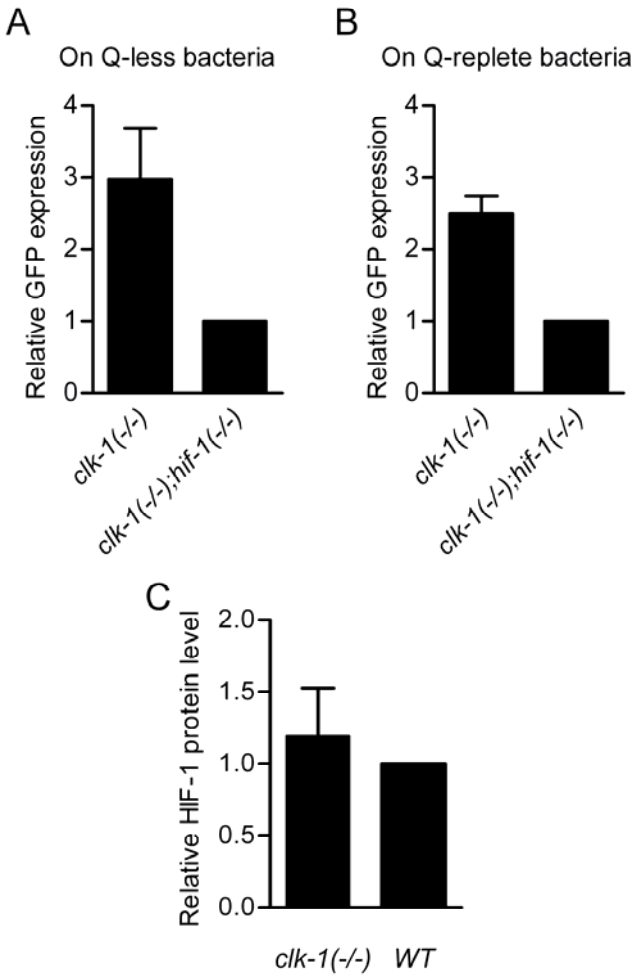

Figure 4_2. Quantification of *Pnhr-57:GFP expression* and HIF-1 protein level in *clk-1* mutants

