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HIF-INDEPENDENT RESPONSES IN HYPOXIA

Divya Padmanabha

APPENDIX 2-A

APPENDIX 2-B

HIF-INDEPENDENT RESPONSES IN HYPOXIA

A dissertation submitted in partial fulfillment of the requirements for the degree

of Doctor of Philosophy at Virginia Commonwealth University.

By

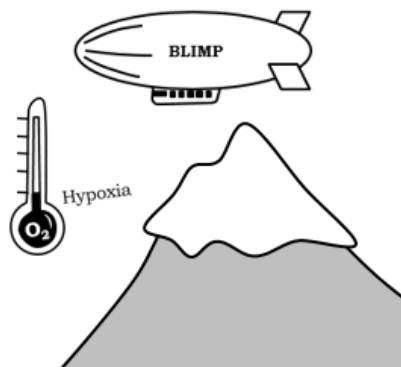## DIVYA PADMANABHA, M.S.

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APPENDIX 2-C

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LIST OF ABBREVIATIONS

ATP	Adenosine tri phosphate
BLIMP-1/ BLMP-1	B-lymphocyte maturation protein
ChIP	Chromatin immunoprecipitation
CHO	Chinese hamster ovary
CoCl2	Cobalt Chloride
DNA	Deoxyribonucleic acid
ER	Endoplasmic Reticulum
ETC	Electron transport chain
GFP	Green Fluorescent Protein
HIF	Hypoxia Inducible Factor
IP	Immunoprecipitation
MTA-1	Metastasis Tumor Antigen-1
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
qPCR/ qRT-PCR	Quantitative realtime PCR
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Room temperature
SE	Standard error
SEM	Standard error of mean
TCA	Tricarboxylic acid cycle
UPR	Unfolded protein response
WT	Wild type

APPENDIX 2-F
ABSTRACT

HIF-INDEPENDENT RESPONSES IN HYPOXIA

By Divya Padmanabha, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2015.

Major director: Keith D. Baker, Ph.D, Assistant Professor, Biochemistry and Molecular

Biology

The adaptive response to hypoxia is accompanied by widespread transcriptional

changes that allow for prolonged survival in low oxygen. Many of these changes are

directly regulated by the conserved hypoxia-inducible factor-1 (HIF-1) complex;

however, even in its absence, many oxygen-sensitive transcripts in *Caenorhabditis*

elegans are appropriately regulated in hypoxia. To identify mediators of these non-HIF-

dependent responses, I established a *hif-1* mutant reporter line that expresses GFP in

hypoxia or when worms are treated with the hypoxia mimetic cobalt chloride (cobalt

chloride). The reporter is selective and HIF-independent, in that it remains insensitive to

a number of cellular stresses, but is unaffected by mutation of the prolyl hydroxylase

egl-9, suggesting that the regulators of this response pathway are different from those

controlling the HIF pathway. I used the HIF-independent reporter to screen a

transcription factor RNAi library and identified genes that are required for hypoxia

sensitive and cobalt chloride-induced GFP expression. Three mediators of the HIF-

independent response zinc finger protein BLMP-1, chromatin remodeling factor LIN-40,

and T-box transcription factor TBX-38 were isolated as mediators of the HIF-

independent response. First, we show that mutation of *blmp-1* renders animals sensitive

to hypoxic exposure and that *blmp-1* it is required for appropriate hypoxic-induced

expression of HIF-independent transcripts. Further, we demonstrate that BLMP-1 is

necessary for an increase of hypoxia-dependent histone acetylation within the promoter

of a non-HIF-dependent hypoxia response gene. Additionally, we explore BLMP-1's role

in two hypoxia-regulated physiological processes namely unfolded protein response and

collagen formation. We also briefly investigate the role of LIN-40 in the hypoxia

response.

General introduction and thesis organization

Oxygen homeostasis is one of the most fundamental physiological processes necessary for aerobic animals. All metazoans utilize molecular oxygen to generate metabolic energy for normal growth and for maintaining intracellular bioenergetics including oxidative phosphorylation. During evolution, multicellular organisms have developed complex networks for oxygen homeostasis at both systemic and cellular levels (Semenza, 2014). The condition when cellular oxygen levels do not meet the physiological demand is termed as hypoxia, and it occurs at oxygen concentrations below <2%. Hypoxia occurs during development, homeostasis, as well as many pathological conditions, such as cancer, ischemia, and stroke. In humans, systemic, local, and intracellular homeostatic responses are triggered in response to hypoxia in conditions like anemia owing to the reduced oxygen carrying capacity of blood, high altitude-related low oxygen partial pressure in arterial blood, neovascularization in ischemic myocardium, and pulmonary disease, or in toxin-mediated oxygen limitation (Iyer et al., 1998; Semenza, 2003). Hypoxia dependent responses have been largely studied with respect to the tumor microenvironment, owing to its irregular vascular supply and altered metabolic and proliferative status (Dewhirst, Cao, & Moeller, 2008). Laboratory and clinical evidence strongly associate hypoxia with tumor development and the hypoxia phenotype is strongly associated with adaptive responses such as decreased proliferation and protein synthesis, high rates of glycolysis and anaerobic metabolism, increased secretion of angiogenic factors, increased metastatic potential, and poor response to therapy (Wouters & Koritzinsky, 2008). Given the importance of hypoxia related responses, we sought to isolate new mediators of this response.

First, I give an introduction to hypoxia mediated cellular changes with an emphasis on the function of Hypoxia Inducible Factor, the most widely studied effector of hypoxia responses. Although my thesis examines HIF-independent factors, understanding the regulation of HIF-1 is critical. Owing to the pervasive nature of HIF-1, it is highly likely that HIF-1 and the HIF-independent regulator-BLMP1 in this study-work synergistically or antagonistically to orchestrate the hypoxia pathways. It is also possible that the oxygen sensors that mediate the HIF-1 response also mediate the HIF-independent response. Next, I introduce the hypoxia adaptation pathway in *C.elegans* and highlight some differences in the regulation of hypoxia in the worm. I then try to summarize the current literature on HIF-independent processes in an effort to highlight that a lot is unknown with respect to hypoxia mediated molecules.

In the results, I elaborate on the details of the RNAi screen conducted to identify the HIF-independent molecule. There is also a section on the construction of *C.elegans* hypoxia chamber. I then describe the identification and role of BLMP-1 in hypoxia, its downstream targets, and its importance for survival in hypoxia. I also try to understand how BLMP-1 is regulated and its physiological role in hypoxia.

Apart from the BLMP-1 story, I also worked on a short study in alternative fat oxidation pathways in *Drosophila.* The results from this shorty study are very interesting and they lay the foundation to a novel aspect of long chain fatty acid oxidation.

1. Introduction

1.1 History of hypoxia studies

The beginning of hypoxia studies was as early as 100 years ago when Schwarz and colleagues noticed that normal mammalian cells under hypoxia were less sensitive to irradiation than those irradiated in the presence of oxygen (Bertout, Patel, & Simon, 2008). A cytological evaluation of damage in patient tumors after radiation revealed that patients allowed to breathe hyperbaric oxygen at 3 atmospheres pressure showed better prognosis than patients allowed to break room air (Mcewen, 1965). Numerous other studies evaluated the effects of hypoxia on transplanted or spontaneous tumor regression after irradiation in mice and established that human tumors contained radioresitant hypoxic cells. During the 1990s, it was found that hypoxic cells could be targeted directly with hypoxia-specific cytotoxins, and a combination of radiation with a hypoxia-specific cytotoxin would thus have the potential to destroy the entire tumor (Michael Hockel, 2001).

Thereafter, the expression of a number of genes, including glucose-regulated proteins and O2-regulated proteins such as heme oxygenase and erythropoietin, was found to be oxygen dependent (Roll, Murphy, Laderoute, Sutherland, & Smith, 1991). Several studies then explored the role of hypoxia in key physiological processes, including angiogenesis (Hoeben et al., 2004), DNA replication (Young, Marshall, & Hill, 1988), and metastasis (Brenk, Moore, Sharpington, & Orton, 1972) However, the mechanisms by which gene transcription was regulated by hypoxia was not elucidated until the discovery of HIFs in the 1990s.

1.2 Hypoxia-inducible factor

1.2.1 Discovery of HIF-1

Systematic characterization of erythropoietin (EPO) by multiple groups led to the identification of a cis-acting hypoxia response element (HRE) (5'-G/ACGTG-3') in the 3' flanking region of this locus that confers O2 regulation of EPO expression. Hypoxia inducible factor (HIF-1) was subsequently described as a hypoxia-dependent transcription factor that binds to EPO HRE and activates EPO transcript in hypoxic cells (G. L. Wang, Jiang, Rue, & Semenza, 1995). This was followed by the purification of HIF-1 and it was discovered that HIF-1 is a heterodimeric transcription factor composed of an α and a β subunit. Both subunits are members of the basic helix-loop-helix (bHLH) transcription factor family that contains a PAS domain, which was initially characterized using the *Drosophila* proteins PER and SIM and the mammalian protein aryl hydrocarbon nuclear translocator (ARNT) **(FIGURE 1.1)**. While HIF-1β subunit is generally expressed, the stability of HIF-1α subunit is tightly controlled by cellular oxygen level (Jiang, Guo, & Powell-Coffman, 2001).
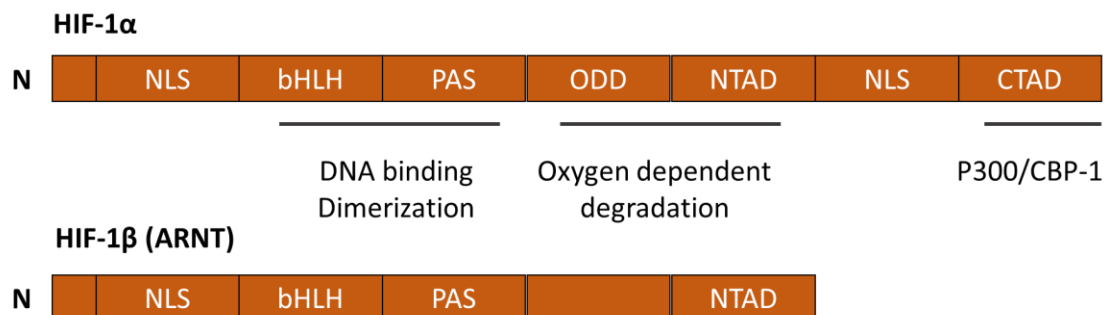
Figure 1.1: Protein domains of HIF-1α and HIF-1β. Functional domains and binding domains are shown, bHLH, basic helix-loop-helix; ODD, oxygen-dependent degradation domain; TAD and CTAD, N- and C-terminal transactivation domain; NLS, nuclear localization signal; PAS, Per-ARNT-Sim.

The dimeric HIF-1 complex binds to conserved HREs associated with numerous transcriptional target genes. More than 100 genes are shown to be putative HIF-1 target genes in mammals. The HIF-1 target genes encode protein products which are involved in angiogenesis/vascular remodeling, energy metabolism, erythropoiesis, cell proliferation and viability, which makes HIF-1 a major mediator of physiological and pathophysiological responses to hypoxia. (Iyer et al., 1998; Wouters & Koritzinsky, 2008).