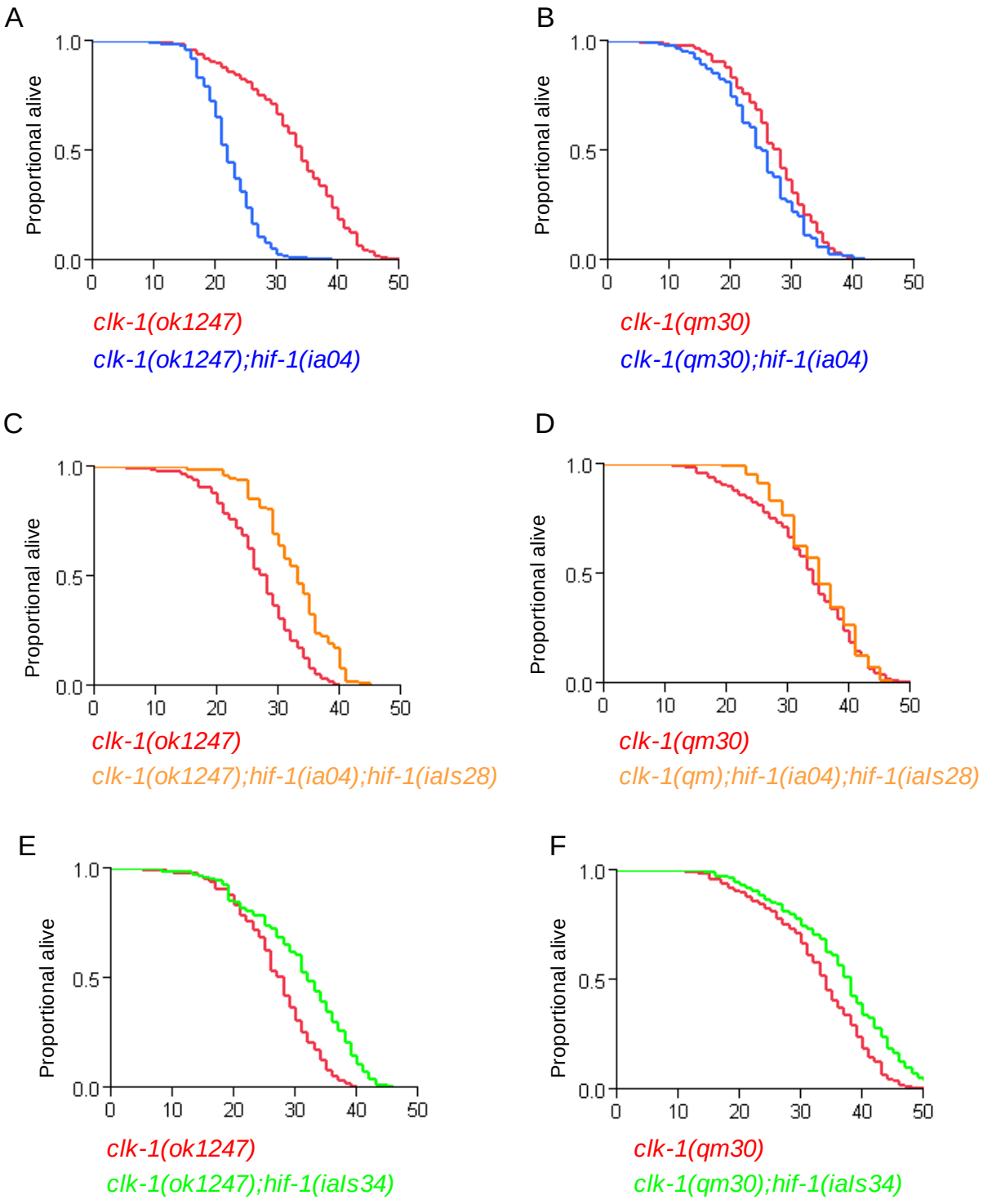

Figure 4_3. Effect of HIF-1 function on the longevity of *clk-1* mutants

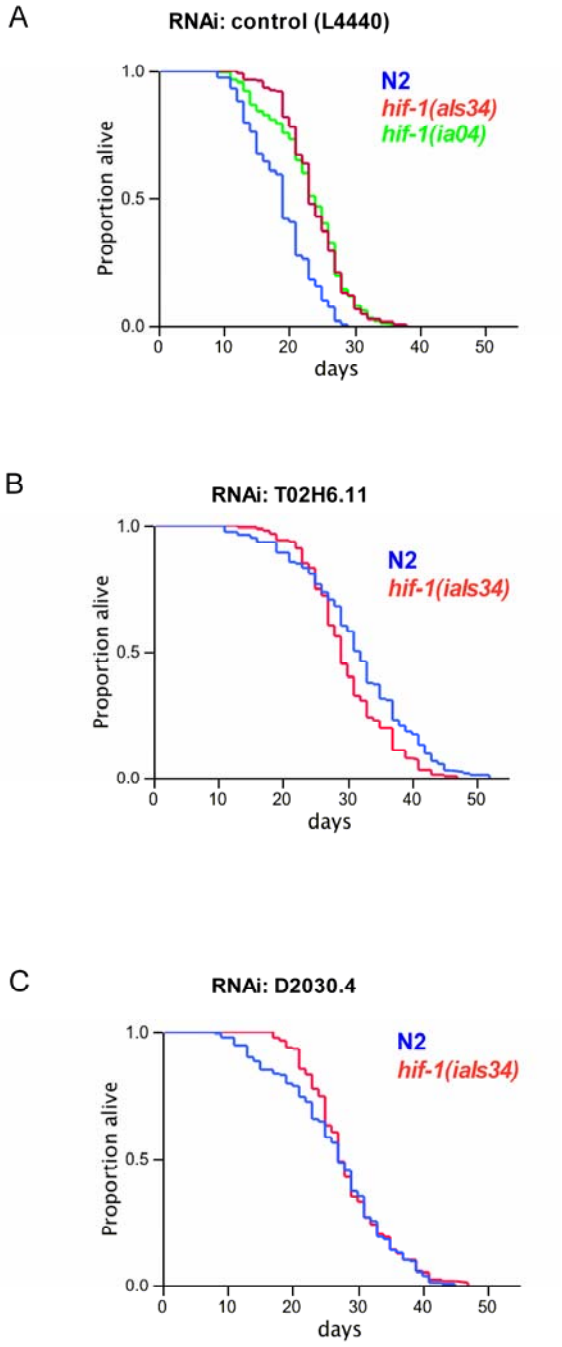

Figure 4_4. Effect of HIF-1 function on the longevity of worms with mitochondrial function deficiency

CHAPTER 5: GENERAL CONCLUSION

In the last decade, *C. elegans* has proved to be an excellent genetic model system in studying both the regulation and function of HIF-1. In 2001 Jiang et al. first identified HIF in *C. elegans* and demonstrated that HIF-1 was regulated by oxygen level and essential for *C. elegans* to survive the hypoxia (Jiang et al., 2001). In the same year, Epstein et al. first identified HIF prolyl hydroxylase in *C. elegans*, which provided the direction for the discovery of PHDs in mammals (Epstein et al., 2001). Since then *C. elegans* hypoxia signaling has been studies intensively. It provides important insight into the clinical therapy of human disease associated with improper function of HIFs (Semenza, 2009).

Genomic RNAi screen for HIF-1 regulators

C. elegans genome RNAi library was constructed by Julie Ahringer's group in 2001. Since then it has been a powerful genetic tool for the study of cellular signaling network in *C. elegans*. Using this bacteria-mediated RNAi library we completed a genome-wide screen to discover the negative regulators of HIF-1. The screen was based on the activation of *Pnhr-57:GFP* reporter. 179 RNAi clones were identified to induce the GFP reporter. Remarkably, 89 genes were predicted to have mitochondrial or metabolic functions, and the majority of these were electron transport chain components (42 genes) or subunits of mitochondrial ribosomes (24 genes). In agreement with this, a recent study showed that inhibition of mitochondrial replication via *par-2.1 / mtssb-1* RNAi or ethidium bromide treatment caused a strong induction of hypoxia-responsive genes, including *nhr-57* (Sugimoto et al., 2008). The second largest

category of genes are involved in protein folding or turnover (33 genes), and the majority of these are proteasomal components. The remaining major categories include vesicular transport, transporters and channels, signaling and cytoskeleton, transcription and DNA or RNA processing.

Most of the 179 RNAi treatments increased the expression of the reporter in *hif-1* deficient strains, suggesting that these genes may not regulate HIF-1 function. However the results are still informative to the study of *nhr-57*. For example in the study of *C. elegans* immunity, Bellier et al. found that HIF-1-mediated induction of *nhr-57* helped to protect *C. elegans* from the lethal effects of pore-forming toxins (PFT) (Bellier et al., 2009). Knockdown of *nhr-57* alone significantly reduced the resistance to PFT. These findings suggested that *nhr-57* is essential factor that mediated hypoxia signal to defend worms from PFT.

The genes identified from our screen showed significant overlap with the genes discovered from other independent RNAi screens. First almost all the mitochondrial longevity genes identified from previous screens are among these 179 genes (Curran and Ruvkun, 2007; Hamilton et al., 2005; Hansen et al., 2005; Kim and Sun, 2007; Lee et al., 2003). Second many of our genes that encode proteasomal components had been shown to regulate polyglutamine toxicity (Nollen et al., 2004) and function in response to osmotic stress and glycerol production (Lamitina et al., 2006). It suggests interactions between hypoxia signaling pathways and other stress response pathways.

We then focused on genes that had been shown to have evolutionarily conserved roles in stress response, as they might be involved in the cross-talk between HIF-1 and other key pathways.