*hif-1*α -/- mice die by E10.5 with severe vascular defects, underscoring the essential role of HIF-1 in controlling the embryonic responses to variations in oxygen concentrations in the microenvironment (Ryan, Lo, & Johnson, 1998). The significance of the transcriptional changes regulated by HIF-1 is highlighted by its role in cancer progression (Giaccia, Siim, & Johnson, 2003; Semenza, 2003). HIF-1 accumulates in a broad array of human cancers (Maxwell, Pugh, & Ratcliffe, 2001). HIF-1 accumulation has been associated with poor prognosis in breast cancer, cervical cancer, ovarian cancer, and neuroblastoma (Semenza, 2014). Some of the promising interventions for anti-cancer therapies involve mechanism for downregulating HIF-1, either by inhibiting translation or promoting degradation, or inhibiting key HIF target genes, such as VEGFA (Giaccia et al., 2003).

The complexity of HIF-1 arises with the discovery of the HIF-1α paralogues. HIF-2α (also known as endothelial PAS domain protein 1) is also oxygen-regulated and can dimerize with HIF-1β to mediate hypoxia responses. While HIF-1α is expressed ubiquitously, both HIF-2α and HIF-3α are expressed in more restricted, but partially overlapping, cell types. HIF-1α and HIF-2α activate transcription of an overlapping but

distinct set of genes (M. Wang et al., 2005). Another paralogue, HIF-3α inhibits the transcriptional activity of HIF-1α by competing with HIF-1β (Makino Y et al. 2001)

1.2.2 REGULATION OF HIF-1

The discovery of prolyl hydroxylases is a great example for the utility of *C. elegans* in dismantling the mechanism of HIF regulation. After the molecular characterization of the HIF1 complex in mammals, the HIF1α subunit was found to be ubiquitinated and degraded in normoxia by von Hippel-Lindau tumor suppressor (VHL). Using *C.elegans*, *egl-9* or prolyl hydroxylase (PHD) was found to hydroxylate specific proline residues on the oxygen-dependent-degradation (ODD) of the HIF-1α subunit (Epstein et al., 2001). Using oxygen and 2-oxoglutarate as co-substrate, iron and ascorbate as cofactors, PHDs sense the intracellular oxygen tension as well as aerobic metabolites to regulate the degradation of HIF-1α subunit (Figure 1.2).
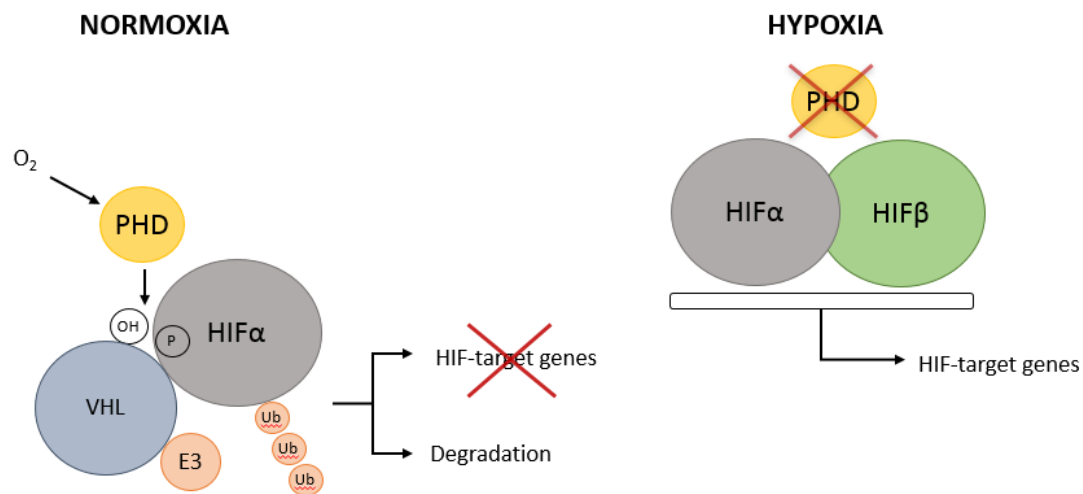
Figure 1.2: Regulation of HIF-1 is tightly controlled by PHD and VHL-1. Details in the text.

HIF-1/PHD/pVHL forms an evolutionarily conserved negative feedback loop to regulate oxygen homeostasis. Interestingly, in *C.elegans*, expression of *egl-9* mRNA was found to be induced by hypoxia in a HIF-1-dependent manner (C. Shen, Nettleton, Jiang, Kim, & Powell-Coffman, 2005). Mammalian PHDs also contain hypoxia response elements and are induced by HIF-1 during hypoxia (N. Chen et al., 2011).

The regulation of HIF also occurs at levels independent of HIFα stability. Factor inhibiting HIF (FIH) mediates hydroxylation of an asparagines residue in the carboxy-terminal of the HIFα subunit, which blocks its interaction with transcription co-activator p300 (C/EBP), thereby attenuating HIF-1 transcriptional activity (Mahon, Hirota, & Semenza, 2001). FIH is also an iron and 2-oxoglutarate dependent dioxygenase and could function as the oxygen sensor. Moreover, PHD regulates HIF-1 activity in a VHL-independent manner by interacting with a MAPK protein (Huang, 2005).

1.3. *C. elegans* and hypoxia

C. elegans is a soil nematode and is found on rotting vegetation in the wild (Duveau & Félix, 2012). Under favorable environmental conditions (abundant food, low population density and moderate temperature) *C. elegans* develops from egg through four larval stages to reach reproductive maturity within 3-5 days. After hatching, worms of the L1 stage comprise 558 cells and develop through 3 more larval stages, L2—L4, each marked by ecdysis and stage-specific patterns of cellular events. The cellular events including neuronal, epidermal, and gonadal cell lineage cell divisions and morphogenetic events like gonadal and vulval formation lead to the final 959 somatic cells in hermaphrodite animals. The small body size, ease of culture, short generation

time, and relatively easy forward and reverse genetics makes *C.elegans* a very desirable genetic system (Powell-Coffman, 2010).

Environmental oxygen might be limited or fluctuating in natural habitats enriched for microbial food especially deep in the soil (Voorhies & Ward, 2000). *C. elegans* do not have complex respiratory and circulatory systems and the cylindrical body and simple gut design favors diffusion of oxygen across the gut lumen and cuticle. *C. elegans* are able to sense various oxygen tensions and prefer a 5-12% O_2 concentration (Gray et al., 2004). Although it is sensitive to low concentrations of oxygen, the worm has evolved several mechanisms to survive the stress of hypoxia/ anoxia. Adult worms are able to maintain a steady metabolic rate even when exposed to a range of decreasing oxygen tensions. Metabolic rates begin to drop for young adults at oxygen tension lower than 3.6 kPa (~2%) (Anderson, 1976; Voorhies & Ward, 2000). At concentrations below 0.2% hypoxia, metabolic rate drops to as low as 5% of that in normoxic conditions (Voorhies & Ward, 2000). At every stage of development *C. elegans* survive 24 hours of severe hypoxia at a rate of 90% or greater (Padilla, Nystul, Zager, Johnson, & Roth, 2002; Voorhies & Ward, 2000).

So, how is the worm so adept at dealing with hypoxia? When confronted with oxygen deprivation the worm responds by remaining animated or entering into suspended animation depending on oxygen concentration and metabolic state (Nystul & Roth, 2004). Worms remain active in hypoxia, while they enter a state called suspended animation in anoxia and severe hypoxia (≤0.1%). This 'suspended animation' response is mediated by the spindle checkpoint protein SAN-1 in a HIF-independent manner in embryos and the ceramide synthase HYL-2 in adults although the factors critical in this

decision are unclear (Budde & Roth, 2011). After non-lethal exposures to anoxia the animals will resume biological processes such as cell division, development, eating, movement, and offspring production (Miller & Roth, 2009). Survival in severe hypoxia/ anoxia depends upon developmental stage, growth temperature, diet, and genotype. *C. elegans* are more sensitive to hypoxia if the temperature during exposure is increased (>24°C) or if the duration of anoxia exposure is increased (Mendenhall, LaRue, & Padilla, 2006). The most severe hypoxia/ anoxia tolerant developmental stages are dauer larvae and embryos (Padilla et al., 2002).

Extended survival in severe hypoxia and anoxia necessitates metabolic changes and cell cycle in the embryo. When exposed to severe hypoxia, the embryo arrests cell cycle progression at either the interphase, last prophase, or metaphase stages and modifies its metabolic pathway to adapt to the reduction in the ratio of ATP/AMP (Padilla et al., 2002). Indeed carbohydrate homeostasis is critical for survival in severe hypoxia/ anoxia; for instance, a 24-hour anoxia treatment resulted in a decrease in glycogen levels to approximately 20% of the initial levels (Iii & Roth, 2010), suggesting that glycogen perhaps plays an important role in maintaining metabolism during anoxia exposure. Additionally, knockdown of *gsy*-1, the glycogen synthase, renders the embryos sensitive to the severe hypoxic. Moreover, the environment to which the parent is exposed can influence the survival rate of its embryos; the embryos of adults treated to a high salt environment (300 mM sodium chloride) are sensitive to anoxia insult (Iii & Roth, 2010). The uterus of an adult provides unique protective abilities in severe hypoxia. Embryos within the uterus of gravid adults exposed to 0.1% of hypoxia the embryos survive by arresting and going to suspended animation (Miller and Roth,

2009). In contrast, embryos that are laid on agar plates and then treated to severe hypoxia for 48 hours do not survive (Budde & Roth, 2011).

1.3.1 *C.elegans* HIF-1

In terms of HIF function and regulation, *C.elegans* has several advantages. The genes and pathways that govern hypoxia, including the oxygen-dependent stability of HIF-1 are conserved, and there is no redundancy in the corresponding counterparts. For instance, *C. elegans* has a single HIFα homolog, termed HIF-1; and a single HIFβ homolog, termed AHA-1 (Jiang et al., 2001). Compared to three paralogues PHDs in mammals, there is one homolog, EGL-9, in *C. elegans*, while VHL-1 is the single VHL homolog in *C. elegans* (Epstein et al., 2001). The reduced complexity of the HIF-pathway facilitates interesting genetic analysis (Epstein et al., 2001; Powell-Coffman, 2010). Further, while the *hif-1*α -/- mouse dies by E10.5 with severe vascular defects, *hif-1(ia4)* mutants are viable in normoxia (Powell-Coffman, 2010; Ryan et al., 1998). *C.elegans hif-1* gene has 42% identity and 67% similarity to human HIF-1α in the bHLH domain, and it has 30% identity and 55% similarity to HIF-1α in the two PAS core domains *C. elegans hif-1*(*ia04*) loss-of-function mutant is a 1,231-bp deletion of the second, third, and fourth exons, resulting in the deletion of most of the bHLH and PAS domains, a shift in frame, and a premature translational stop.