sbp-1 encodes the *C. elegans* Sterol Regulatory Element Binding Protein (SREBP) and plays a central role in in maintaining lipid homeostasis in *C. elelgans* (McKay et al., 2003; Taghibiglou et al., 2009; Yang et al., 2006). In our research we showed that RNAi knockdown *sbp-1* induced *Pnhr-57:GFP* reporter through *hif-1*-dependent mechanism. Further study showed that *sbp-1* RNAi had no effect on HIF-1 protein levels. It suggests that SBP-1 may inhibit HIF-1 transcriptional activity through direct or indirect mechanism. *C. elegans sbp-1* mutants are pale and skinny. They lack of fat stores and arrest at larval stage. MaKay et al. also conducted a small scale RNAi screen for the genes that regulate fat storage in *C. elegans*. They found 8 genes in which RNAi knockdown reduced fat accumulation in worms. Interestingly 3 of these gene also induced *Pnhr-57:GFP* reporter upon RNAi (McKay et al., 2003). Moreover SBP-1 has been also shown to be activated in response to anoxia (0% oxygen) (Taghibiglou et al., 2009). Although it is still unknown whether SBP-1 activity could also been induced by hypoxia (0.1%~5% oxygen), the inhibition of HIF-1 by SBP-1 suggests that HIF-1 may function in lipid metabolism.

SKN-1 negatively regulates HIF-1 activity in *C. elegans*.

SKN-1 is a key transcriptional regulator of phase II detoxification enzymes. In the RNAi screen, knockdown of *skn-1* induced *Pnhr-57:GFP* expression. In the further characterization, I first found that SKN-1/Nrf negatively regulated HIF-1 protein levels in *C. elegans*. Later we demonstrated that SKN-1 acts through a putative SKN-1 binding site on the promoter of *egl-9* to increase expression of the EGL-9 prolyl hydroxylase thereby attenuating HIF-1 protein levels and HIF-1 activity. It is the first study that showed the direct interaction between hypoxia signaling (HIF-1) and SKN-1/Nrf detoxification pathway.

egl-9 mRNA was up-regulated by hypoxia and the induction required HIF-1 function (Shen et al., 2005). It suggests that *egl-9* is regulated by both HIF-1 and SKN-1. Besides *egl-9*, a few genes are also regulated by both transcription factors, including K10H10.2, F57B9.1, M05D6.5 and *rhy-1*(Murphy et al., 2003; Shen et al., 2005). Quantitative PCR also confirmed that K10H10.2 mRNA levels were induced by both *skn-1* RNAi and hypoxia, whereas the expression of F22B5.4, a *hif-1*-dependent gene was only regulated by hypoxia, not *skn-1* function. In this study we concluded that SKN-1 modulates HIF-1-mediated gene expression is dependent upon developmental and environmental cues and upon promoter context.

The significance of this finding is presented in chapter 3. The coordination among different signaling pathways is essential for human health and fittness. The basic understanding of cellular signaling cascade is important in drug design and clinical therapy.

HIF-1 mediate the increased longevity associated with mitochondria deficiency

The studies of mitochondria function in the mammals and *C. elegans* have suggested a role of mitochondria in HIF-1 regulation in *C. elegans* (Sugimoto et al., 2008; Wang et al., 2010). In this thesis study I did find that HIF-1 activity was induced in *clk-1* mutants and HIF-1 function was required for the life span extension associated with *clk-1* knockout. It is the first time to show the regulation of HIF-1 by mitochondria function in *C. elegans*. Mitochondria, as the major oxygen consumption organelle in the cell, play an important role in HIFs regulation in both *C. elegans* and mammals.

Furthermore, Curtis et al. also showed that the low-energy sensing AMP-activated protein kinase AMPK/*aak-2* was partially required for the life span extension in *clk-1* mutants (Curtis et

al., 2006). It indicated that HIF-1, CLK-1 and AAK-2 may act in a same pathway to modulate the life span. In *Pnhr-57:GFP* reporter assay, no difference on GFP expression by *aak-2* RNAi treatment was observed in the worms that overexpressed HIF-1. But it did not rule out the possibility that *aak-2* may work downstream of HIF-1 in the regulation of longevity.

HIF-1 may be required for the viability associated with mitochondria malfunction in *C. elegans*

Based on "Mitochondrial Threshold Effect Theory", the moderate dysfunction of mitochondria function promotes longevity and the severe interruption of mitochondria ETC results in larval-arresting and sterility in *C. elegans*(Ventura et al., 2006). In this thesis research I showed that RNAi inactivation of mitochondria ETC genes extended *C. elegans* life span. Further knockout of *hif-1* made these long-lived worms arrest at larval stage. My primary observation suggested that *hif-1* function may be required for the viability of the worms associated moderate mitochondria dysfunction. The alternative explanation is that mutation of *hif-1* may enhance general RNAi efficiency to *C. elegans*. In *hif-1* mutants, mitochondria gene RNAi may cause severe dysfunction of mitochondria, leading to developmental cessation at larval stage.

In the second part of this thesis study I confirmed that *C.elegans* mitochondria also play an important role in HIF-1 regulation by both biochemical analysis and longevity assay. Further studies on the mechanism of HIF-1 induction mediated by mitochondria dysfunction in *C. elegans* may shed light on the research in mammals.

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APPENDIX: HEAT SHOCK ACTIVATION OF HSF-1 NEGATIVELY REGULATES HIF-1 THROUGH *VHL-1* INDEPENDENT MECHANISM.

BACKGROUND

In the transition from normal culture condition to heat stress condition, the expression of heat shock proteins significantly increases in response to up-regulated misfolded proteins and denatured proteins. Heat shock factors (HSF) is a family of transcription factors, plays a central in the activation of other *hsp*s and mediation heat stress response. In mammal there are three HSFs: HSF1, HSF2 and HSF4, whereas in invertebrate there is only one single heat shock factor that mediates the heat stress response. Mammalian HSF1 is the homologue of HSF in invertebrates and essential in response to heat stress (McMillan et al., 1998; Xiao et al., 1999), whereas HSF2 and HSF4 played important role in differentiation and development (Bu et al., 2002; Fujimoto et al., 2004; Kallio et al., 2002; Wang et al., 2003).

It is a common agreement that HSF1 can be activated by nonnative proteins in different conditions including many developmental cues and environmental stresses (Morimoto, 1998). The injection of denatured proteins can activate HSF1 (Ananthan et al., 1986). HSF1 can be activated through different mechanisms in response to different stressors and HSF1 mediates stress response through transcriptional activation of other heat shock proteins such as hsp70 (Hahn and Thiele, 2004; Liu and Thiele, 1996; Tamai et al., 1994).

In *C. elegans*, there is only one heat shock factor, HSF-1. As previously reviewed in Chapter 1, HSF-1 function is required for the immunity to bacterial pathogens (Garigan et al., 2002a; Garigan et al., 2002b; Singh and Aballay, 2006a; Singh and Aballay, 2006b). RNAi inactivation of *hsf-1* results in increased susceptibility to *P. aeruginosa* and shortens the life span of wild type N2, *daf-2* mutant or *age-1* mutant worms (Hsu et al., 2003; Morley and Morimoto, 2004).

RESULTS

It has been shown in chapter 2 that we conducted a genome-wide RNA interference screen to discover the genes that negatively regulate HIF-1 activity. In the primary screen, we found that RNAi inactivation of heat shock factor 1 (*hsf-1*) induced the expression of *Pnhr-57:GFP*, a HIF-1-dependent reporter (data not shown). It suggested that HSF-1 may negatively regulate HIF-1 function.

Heat-shock-activation of HSF-1 suppressed HIF-1 function

We examined the HIF-1 protein level associated with *hsf-1* knockdown. Since HSF-1 function can be induced by heat stress, we conducted the RNAi feeding experiment at both normal condition and heat stress condition. Worms were fed on *hsf-1* RNAi bacteria or control RNAi bacteria from hatchery to L4 stage at 15ºC or 20ºC. Then half of the samples were transferred to 25ºC incubator and cultured for 18 hours before being harvested. The rest of worms remained at 15ºC or 20ºC and were harvested 8 hours after the sample at 25ºC were collected. At 15ºC RNAi inactivation of *hsf-1* increased HIF-1 protein by 2-fold compared to that in worms fed on control RNAi bacteria (Figure appendix_1 column 1). Most strikingly,

HIF-1 protein level was increased more than 10-fold in *hsf-1* RNAi knockdown worms after the heat shock (Figure appendix_1 column 2). The observations at 20ºC were similar (Figure appendix_1 column 1 and column 2). The results suggested that HIF-1 activity was suppressed by HSF-1 function at normal condition or in response to heat stress.