Strain: ZG31

Figure 1.3: Genomic structure of *C.elegans hif-1* and *hif-1(ia4)* mutant: *C.elegans hif-1* has conserved PAS and bHLH domains. The *ia04* mutation is a 1,231-bp deletion of the second, third, and fourth exons. This introduces a frameshift and premature stop in the mutant mRNA.

hif-1(*ia04*) mutants are viable under normal conditions, although they fail to adapt to hypoxia (0.5% or 1% oxygen a 20°C) (Jiang et al., 2001; Padilla et al., 2002). A microarray study of the L3 larval stage treated to 0.1% hypoxia identified 62 mRNAs, including transcription factors, metabolic enzymes, and signaling molecules, that were differentially regulated by HIF-1 (C. Shen et al., 2005). HIF-1 also modifies neuron function and development in low oxygen conditions. A short exposure to hypoxia modifies the aerotaxis of worms from the preferred 10% oxygen to 8% oxygen in a HIF-1-dependent manner (Chang, Chronis, Karow, Marletta, & Bargmann, 2006). Moreover, HIF-1 induces the expression of TYR-2 tyrosinase in the ASJ neurons to antagonize germline apoptosis triggered by CEP-1, the homolog of p53 (Sendoel, Kohler, Fellmann, Lowe, & Hengartner, 2010). In a manner similar to aberrant brain development in hypoxia in mammals, hypoxia induced HIF-1 stabilization led to defective axon guidance and neuronal migration in *C. elegans* (Pocock & Hobert, 2008).

1.3.2. Regulation of *C.elegans* HIF-1

The PHD/VHL-1 pathway regulating HIF-1 protein stability is conserved from mammals to *C.elegans* (Epstein et al., 2001; Jiang et al., 2001). Besides the regulation of the HIF-1 protein, the expression of *hif-1* s also tightly regulated. In normoxia, the HIF-1 subunit is regulated by EGL-9, VHL-1, protein scaffolding protein SWAN-1, and the transmembrane acyl transferase RHY-1 (Shao, Zhang, Ye, Saldanha, & Powell-Coffman, 2010; C. Shen, Shao, & Powell-Coffman, 2006). The expression of HIF-1 target genes was markedly higher in *egl-9(sa307)*, *vhl-1* (*ok161*), *swan-1 (ok267)*, and *rhy-1*(*ok1402)* mutants (Shao et al., 2010; C. Shen et al., 2005, 2006).

1.3.3. Non-traditional roles of *C.elegans* HIF-1

In addition to mediating hypoxia adaptation, *C.elegans* HIF-1 is important for combating several stresses. HIF-1 mediated heat acclimation at 25°C protected worms from severe heat stress (35°C), sodium azide treatment, and heavy metal stress (cadmium) (Treinin et al., 2003). Interestingly, mammalian HIF-1 also contributes to heat acclimation-induced neuroprotection (Shein, Horowitz, Alexandrovich, Tsenter, & Shohami, 2005). *C. elegans* HIF-1 is involved in pathogen responses (Shao et al., 2010), dauer formation (C. Shen et al., 2005), protection against bacterial pore-forming toxins (Bellier, Chen, Kao, Cinar, & Aroian, 2009),.and resistance to high concentrations of hydrogen sulfide and hydrogen cyanide (Budde & Roth, 2010, 2011). Several studies have also explored the HIF-1's role in modulating *C. elegans* lifespan, although the results vary from one lab to the other. Moderate over-expression of HIF-1 increases lifespan, but the effects of loss of function mutation of *hif-1* is variable depending on temperature, food conditions, and small differences in hypoxia concentration (D. Chen,

Thomas, & Kapahi, 2009; Mehta et al., 2009; Muller, Lustgarten, Jang, Richardson, & Van Remmen, 2007).

1.4. Beyond HIF-1: Exploring HIF-independent pathways in hypoxia

It is evident that the HIF-1 is a crucial gene regulating diverse responses for hypoxia adaptation. However, there are several gaps in our knowledge of how cells combat low oxygen challenges.

Though the HIF-1 pathway is thought to be the primary regulator of hypoxic responses, its actions in any system do not account the entire hypoxic response. Data from mammalian cells show that HIF-1α is dispensable for hypoxic upregulation of a host of transcripts (Wood et al. 1998). Studies on CHO cells defective for HIF-1 function showed that well recognized oxygen-regulated proteins including GRP78 and heme oxygenase 1 (HO-1) were regulated independent of HIF-1 (Dachs & Stratford, 1996; Roll et al., 1991) Despite the ubiquitous presence of the HIF pathway, the hypoxia responses vary significantly from cell-type to cell-type, suggesting that HIF-1 and other factors collectively refine hypoxic responses in accordance with cell-specific needs (Ryan, Lo, and Johnson 1998; Carmeliet et al. 2014). For instance, VEGF expression in colon cancer in hypoxia is dependent on the k-*ras* and not *hif-1.* In contrast, the regulation of VEGF in pancreatic and hepatocellular cancer cells is entirely *hif-1* dependent (Mizukami et al., 2004). Considering some hypoxia-response genes are induced in the presence and absence of HIF-1 as in the case of HO-1, it is likely that the same gene is regulated by HIF-1 and other HIF-independent factors depending on the cell type and oxygen concentration (Roll et al., 1991; Wood et al., 1998). It is interesting

to nore that regulators of immune function—c-Myc, NF-KB, and AP-1—also regulate hypoxia- responsive genes in a HIF-1-independent manner in hypoxia (Mizukami, Kohgo, & Chung, 2007).

Largely explored in the context of cancer, two pathways are involved in a HIF-independent hypoxia response to influence gene expression and cell behavior in mammals. The first is mammalian target of rapamycin (mTOR) pathway that mediates the hypoxia response via several pathways. In response to growth and metabolic signals, mTOR, as part of mTOR complex 1 (mTORC 1), controls the translational machinery by phosphorylating ribosomal protein S6 kinase (p70S6K), eukaryotic elongation factor 2 kinase (eEF2 kinase) and eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) (Hay & Sonenberg, 2004). The mechanism by which hypoxia regulates mTOR is not clearly defined, although it is suggested that the activation of the tuberous sclerosis protein I (TSC1)-TSC2 complex and hypoxia-response gene REDD1 contribute to the inhibition (Brugarolas et al., 2004). The final outputs are reduced protein synthesis and induction of apoptosis; however, the molecules mediating the this response are yet to be uncovered (Wouters & Koritzinsky, 2008).

The lack of oxygen and hence, the lack of a terminal electron acceptor, impairs disulphide bond formation and impairs protein folding in the ER. This subsequently activates the unfolded protein response (UPR), although little is known about the molecules that sense changes in oxygen and mediate the response (Ron & Walter, 2007). By an unknown mechanism, hypoxia activates ER stress sensors, including PKR-like ER kinase (PERK) and inositol-requiring protein1 (IRE1) (Koumenis et al.,

2002). Several studies have established a role of XBP-1, the downstream target of IRE-1, with the pathogenesis of neoplasias. For instance, XBP-1 mediates the UPR in solid hypoxic tumors and is essential for hypoxia survival (Romero-ramirez et al., 2004). Consequently, owing to hypoxia derived ER stress, translation is inhibited thereby preventing the accumulation of unfolded proteins in ER, and ER-associated degradation (ERAD) and autophagy are induced to remove misfolded proteins (Bernales, McDonald, & Walter, 2006; Koumenis et al., 2002; Romero-ramirez et al., 2004)

Compared to studies in mammals, more progress has been made in isolating HIF-independent mediators in hypoxia in model organisms. Shen et al. (2005) found 110 hypoxia response genes in *C. elegans*, 47 of which were induced in the absence of HIF-1 (C. Shen et al., 2005). These HIF-1-independent genes included genes involved in energy metabolism, signaling, extracellular matrix modeling, and heat shock proteins. Moreover, Lee et al (2013) reported that the HIF-independent gene *hsp-16.1* is regulated by hypoxia-specific chromatin remodeling involving chromatin modifiers *isw-1* and *hda-1*, *histone H4*, and *NURF-1* (J. Lee & Lee, 2013).

We had previously conducted studies in *Drosophila* to identify HIF-dependent and HIF-independent transcripts in hypoxia. Our studies showed that like HIF-1, the orphan nuclear hormone receptor Estrogen-Related receptor (dERR) regulates a large set of regulated by HIF-1, but a sizeable number of transcripts regulated by ERR were HIF-1-independent. This result was in concert with the role of mammalian ERR in hypoxia; ERR was found to participate in hypoxic responses with HIFs (Ao, Wang, Kamarajugadda, & Lu, 2008), as well as separately from HIF-1 upregulating VEGF and

angiogenesis (Narkar et al., 2011). Moreover, the hypoxic regulators of a large set of ontologically diverse transcripts, are unaccounted for.

In conclusion, there remain substantial gaps in our knowledge regarding the regulators of hypoxic-induced transcription. The regulation of a large cohort of hypoxia response genes in *C.elegans*, *Drosophila*, and mammalian cells cannot be explained by the actions of HIF alone and existing data strongly suggest a HIF-independent player that works parallel with HIF-1 in hypoxia. What is the mediator of the HIF-independent response? Is this mediator regulated in the same manner as HIF1? Does this mediator function in concert with HIF-1 or does it regulate a separate pathway for hypoxia adaptation? This thesis describes my efforts to resolve some of these unresolved questions.

Aims

Graphical representation of hypothesis
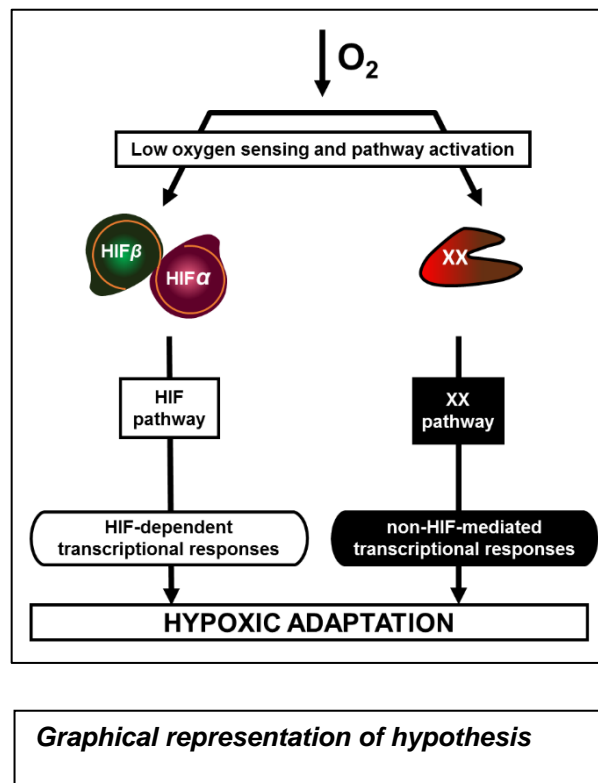
I hypothesized that an unidentified factor (xx) acts in parallel to but separate from the HIF-1 pathway to regulate transcriptional responses for hypoxia adaptation (Figure 2.1). We proposed to use the *C.elegans* to test this hypothesis because of the following reasons: (1) superior tools available for genetic manipulations; (2) conservation of hypoxia responses in worms and mammals; (3) viability of *hif-1(ia4)* mutants in normoxia. Since the well-established regulators of hypoxic transcription fulfil the same functions in mammals and *C.elegans*, we anticipated that the newly identified players in *C.elegans* is likely conserved in mammals. Given the pervasive influence of HIF in disease progression, we expected the HIF-independent transcription factors to also play similarly influential roles.