HSF-1 regulates HIF-1 in *vhl-1* independent manner

Previous studies showed that HIF-1 is negatively regulated by EGL-9/VHL-1 proteasomal degradation pathway in *C. elegans*(Bishop et al., 2004; Epstein et al., 2001). Here the dramatic induction of HIF-1 protein may be caused by the malfunction of 26S proteasomal degradation pathway, other than *hsf-1* knockdown. So we repeated the previous assay in *vhl-1* loss-of-function-mutant animals. In *vhl-1* mutants, HIF-1 protein is strongly induced at normoxia (Epstein et al., 2001; Shen et al., 2006). We found the similar results in *vhl-1* mutants as in the animals carrying wild type *vhl-1* at normal culture condition or after heat shock (15ºC->25ºC and 20ºC->25ºC). In *vhl-1* mutant background, RNAi knockdown of *hsf-1* can further induce HIF-1 protein level by 2-fold in comparison to that in worms fed on RNAi control bacteria at 15ºC (Figure appendix_1 column 4). The induction of HIF-1 protein by *hsf-1* RNAi knockdown is more than 8-fold after heat shock treatment (Figure appendix_1 column 3). We also got the similar finding in the experiment that carried out at 20ºC (Figure appendix_2 column 3 and column 4). The results suggested that the mechanism of the HIF-1 induction associated with *hsf-1* knockdown is genetically independent of EGL-9/VHL-1 proteasomal degradation pathway.

hsf-1 RNAi knockdown did not affect *hif-1* mRNA level in response to heat stress

Since the regulation of HIF-1 by HSF-1 is independent of the posttranslational regulation pathway (EGL-9/VHL-1 pathway), HSF-1 and other HSF-1-dependent HSPs may directly repress the transcription of *hif-1* during heat shock. With the same experimental setup, we assayed the mRNA levels of *hif-1* in worms fed on *hsf-1* or control RNAi bacteria and at 15ºC or by 15ºC ->25ºC heat shock treatment. The quantitative PCR results showed that *hif-1* mRNA did not change by either *hsf-1* RNAi treatment or heat shock (Figure appendix_3). It indicated that HSF-1 and other HSF-1-dependent HSPs didn't suppress *hif-1* transcription.

CONCLUSION AND FUTURE DIRECTIONS

HSF-1 and HIF-1 are both *C. elegans* transcription factors that mediate stress response and modulate longevity. Previous studies have demonstrated that HIF-1 can be activated by heat acclimation (Maloyan et al., 2005; Treinin et al., 2003). In this study, we demonstrated that HSF-1 negatively regulated HIF-1 activity at normal culture condition or in the response to heat stress. We also showed that the mechanism of HIF-1 protein induction associated with *hsf-1* RNAi knockdown is genetically distinguished from EGL-9/VHL-1 proteasomal degradation pathway. Moreover HSF-1 and other HSF-1-dependent HSPs did not suppress the transcription of *hif-1* in *C. elegans*.

There are numbers of different ways by which HSF-1 suppresses HIF-1 activity. It has been shown that heat shock protein inhibited the translation machinery, which could limit the accumulation of misfolded protein during the heat stress (Morimoto, 1998; Panniers, 1994). So HSF-1 and other HSPs may repress the translation of *hif-1* mRNA. It was also reported that

HSP90 was involved in VHL-independent posttranslational regulation of HIF-1α at normoxia in mammal (Liu et al., 2007; Liu and Semenza, 2007). So it is possible that HSF-1 negatively regulates HIF-1 through a VHL-1/EGL-9 independent proteasomal degradation mechanism.

Both HSF-1 and HIF-1 are part of stress response network and modulate life span in *C. elegans*. The coordination among different stress response pathways plays an essential role in survival and proper function in living organisms. The better we know about the interactions among these signaling pathways, the better we can understand the causes of different diseases and help in the drug design or other therapy methods.