Aim 1. Establish hypoxic sensor animals that report on HIF-dependent and HIF-independent actions. I planned to use hypoxia-sensitive genes that are expressed in a HIF-independent manner to generate animals that report on pathway-specific hypoxic activation. Generation of these transgenic animals will enable us to view how specific treatments or genetic backgrounds influence hypoxia responses and allow for the targeted disruption of one hypoxic response pathway over another.

Aim 2. Identify essential regulators of non-HIF-mediated hypoxia responses by RNAi screen. Using hypoxia, I planned to conduct an RNAI screen for factors that specifically target non-HIF-mediated responses, while leaving the HIF pathway functionally intact. I then sought to explore downstream targets of the HIF-independent transcription factor and determine how hypoxia adaptation is compromised in loss of function mutations of the HIF-independent factor

3. Materials and Methods

3.1: Custom hypoxia chamber for *C.elegans*

Our lab had previously used hypoxia chambers for inducing hypoxia in flies and cells. To understand if the hypoxia chambers available in lab could be used to induce hypoxia in worms, I used a visual assay using a previously generated reporter of HIF-1 function. The expression of nuclear hormone receptor *nhr-57* is induced by hypoxia and a reporter construct in which the expression of GFP is controlled by the promoter of *nhr57:GFP* has been previously established. Upon hypoxia treatment, *nhr-57:GFP* levels increase markedly. The reporters were treated to 0.5% hypoxia in the Hypoxia Incubator Chamber, which was previously used to treat flies. The Hypoxia Incubator Chamber is a sealed chamber with a surface-type seal and an inlet for pre-mixed gas. I used 0.5% oxygen CO_2 premade gas for this purpose. Once the gas is purged in, the O-ring is uniformly compressed by a ring clamp for an air-tight seal. Surprisingly, following hypoxia treatment, the *nhr57:GFP* reporters failed to show GFP induction. Moreover, qPCR experiments showed that there was no induction of *nhr57* mRNA expression upon hypoxia treatment. This suggested that the worms were not experiencing hypoxia in the hypoxia incubator chamber, possibly because the gas was escaping through the O-ring in spite of tight sealing.

C.elegans elicits different physiological responses in different conditions, which highlights the importance of having tight experimental control over the hypoxic concentration of O_2. Hence, I decided to collaborate with Coy Laboratory to design a

custom hypoxia chamber. Coy Labs has developed a wide array for hypoxia and anoxia chamber for cell culture. We decide to modify the chamber in the following manner:

The Coy Lab hypoxia chamber for cells has a small glove box that is connected to a bigger hypoxia compartment with a door. All the manipulations are conducted in the glove box to minimize gain of oxygen from the bigger compartment. The hypoxia chamber contains the regulators and flow meters and the fixed oxygen sensor consisting of a galvonic electrode with a gold cathode to monitor the oxygen level. For inducing hypoxia in worms, we decided to adapt the hypoxia chamber to only include the glove box. Hence, the regulators, flow meters, and sensor is now on the glove box. Owing to its smaller volume and omission of the temperature and carbon dioxide inlets, the system became cheaper and more effective at maintaining low oxygen concentrations. Gases are introduced through a unique gas tube shower system that, along with the internal fan, provides the circulation required for uniform gas distribution and fast response of the controller.
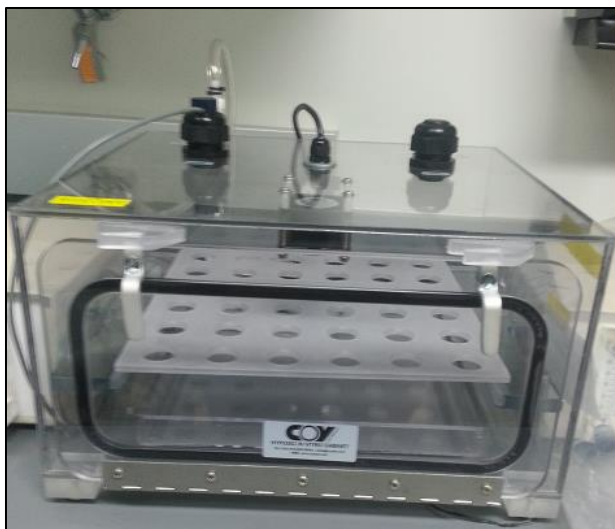
Figure 3.1: Modified Coy Lab hypoxia chamber for *C.elegans*

Instead of pumping pre-made oxygen gas and then starting the system, we decided to apply a continual flow method using a nitrogen cylinder. We found that apart being far cheaper (200$ per oxygen tank to 12$ per nitrogen tank), a continual system ensures rapid equilibration of the chamber and increases the stability of the system.

Setting up hypoxia chamber: One-eighth-inch outer-diameter tubing was used with snap connectors for all connections. The tubing has to be unreactive with O2 and we used such as fluorinated ethylene propylene. The compressed gas tank is connected to a rotameter (Aalborg) that acts as a flow control device. The upstream pressure from the tank must be within the range of the flow device and the hose barb fittings.

Treating worms to hypoxia: Worms are transferred onto the plates and placed in the chamber. The oxygen concentration is then adjusted. For short-term studies (24 hours), plate dessication is not necessary, but for long-term studies, a plate with distilled water must be placed in the chamber. Once the treatment is over, plates should be removed and worms immediately collected within a minute for further applications. For qPCR experiments, collect worms with cold M9.

Verifying P*nhr57::GFP* expression in hypoxia: To ensure that the chamber induces hypoxia appropriately, I used the HIF-dependent reporter P*nhr57::GFP* as a visual assay. I saw consistent P*nhr57*::GFP expression from 0.1%--0.5% hypoxia, thus verifying the utility of the hypoxia chamber. A representative experiment is shown in Figure 3.2.
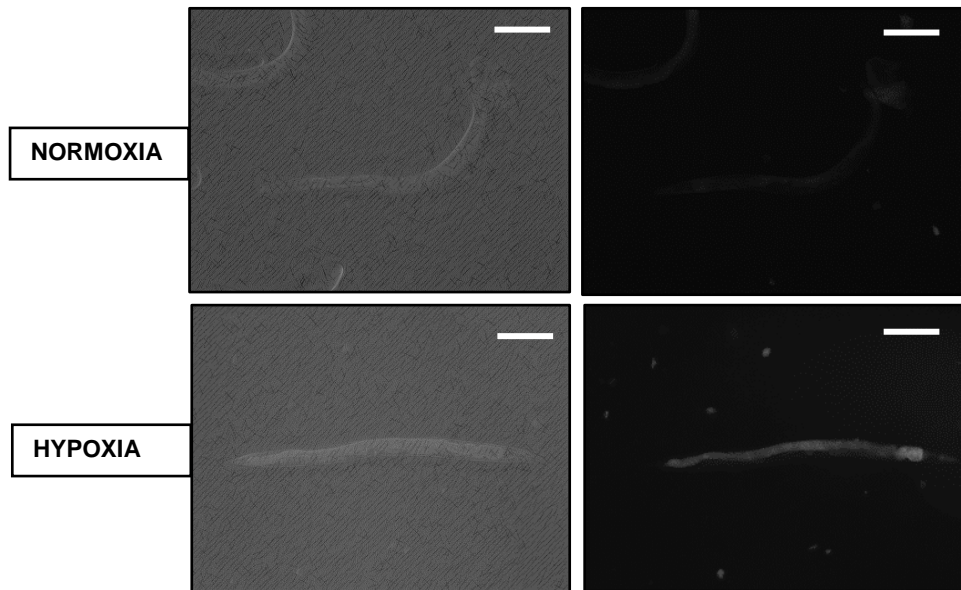
Figure 3.2: The HIF-dependent reporter Pnhr-57:GFP is induced following 0.1% hypoxia treatment Reporters were treated for 5 hours for a range of hypoxia concentrations from 0.1—0.6% and the reporters showing GFP fluorescence were scored. The HIF-1 dependent reporter was induced reliably until 0.5% hypoxia (as shown in the figure). The images on the left image are DIC, while those right are taken using fluorescence microscope Scale bar: 10uM

3.2: Strains

Worms were maintained as described (Sulston and Hodgin 1988) with the following modifications: grown on NGMSR plates (Avery 1993) at 20° on *E. coli* strain

HB101 unless indicated differently.

The wild type strain was *C. elegans* variant Bristol, N2. The *blmp-1* (*tm548*) mutant was generated by the National Bioresource Project in Japan; it contains an 810 bp deletion that has been described elsewhere (Huang *et al.* 2014b). We acquired it from Dr. Horvitz then out-crossed it 10 times against N2 (the outcrossed strain is calledYJ55). The *hif-1* mutant strain is ZG31 *hif-1(ia4)*, carrying a 1,231 bp deletion (JIANG *et al.* 2001)*.

The *egl-9* allele (*sa307*) contains a 243 bp deletion *(Darby et al. 1999).*

The *3kb-F45D3.4::GFP* reporter plasmid was generated by cloning the promoter

of *F45D3.4* into pPD95.69 using the following primers: 5′- ataagctcacttgttaggtccaattggc-3′ (forward containing a HindIII site) and 5′- atgagctcgtttatttcgagcggttgtg-3′ (reverse containing a SacI site). The plasmid was injected into either the wild type or *hif-1(ia4)* background to generate the respective reporter strains (YJ28 [*uyEx3[myo-2::mCherry pF45d3.4::GFP]* and YJ36 [*hif-1(ia4);uyEx3[myo-2::mCherry pF45d3.4::GFP]]*). mCherry, under the control of a *myo-2* promoter, was used as a transgenic marker. The *egl-9* reporter strain (YJ204) was generated by crossing YJ36 with CB6088 *egl-9(sa307) hif-1(ia4)*. For hypoxia treatment, animals were cultured on NGMSR plates in a Coy hypoxia chamber with oxygen analyzer (Coy Laboratory Products, Grass Lake, MI). Oxygen was kept constant using a continuous nitrogen gas source. For cobalt chloride

treatment, a 100 mM stock solution was prepared and filter sterilized using a 0.22-µm filter. 500 µl of the stock solution was added onto a 10 ml plate to make a final concentration of 4.76 mM. The effect of cobalt chloride on worms was examined either by a western blot assay to measure HIF-1 protein, by qRT-PCR to assess transcript level, or by fluorescence microscopy to detect reporter gene expression induced by hypoxia. cobalt chloride-induced GFP fluorescence lasts for at least 48 hours in our reporter strain.

3.3. Hypoxia sensitivity assay

Assays for embryo viability at 0.5% or 2.0% O_2 were performed as described previously (PADILLA *et al.* 2012). In short, 15-20 1-day-old adults were allowed to lay eggs on a NGM plate seeded with OP50 for 1 hour and then removed. The eggs were counted and incubated in hypoxia (0.5% or 2.0% O_2, 20°) for 96 hours using a hypoxia

glovebox workstation (Ruskinn Inc). Immediately after treatment, animals were examined to determine the number of animals that survived the hypoxia treatment and the developmental stage of survivors. At least three independent experiments were performed for each condition.

3.4. RNAi screen

A bacteria-mediated feeding RNAi screen was performed as described (Fraser

et al. 2000) with the following modifications: The YJ36 strain was screened with the clones of transcription factor genes from the Ahringer feeding library (Kamath and

Ahringer 2003). The plates contained NGM agar with 1 mM IPTG and 100 µg/ml carbenicillin that were inoculated with bacterial cultures grown 16-18 hours for each

targeted gene. L1 stage worms were then transferred onto clonal plates and left at 20⁰.

Adults were then subjected to hypoxia or cobalt chloride treatment.

3.5. Quantitative RT-PCR

For total RNA preparation, 1-day-old adults were grown on NGMSR plates at

20°, washed with M9 buffer and re-suspended in Trizol (Bioline). RNA extraction was

carried out by freeze-thawing 4 times in liquid nitrogen and then 250μL chloroform was

added. Samples were vortexed for 30 seconds and spun down at 13,200 rpm for 10

minutes at 4°C. The aqueous phase was taken and 500μL of isopropanol was added.

Samples were vortexed and then incubated at -20°C for 30 minutes. RNA was pelleted

at 13,200 rpm for 15 minutes at 4°C. The supernatant was removed and the RNA pellet

was washed with 75% ethanol. The samples were then centrifuged at 13,200 rpm for 10

minutes at 4°C. Supernatant was removed and RNA pellet was dried at 37°C. RNA was

then re-suspended in the appropriate amount of DEPC H_2O depending on pellet size

and then incubated at 55°C for 10 minutes. Isolated RNAs were treated with DNase

(Promega, Madison, WI) in a 1:10 ratio with DNase and Buffer and incubated at 37°C

for 30 minutes. Following ethanol precipitation and air-drying, the pellet was dissolved in

DEPC water. cDNA was synthesized from 2 µg of RNA (Applied Biosystems, catalog

number# 438706). 2μg RNA, 1μL Random Hexamer Primer Mix, 1μL 10mM dNTPs mix

were preheated at 95°C for 1 minute then cooled to 4°C. Then the 5x RT Buffer, 200U

Reverse Transcriptase, 10U RNase Inhibitors, and DEPC H_2O was added to a final

volume of 20 μL. The PCR was set as follows: 42°C for 50 minutes, 70°C for 15 minutes

and then cooled to 4°C.

Real-time qPCR was performed using SensiMix SYBR and Fluorescein Kit (Bioline, Taunton, MA) according to manufacturer's instruction. Briefly, the cDNA was diluted to 1:20 ratio and then 5μL of the cDNA was used as the template for the reaction. The reaction mixtures for the genes were prepared as follows: 12.5 μL of SYBR Green mix, 1 μL 5 μM forward primer, 1 μL 5 μM reverse primer, and 5.5 μL of dH_2O. All the samples were assayed in three biological replicates and analyzed using a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). *ama-1* (RNA polymerase II) was used for normalization.

3.6. Western blotting

Equal amounts of protein were mixed 1:1 with 2X Laemmli sample buffer containing 1 mM β-mercaptoethanol. Samples were incubated at 95°C for 5 mins and separated using SDS-PAGE electrophoresis (running buffer: 25 mM Tris, 192 mM glycine, 0.1%SDS). The proteins were transferred to a nitrocellulose membrane and soaked in transfer buffer (70% methanol, Tris-Hcl) (Millipore, Billerica, MA) using a wet transfer apparatus (Bio-Rad, Hercules, CA). After trans-blotting, membranes were incubated in blocking buffer (5% nonfat dry milk and 1% BSA in 0.5% TBST) overnight. Membranes were incubated with antibody in 0.5% TBST (1% milk, 0.1% BSA). Antibodies used were: anti-Ce-HIF-1 antibody (a gift from Dr. Peter Ratcliffe, Oxford; 1: 5,000 dilution); AA4.3 anti-tubulin antibody (developed by Charles Walsh and obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242, 1:1000 dilution); anti-rabbit antibody IgG conjugated with HRP (Santa Cruz SC2030, 1: 5000 dilution); and anti-mouse antibody conjugated with HRP. The bands were detected

using an ECL plus kit (GE Healthcare, catalog# RPN2133).

3.7. Microscopy

GFP-expressing worms were observed using a Zeiss Axio A2 Imager. Images were acquired using Zeiss Axiovision software.

3.8. Chromatin Immunoprecipation (ChIP) assay

The ChIP assays were performed as described, with minor modifications (Niu et al., 2011). L1 stage worms were grown at 20° and then treated with hypoxia at L3 or 1-day adult stages. After treatment, worms were collected and washed and treated with M9 containing 750 µl formaldehyde for crosslinking. The samples were incubated at RT on a shaker for 30 minutes. To stop crosslinking, 1M Tris-HCl was added and samples were incubated on a shaker at room temperature for another 10 mins. The samples were then spun down at 4°C, 450xg for 10 minutes. The supernatant was removed and the pellet lysed in FA buffer (50 mM HEPES-KOH, pH7.5, 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS and protease inhibitor 10 cocktail (Roche, 11836170001) by sonication (5 times at 10 s intervals). The samples were then spun 4°C, 7000 rpm for 10 minutes. Supernatant (chromatin) was pre-cleared as follows: 100 µl supertanant + 1 ml IP buffer (0.01%SDS, 1.1% Triton X, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.0, 16.7mM NaCl) with inhibitors + 10µl ChIP beads (3X washed protein G sepharose beads resuspended in equal volume of ChIP buffer with 10 µl of 1 mg/ml BSA per 100 µl beads and 5 µl of 10 mg/ml red herring DNA per 100 µl beads) on rotator at 4°C for 30 minutes. Beads were pelleted to give a pre-cleared chromatin. 200 µl was saved as input. The lysates were incubated overnight at 4° with

either anti-GFP (Abcam ab290) or IgG (Millipore). The next day, 50µl ChIP beads were added and samples were incubated on a rotator at 4°C for 2hrs. Beads were spun down at 6000 rpm at 4°C for 1 min and were washed 2X (10 mins rocking at 4°C followed by a spin at 6000 rpm for 1 min) with each of the following buffers: ChIP buffer I (0.1% SDS, 1% Triton X, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150mM NaCl), ChIP buffer II (0.1% SDS, 1% Triton X, 2mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), ChIP buffer III (0.25 M LiCl, 1% NP.-40, 1% Sodium Deoxycholate, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0) and ChIP buffer IV (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA was then eluted from the beads by adding 200 µL ChIP elution buffer (1% SDS, 0.1 M $NaHCO_3$) and rocking for 15 mins at room temperature. The beads were pelleted at 6000 rpm. Cross linking was reversed by adding 8 µl 5M NaCl followed by incubating the supernatant (and also the input sample that was saved previously) overnight at 65°C. 1 ml PB buffer was added and samples were purified on miniprep columns. Final DNA was eluted with 50 µl buffer watee. DNA was recovered by phenol-chloroform extraction, precipitation, and then eluted.

3.9. Tunicamycin treatment

Synchronized eggs of wild-type and *blmp-1* mutants were plated onto standard plates, counted, and allowed to hatch. After 24 hours, the L1s were treated with tunicamycin (1 ug/ml or 5 ug/ ml) for 24 hours and the adults were counted after 3 days. Three independent experiments were conducted.

3.10. Statistical analysis

Results are presented as the mean ± SE. Statistical comparison was performed using two-tailed Student's t-tests. A p-value less than 0.05 was considered statistically significant and annotated by *.

3.11. Primers used:

qRT-PCR	Sequence
mod-5 Forward	5'-tgggattccgaagttccgtg-3'
mod-5 Reverse	5'-tgacggagtggtcaatggtg-3'
zip-1 Forward	5'-ccgcctcatccaataaccga-3'
zip-1 Reverse	5'-tcctcctcggctggtatgaa-3'
F44E5.5 Forward	5'-ggaggaggtacatttgatgtttc-3'
F44E5.5 Reverse	5'-tcgttgatcaaagtcttctcct-3'
icl-1 Forward	5'-agcagaccagattgaagcctgtga-3'
icl-1 Reverse	5'-cagccaaacgagacgcattcaagt-3'
ama-1 Forward	5'-aacgcggtaccatcagtttc-3'
ama-1 Reverse	5'-ggacgacgtgttcctacgat-3'
fmo-12 Forward	5'-aacggtacagtcagaatcaagccaa-3'
fmo-12 Reverse	5'- actaactggaaccaactttccgtgt-3'
egl-9 Forward	5'-atccggaaatctactgactcgggagta-3'
egl-9 Reverse	5'-tgggagcccttcgtctagatctt-3'
nhr-57 Forward	5'-gtatgccggaaaaagaacga-3'
nhr-57 Reverse	5'-atgcaggggaagatgaacag-3'
ChIP primers	
cF45 F	5'-agacagtgatagaggaatgtaaacac-3'
cF45 R	5'-atattgccgccctgtagtcgt-3'
Cloning	
F45D3.4 3kb Forward	5'-ataagctcacttgttaggtccaattggc -3'
F45D3.4 3kb Reverse	5'- atgagctcgtttatttcgagcggttgtg-3'
F45D3.4 5kb Forward	5'- gatc aagctt cga aaa cga caa cac cga atg cg–3
F45D3.4 5kb Reverse	5' - aatt ccg cgg gtt tat ttc gag cgg ttg tg – 3'
Genotyping	
HIF Geno Forward	5'- gat tga agg tgg agt agc gcc –3'
HIF Geno 1 kb Reverse	5' -cgg agc cag atc cac atc gaa t-3'
HIF Geno in Reverse	5'- gttttctattgctcacctgt – 3'

4. Results

4.1: *C.elegans hif-1* mutant phenotype

Organismal effects of hypoxia have been explored by examining the viability to adulthood of *C. elegans* embryos. Embryos laid by wild-type (N2) and *hif-1* deletion mutants survive in house air O_2 concentrations (21% O_2). N2 worms are able to adapt and survive to adulthood in 0.5% O_2, while *hif-1* embryos cannot, thereby underscoring the importance of HIF-1 for hypoxia adaptation (Iii & Roth, 2010). However, another lab reported the *hif-1* mutants can survive 0.5% O2 but cannot survive 0.3% O2 (C. Shen et al., 2005). Owing to these discrepancies, I sought to find the concentration at which HIF-1 is stabilized for hypoxia adaptation. I conducted a dose response study with wild type and *hif-1* mutants and scored for the lethality phenotype of *hif-1* mutants.

Some labs conduct hypoxia lethality studies by placing embryos in continuous hypoxic treatment of worms for 5 days (Padilla, Goy, & Hajeri, 2004). Our chamber is inefficient at maintaining hypoxia concentrations for extended periods of time (oxygen sensor needs to be recalibrated after 48 hours continuous treatment), and hence, I modified the lethality assay to include shorter but harsher treatment to the embryos for 24 hours.

Accordingly, embryos from wild type and *hif-1* mutants were treated to hypoxia for 24 hours, and then reared in normoxia for 3 days. Embryos that survived the insult and became adults were counted. As seen in Figure 3.1, *hif-1* mutant embryos cannot survive harsh hypoxia treatment at 0.1% and the number of surviving *hif-1* mutants correlated with oxygen concentration. The lethality phenotype was not observed after 2% hypoxia and since the Ce-HIF-1 antibody is not commercially available, we were

unable to look at HIF-1 stabilization at the different oxygen conditions. However, we confirmed via western blotting that HIF-1 is stabilized following 12 hours treatment in 0.1% hypoxia. Thus, we established that the HIF-1 pathway is activated at oxygen concentrations below 2% and there exists a dose dependent relationship between survival of *hif-1* mutants and oxygen concentration.