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TABLES

Table appendix_1. Inductions of HIF-1 protein by *hsf-1* RNAi knockdown at 15ºC or with 15ºC->25ºC treatment

		ZG429(*hif-1-/-; iaIs28)*				ZG431*(vhl-1 -/-; hif-1-/-; iaIs28)*			
	RNAi	*hsf-1*	L4440	*hsf-1*	L4440	*hsf-1*	L4440	*hsf-1*	L4440
	Heat shock	+	+	-	-	+	+	-	-
EXP1	*hsf-1/L4440*	12.9		1.5		5.6		2.17	
EXP2	*hsf-1/L4440*	7.7		3.7		15		1.22	
EXP3	*hsf-1/L4440*	9.8		1.5		6.10		3.7	
EXP4	*hsf-1/L4440*	11.9		2.17					
EXP5	*hsf-1/L4440*	15		2.1		6.29		2.24	
Average	*hsf-1/L4440*	11.46		2.19		8.25		2.33	

Table appendix_2. Inductions of HIF-1 protein by *hsf-1* RNAi knockdown at 20ºC or with 20ºC->25ºC treatment

		ZG429(*hif-1-/-; iaIs28)*				ZG431*(vhl-1 -/-; hif-1-/-; iaIs28)*			
	RNAi	*hsf-1*	L4440	*hsf-1*	L4440	*hsf-1*	L4440	*hsf-1*	L4440
	Heat shock	+	+	-	-	+	+	-	-
EXP1	*hsf-1/L4440*	6.1		1.2		1.9		1.3	
EXP2	*hsf-1/L4440*	2.6		1.2		2.8		0.9	
EXP3	*hsf-1/L4440*	3.4		1.1		1.6		1.0	
Average	*hsf-1/L4440*	4.03		1.17		2.10		1.07	

Table appendix_3. Effect of *hsf-1* RNAi on *hif-1* mRNA

	Primer	*hif-1 1*	*hif-1 2*	*hif-1 1*	*hif-1 2*	*inf-1*
	Heat shock	+	+	-	-	
EXP1	*hsf-1/L4440*	1.094294	0.641713	1.36604	1.172835	1
EXP2	*hsf-1/L4440*	1.351911	0.534033	0.040667	0.099098	1
EXP3	*hsf-1/L4440*	1.71119	1.448942	1.428994	0.846745	1
Average	*hsf-1/L4440*	1.38	0.87	0.94	0.71	1

FIGURE LEGENDS

Figure appendix_1. Inductions of HIF-1 protein by *hsf-1* RNAi knockdown at 15ºC or with 15ºC->25ºC treatment

The relative HIF-1 protein levels in worms fed on *hsf-1* RNAi to those in worms fed on control RNAi bacteria were calculated at each experimental condition. The experimental condition included the worm strains (*vhl-1* mutants or worms carrying wild type *vhl-1*) and temperatures (at 15ºC or with 15ºC->25ºC treatment).

Figure appendix_2. Inductions of HIF-1 protein by *hsf-1* RNAi knockdown at 20ºC or with 20ºC->25ºC treatment

The relative HIF-1 protein levels in worms fed on *hsf-1* RNAi to those in worms fed on control RNAi bacteria were calculated at each experimental condition. The experimental condition included the worm strains (*vhl-1* mutants or worms carrying wild type *vhl-1*) and temperatures (at 20ºC or with 20ºC->25ºC treatment).

Figure appendix_3. Effect of *hsf-1* RNAi on *hif-1* mRNA

The *hif-1* mRNA levels were normalized to *inf-1* expression each experimental condition. The experimental condition included the RNAi treatments (*hsf-1* RNAi or control RNAi) and temperatures (at 15ºC or with 15ºC-> 25ºC treatments). Two primers which can amplify the *hif-1* cDNA were used to determine mRNA level of *hif-1*. *inf-1* was used as the reference gene. The relative *hif-1* mRNA levels in worms fed on *hsf-1* RNAi to those in worms fed on control RNAi were plotted.