A)

B)
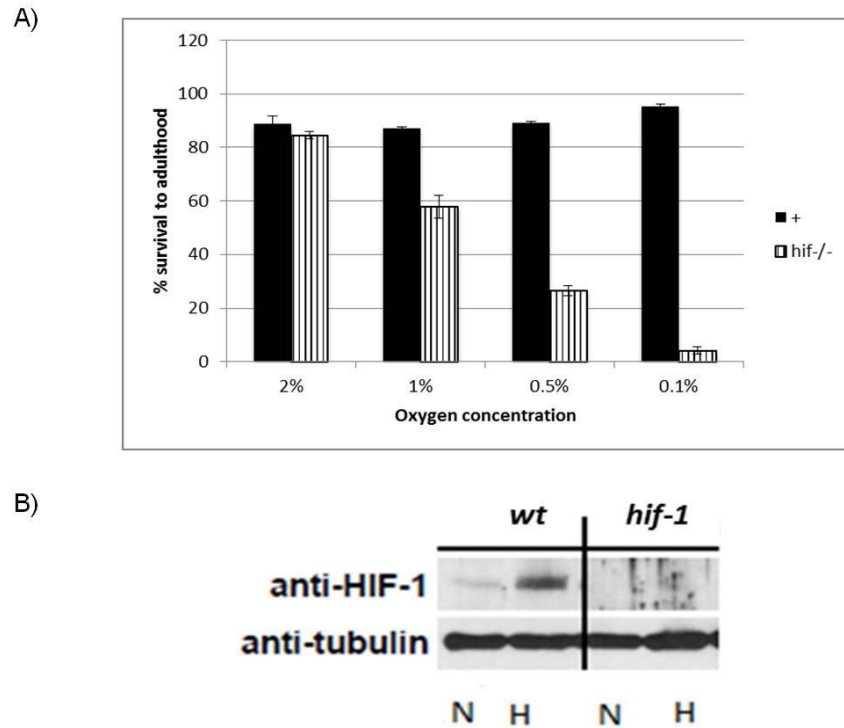
Figure 4.1. Dose dependent response of hypoxia treatment: (A) Wild type and *hif-1*(ia4) mutant embryos were counted and treated to the indicated concentration of hypoxia for 24 hours, following which worms were allowed to proceed to adulthood in normoxia. The percentage of worms surviving to adulthood after hypoxia treatment is plotted. **(B)** Western blot depicting stabilization of HIF-1 protein following 01% hypoxia treatment

4.2. HIF-dependent and HIF-independent genes in hypoxia in *C. elegans*

Previous studies have demonstrated the necessity and importance of non-HIF pathway mediated transcriptional responses in hypoxia (J. Lee & Lee, 2013; Li et al., 2013; C. Shen et al., 2005). By microarray studies, Shen et al (2005) had identified transcripts that were regulated in a HIF-1-dependent and HIF-1-independent manner in L3 larvae treated to 0.1% hypoxia for 4 hours. In order to verify the results of the microarray, I conducted qRT-PCR analysis of 2 candidate genes for each category (Figure 3.2.a). Expressions of *nhr-57,* a nuclear hormone receptor of unknown function*,* and *fmo-2,* a flavin monoxygenase*,* as HIF-1-dependent targets and *F45D3.4* and *F44E5.5*, an hsp-70 protein, as HIF-1-independent targets were evaluated.

We validated that *nhr-57* and *fmo-2* is normally induced in wild-type larvae when they are subject to 0.1% O2 treatment for 12 hours. Further, we confirmed that the response is HIF-dependent by demonstrating that the induction of the genes in hypoxia is diminished in the *hif-1* mutant background. We next validated that the HIF-1-independent genes and found that *F45D3.4* and *F44E5.5* are normally induced in wild-type larvae following a 0.1% O2 treatment for 12 hours. The response is HIF-independent as the induction in hypoxia is maintained in *hif-1* mutants. Moreover, basal values of most of these genes were plotted to ensure that there was no added variability owing to genotype. A representative plot for the basal expression of *F45D3.4* is shown in Figure 3.2.b.
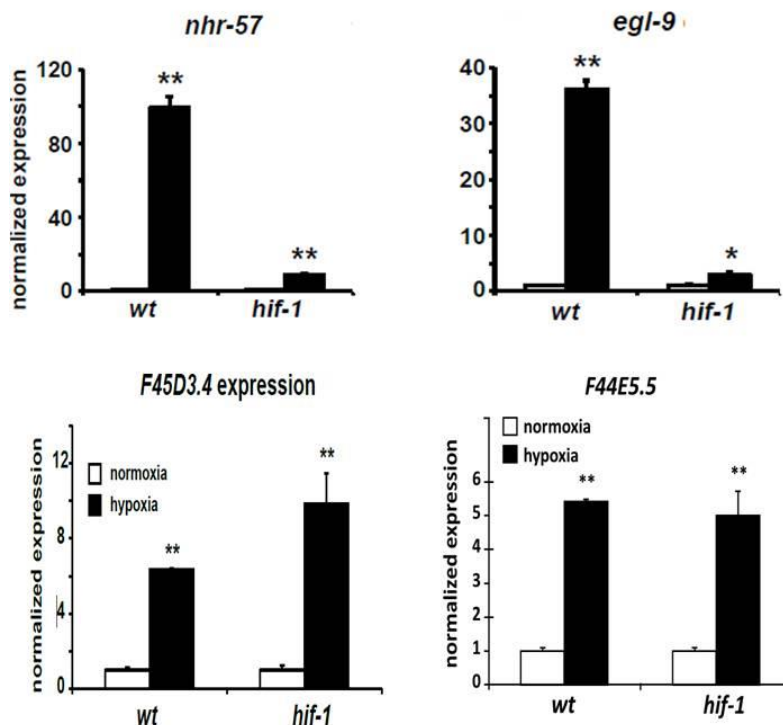
Figure 4.2.a. Hypoxia induces HIF-dependent and HIF-independent genes Wild type and *hif-1* mutant adults were treated to 0.1% hypoxia for 12 hours and qRT-PCR was conducted for 2 HIF-dependent targets (top row) and 2 HIF-independent targets (bottom row) **p<0.01. Data are expressed as mean ± SEM.
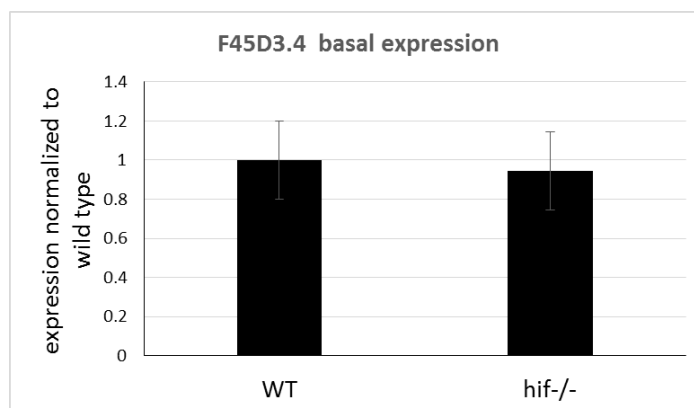
Figure 4.2.b. Expression of HIF-independent gene *F45D3.4* was not altered in basal conditions. The expression levels of *F45D3.4* were determined in wild type and *hif-1* mutant adults at normoxia by qRT-PCR. Data was normalized to wild type normoxia. Data are expressed as mean ± SEM

4.3: *3kb-F45D3.4::GFP* **is a hypoxia-dependent HIF-independent reporter**

To develop a visual assay for HIF-1-independent transcriptional activity, I generated transgenic reporter lines in both the wild-type and *hif-1* mutant backgrounds, where GFP was placed under the control of elements controlling *F45D3.4* expression. I generated 5-kb and 3-kb promoter constructs and found that the *5kb-F45D3.4::GFP* construct was expressed at high levels even in normoxia. The *3-kb-F45D3.4::GFP* construct showed negligible expression in normoxia, and following a 12-hr hypoxic treatment in a chamber containing 0.1% O2, I found that the proximal 3-kb region of the *F45D3.4* promoter was sufficient to drive reporter expression in wild-type and *hif-1* mutant backgrounds (Figure 3.3).

These data confirm that *F45D3.4* is a HIF-independent hypoxia response gene and suggest that the regulatory elements mediating the response localize to a 3-kb region that flanks the *F45D3.4* transcription start site.
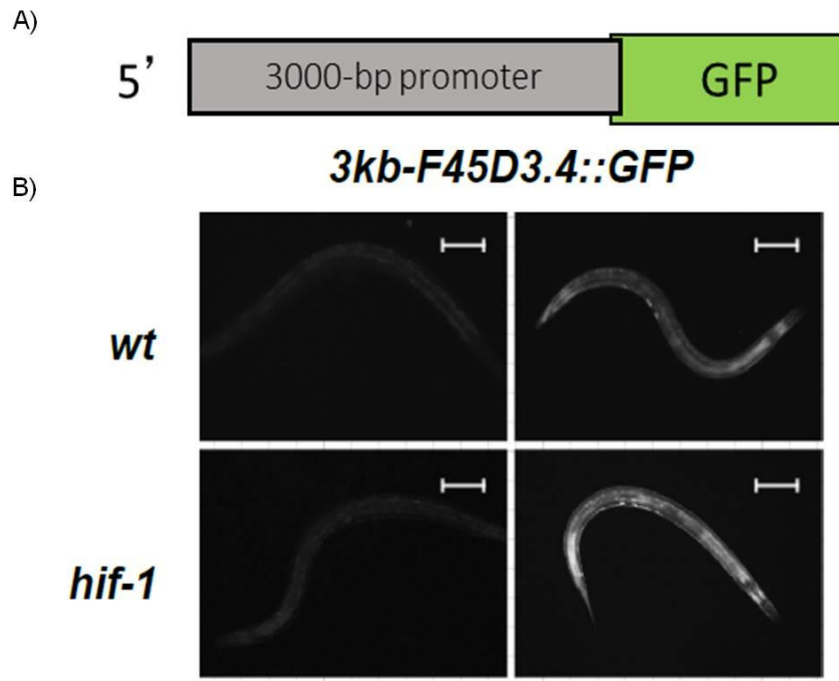
Figure 4.3. HIF-independent transcriptional regulation of *F45D3.4* in hypoxia A) Schematic of HIF-independent reporter *3kb-F45D3.4::GFP* **B)** Expression of GFP in wild type and *hif-1(ia4)* mutant backgrounds carrying the *3kb-F45D3.4::GFP* construct after treatment in normoxia and hypoxia for 12 hours. Scale bars indicate 10 µm.

4.4. Cobalt chloride mimics hypoxia in *C.elegans*

Considering that one of the major goals of the study was to conduct a screen, we found that the size of the hypoxia chamber was restrictive because only a limited number of plates could fit in the chamber. Hence, we decided to explore other options for inducing hypoxia in the worms. Cobalt chloride is widely used as a hypoxic mimetic in cells, because it can elicit normoxic transcriptional responses that greatly resemble those seen in hypoxia (Vengellur & LaPres, 2004); however, its utility in recapitulating transcriptional hypoxic responses in *C.elegans* had not been demonstrated. Since it is easier to treat several plates to cobalt chloride at once as opposed to the hypoxia chamber, we decided to conduct the screen using cobalt chloride as a hypoxia mimetic, and then verify the results with the hypoxia chamber.