FIGURES

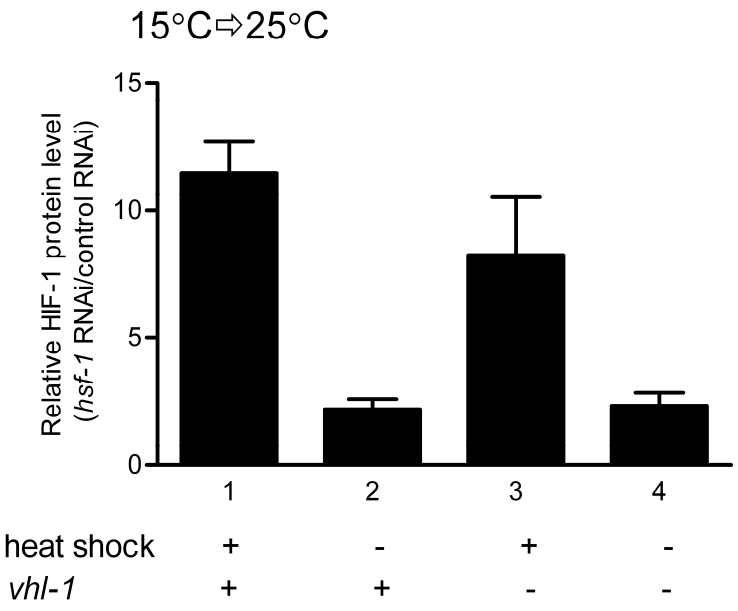

Figure appendix_1. Inductions of HIF-1 protein by hsf-1 RNAi knockdown at 15ºC or with 15ºC->25ºC treatment

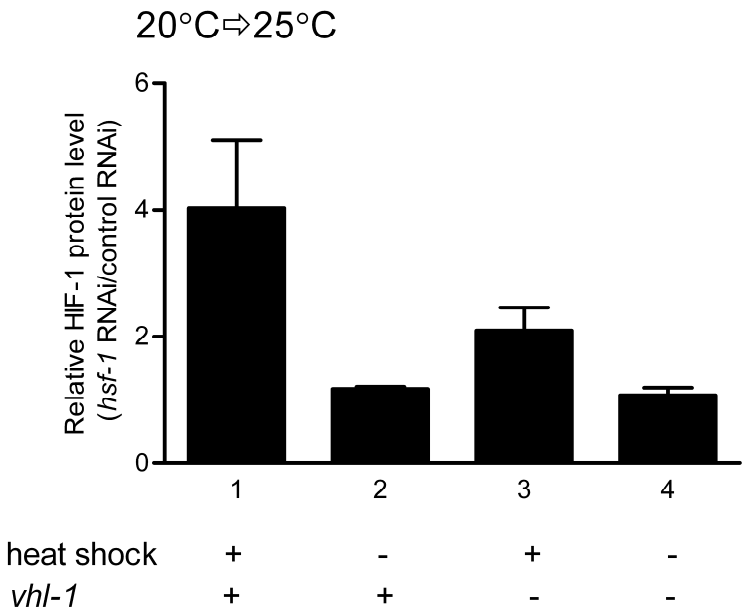

Figure appendix_2. Inductions of HIF-1 protein by hsf-1 RNAi knockdown at 20ºC or with 20ºC->25ºC treatment

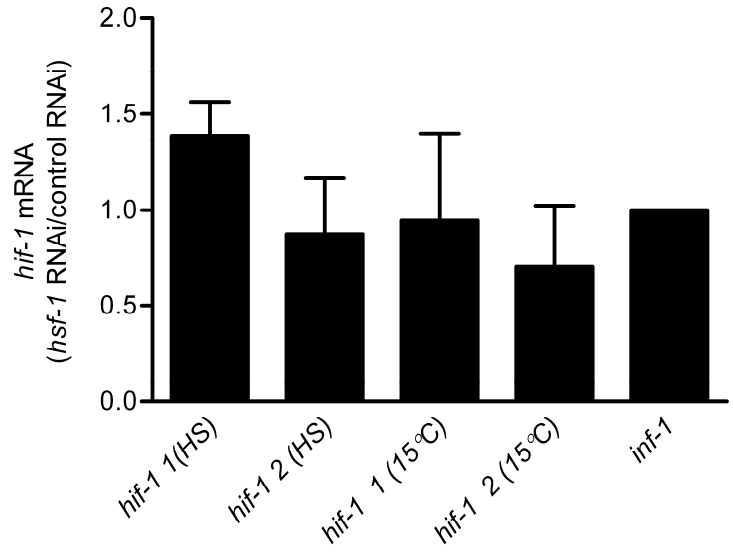

Figure appendix_3. Effect of *hsf-1* RNAi on *hif-1* mRNA

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