The utility of cobalt chloride as a hypoxia mimetic was tested by three approaches: (1) Does cobalt chloride stabilize HIF-1 protein? (2) Does cobalt chloride treatment induce the expression of hypoxia-dependent genes? (3) Does cobalt chloride induce the HIF-independent reporters?

First, I grew worms on plates containing 4.76 mM cobalt chloride for 12 hours and asked if the treatment affected HIF-1 protein levels. We found that cobalt chloride caused a marked increase of HIF-1 protein in control animals that is similar to the accumulation seen in hypoxia. The cobalt chloride treatment also induced a robust transcriptional upregulation of the *F45D3.4* transcript in wild-type animals and *hif-1* mutants in a manner that resembled previous hypoxic treatments. Similarly, a separate HIF-independent transcript, *F44E5.5* (C. Shen et al., 2005), was also upregulated by the treatment. These results suggest that cobalt chloride treatment triggers a transcriptional

response that mimics hypoxic treatment, impacting both HIF- and non-HIF-mediated pathways. Finally, I tested whether the *3-kb-F45D3.4::GFP* reporter responded to cobalt chloride and found the cobalt chloride robustly induced GFP expression in the reporters (Figure 4.4)
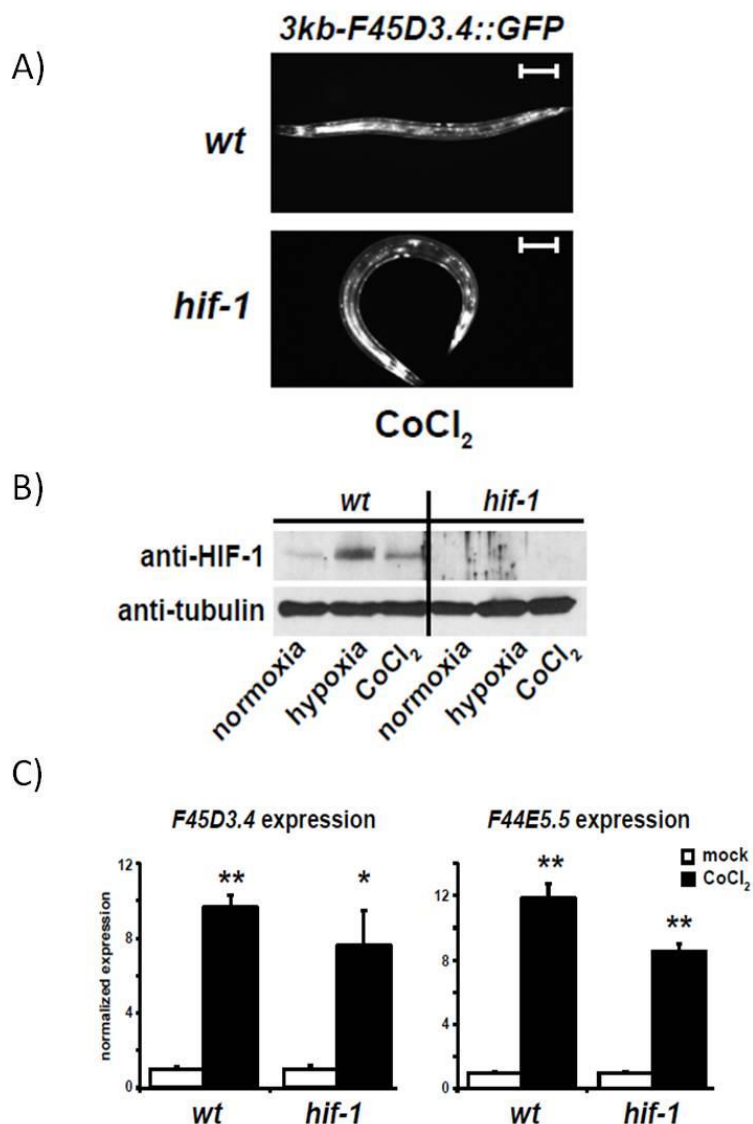
Figure 4.4.a. Cobalt chloride mimics hypoxia treatment in *C.elegans*
A) Expression of GFP in a wild type animal and a *hif-1(ia4)* mutant carrying the *3kb-F45D3.4::GFP* construct after treatment in 4.76 mM cobalt chloride. Scale bars indicate 10 µm **B)** Stabilization of HIF-1 protein was monitored by western analysis following normoxic, hypoxic, and cobalt chloride (4.76 mM) treatments for 12 hours in wild type and *hif-1(ia4)* mutants. Tubulin signal served as a loading control. (**C&D**) Total mRNA was collected from wild type animals and *hif-1(ia4)* mutants after incubation on mock- or cobalt chloride-treated (4.76 mM) plates for 12 hours to measure the expression of the HIF-independent genes *F45D3.4* (C) and *F44E5.5* (D) by qRT-PCR. Normalized values are the average of at least three biological replicates. Error bars are SEM. * indicates a p-value < 0.05, while the p-value for ** is < 0.01. Student's *t-test* was used to determine significance.

Consistent with the effects observed on the endogenous *F45D3.4* transcript the *3-kb-F45D3.4::GFP* reporter animals also responded to cobalt chloride in a concentration-dependent manner in wild type and *hif-1* mutant backgrounds. I found that 4.76 mM induced maximum response in reporters of both background. Next, I looked at percent lethality at this concentration in both reporters. Up to 16 hours of cobalt chloride treatment did not diminish survival but treatments beyond that time tended to negatively impact survival of the wild type and *hif-1* backgrounds equally, suggesting the loss of HIF action was not an important factor in the susceptibility to cobalt chloride treatment. GFP induced by cobalt chloride treatment lasted for at least 48 hours after treatment was stopped.
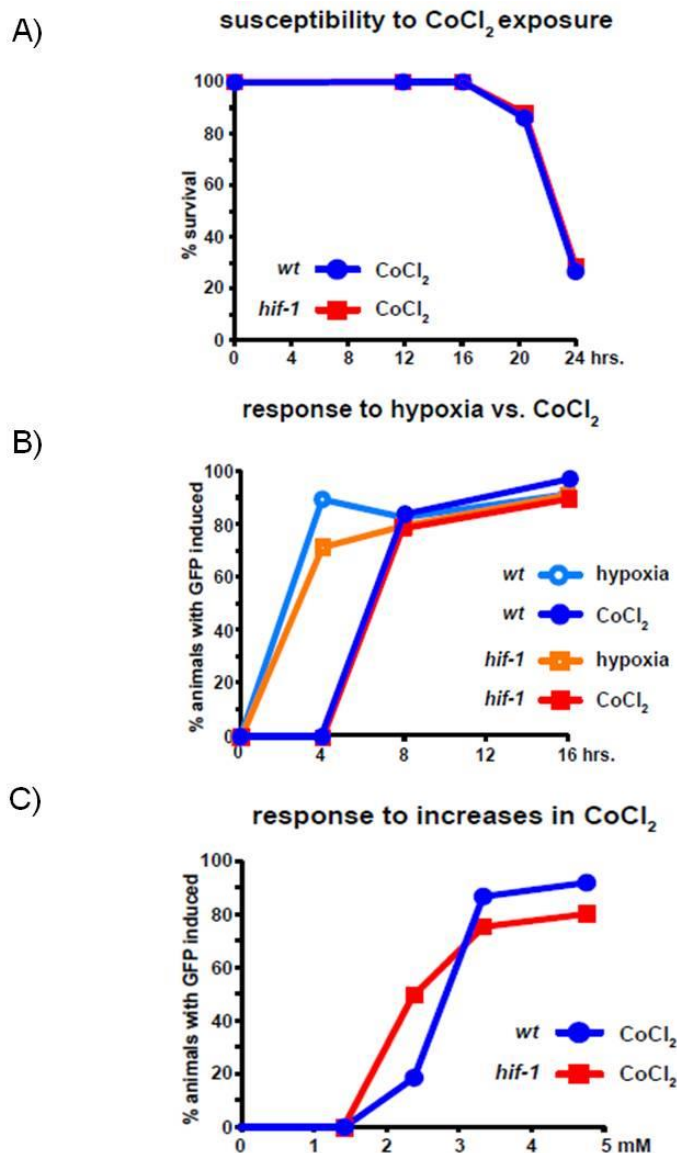
Figure 4.4.b. Testing the cobalt chloride response in *C.elegans*
A) Transgenic *F45D3.4* worms were treated on plates containing 4.76 mM cobalt chloride to measure the effect on lethality of prolonged exposure to cobalt chloride **B)** the hypoxic and cobalt chloride-elicited responses in the indicated backgrounds over time **C)** 4.76 mM is the optimal concentration of cobalt chloride that elicits maximum response in wild type or *hif-1(ia4)* mutants. All data points represent the average of at least 2 different biological pools consisting of at least 20 individual animals.

4.5: Effect of variety of stresses on the HIF-independent reporter

As is the case with cobalt chloride treatment, stimuli other than low oxygen can transcriptionally upregulate hypoxic response genes. To better understand the upstream mediators of the HIF-independent response and to determine how they may differ from those controlling HIF-dependent responses, we subjected *3kb-F45D3.4::GFP* reporter animals to different cellular stresses and compared the responses to results obtained from *nhr-57::GFP* reporter animals, a previously described HIF-dependent reporter line that also responds to hypoxic treatment (C. Shen et al., 2006). Unexpectedly, the *nhr-57::GFP* reporter was not activated by cobalt chloride treatment (1.42 - 4.76 mM) like the *3kb-F45D3.4::GFP* reporter. In spite of the difference in responsiveness to cobalt chloride, the two reporters behaved quite similarly when subjected to atmospheric or low levels of oxygen; neither was active in normoxia (~21% O_2). I wanted to ascertain that the GFP induction was specific to hypoxia and was not a general stress response. I subjected the reporters to various treatments, some of which are known to elicit HIF- and/or non-HIF-pathway responses, and scored for lack of GFP induction. Oxidative stress activates the HIF-1 pathway via reactive oxygen species (Eom, Ahn, Kim, & Choi, 2013). Treatment of *nhr57:GFP* reporters to the herbicide paraquat, which causes mitochondria to generate superoxide anions, resulted in activation of the HIF-dependent reporter. As opposed to the HIF-dependent *nhr57:GFP* control, the HIF-independent reporter did not show GFP expression suggesting that the ROS as those generated by paraquat does not induce this reporter even if HIF-1 is present. Further, Lee et al. (2013) had recently reported that calcium ions are necessary for the induction of a HIF-1-independent gene-hsp-16.1-in hypoxic conditions (J. Lee & Lee, 2013). To test if the

disruption of the cellular calcium balance affected the hypoxia response of reporter, I treated the *3kb-F45D3.4:GFP* reporter with EGTA, which specifically chelates calcium (J. Lee & Lee, 2013). I also looked at some other stresses including hypertonic stress (300 mM NaCl) (Iii & Roth, 2010), heat-shock (37° for 6 hours) (Treinin et al., 2003), and, heavy metal exposure (0.02 mM $NiCl_2$) (Murphy et al., 2011). Interestingly, HIF-1-independent reporters when stressed with starvation (I. Lee, Hendrix, Kim, Yoshimoto, & You, 2012), showed GFP induction even without hypoxia treatment.

Stress	Treatment	GFP reporter induction?	
		F45D3.4-3kb	*NHR-57*
anoxia	0.0% O_2	No	No
hypoxia	0.1 - 0.4% O_2	Yes	Yes
hypoxia	0.5% O_2	No	Yes
$CoCl_2$	1.42 - 4.76 mM	Yes	No
osmotic	300 mM NaCl	No	No
heat	37°C 6hrs.	No	No
ROS	4 mM paraquat	No	Yes
starvation	12 hrs	Yes	No
heavy metal	0.02 mM $NiCl_2$	No	No
calcium chelation	0.4.76 mM EGTA	No	No

Table 1: Effects of stresses on the expression of HIF-independent reporter The reporters were treated as listed and GFP induction scored.

4.6. A transcription factor RNAi screen for identifying candidate mediators of the HIF-independent response.

We aimed to find transcription factors that facilitate non-HIF-mediated hypoxia-sensitive responses. We conducted an RNAi screen using a commercially available library comprising 387 transcription factors in the *C. elegans* genome. Since we were aiming to isolate transcription factors other than *hif-1*, we conducted the screen using the *hif-1;3kb-F45D3.4::GFP* reporter strain. Age matched reporters at L1 stage were fed bacteria containing individual RNAi clones and grown to young adulthood in otherwise normal conditions. At this point animals were treated with 4.76 mM cobalt chloride and assayed 12 hours later for GFP expression. All clones were tested in duplicate on individual batches of 40 animals per trial. Initial positive hits were considered those that robustly inhibited GFP signal in at least 75% of animals (average ≥ 30 of 40 animals per trial). The positive (14 transcription factors) hits were subjected to a secondary screen where at least 100 animals were tested in duplicate.

Based on this screen, 8 transcription factors or 2.1% of the RNAi clones screened showed loss of GFP expression following cobalt chloride treatment (Table 2). Each of these positive RNAi genes was screened for GFP fluorescence following a 12-hour treatment in 0.1% O_2. Only three of the eight RNAi clones—*tbx-38, lin-40,* and *blmp-1*— were capable of extinguishing hypoxia-induced GFP expression (Table 2).
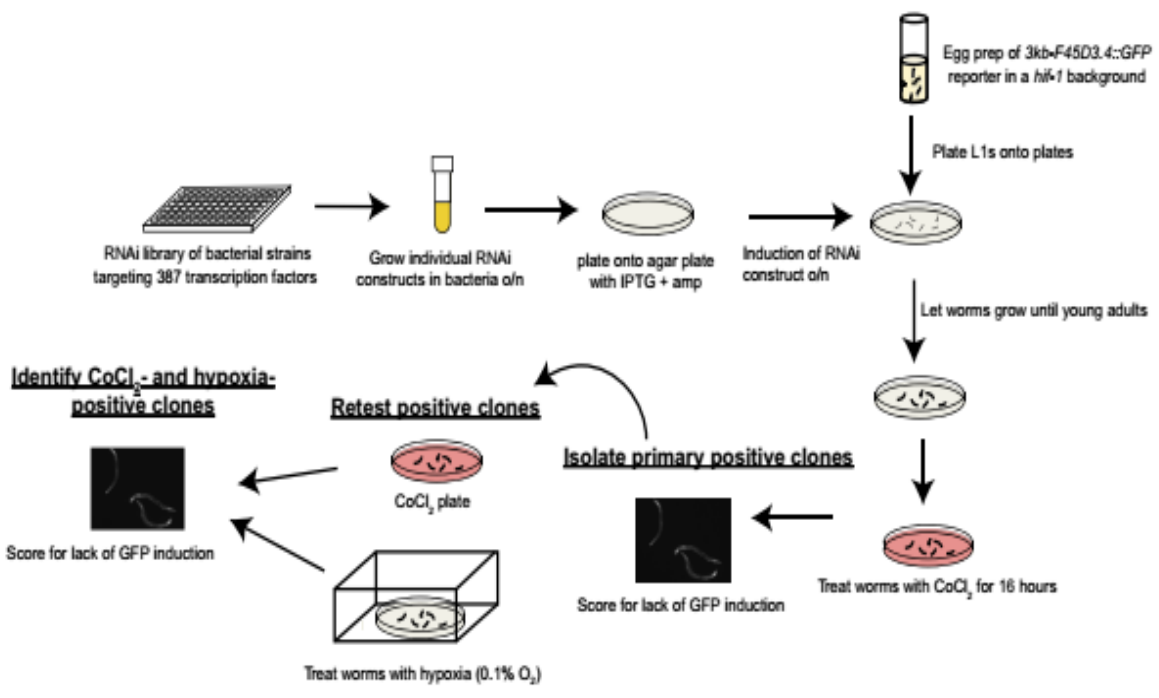
Figure 4.6.a. Schematic for RNAi screen

RNAi target	Function/Process	*F45D3.4-3kb::GFP* expression inhibited in:	
		4.76 mM CoCl$_2$	0.1% O$_2$
tbx-38	T-box transcription factor -- essential for mesodermal induction	Yes	Yes
lin-40	histone deacetylaction, chromatin remodeling	Yes	Yes
blmp-1	zinc-finger protein, putative methyl transferase	Yes	Yes
nhr-31	nuclear hormone receptor -- required for excretory cell function	Yes	No
npp-12	nuclear pore membrane glycoprotein	Yes	No
nhr-89	nuclear hormone receptor	Yes	No
elt-3	GATA transcription factor -- hypodermal cell differentiation	Yes	No
taf-11.2	transcription initiation factor TFIID subunit	Yes	No

Table 2. Results from screen Identified RNAi clones that inhibit GFP expression of the HIF-independent pathway driven by cobalt chloride treatment or hypoxic exposure.

The differential effectiveness of RNAi on suppressing GFP signal following the two treatments, suggests that cobalt chloride and oxygen deprivation target distinct, but overlapping upstream regulators of non-HIF-mediated pathways. We were interested to see the effect of combination of cobalt chloride and hypoxia would result in an additive effect. We tested for expression of *F45D3.4* and *zip-1* in a wild type background in the presence of hypoxia, cobalt chloride, or both together. For *F45D3.4*, both treatments together result in a response that is similar to either treatment alone. For *zip-1*, both together have no apparent effect, whereas either treatment alone did. Hence, the responses seen with the combination treatment seem to vary depending on the gene. More studies exploring how each treatment may or may not interact with

other treatments at specific loci to alter the response are needed.
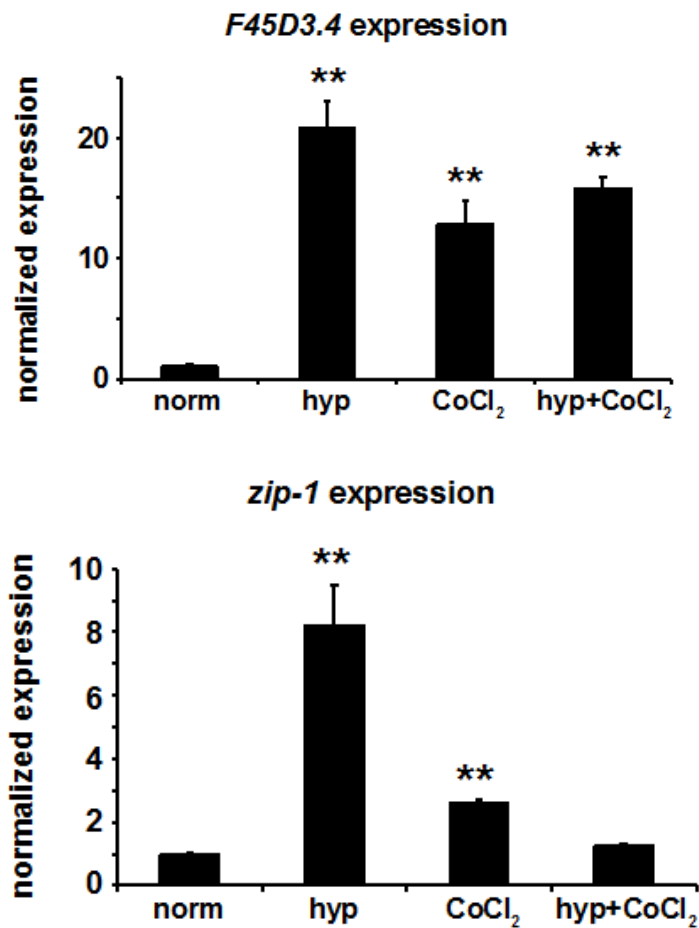
Figure 4.6.b. Treatment with Cobalt chloride and hypoxia yields variable results in *C.elegans* Total mRNA was collected from wild type animals and expression of *F45D3.4* (A) or *zip-1* (B) in normoxia, hypoxia (0.1% O2), cobalt chloride (4.76 mM), or both for 12 hours was measured by qRT-PCR. Error bars are SEM. ** indicates p value <0.01. A student's t-test was used to determine significance.

4.7. *blmp-1* mediates expression of a distinct subset of HIF-1-independent hypoxia response genes

Of the three clones that were effective at mitigating GFP signal in both cobalt chloride and hypoxia, we specifically focused on the zinc finger gene *blmp-1*, which we confirmed was necessary for hypoxic-induced expression of the HIF-independent transcript *F45D3.4.* The *blmp-1* gene encodes a protein with 27% identity to mouse B lymphocyte-induced maturation protein 1 (Blimp-1) and 26% identity to human positive regulatory domain I-binding factor (PRDI-BF1). Both Blimp-1 and PRDI-BF1 are thought to act predominantly as transcription repressors and are essential for the terminal differentiation of B and T cells (Turner, Mack, & Davis, 2010), (Nutt, Fairfax, & Kallies, 2007).

Figure 4.7.a. Graphical representation of mammalian BLIMP-1 gene. BLMP-1 binds to DNA using its zinc finger domain. Unlike other SET domain containing proteins, the PR domain in BLMP-1 does not have methyltransferase activity

BLMP-1 is predicted to contain a positive regulatory (PR) domain, a nuclear localization signal (NLS), and five Kruppel-type [(Cys)2-(His)2] zinc fingers (Figure 3.7.a). The zinc fingers of both Blimp-1 and PRDI-BF1 have been shown to bind to target DNA and are essential for their transcriptional repression activities (Lin, Wong, & Calame, 1997)

(Ghosh, Gyory, Wright, Wood, & Wright, 2001). *C. elegans* BLMP-1 and mammalian BLIMP-1 show striking domain conservation, with 43% similarity in the SET domain and 70% similarity in the Zn fingers (Tunyaplin et al., 2000). The deletion allele *tm548*, which was isolated by a reverse genetic approach (National Bioresource Project), has an 810 bp deletion, removing part of exon 3 and part of intron 3 and may result in a truncated BLMP-1 protein containing the first 254 amino acids of BLMP-1 and 17 amino acids encoded by the third intron.

To determine if and to what extent BLMP-1 may participate in the hypoxic induction of other transcripts we treated *blmp-1* mutant worms with hypoxia and surveyed the expression of five other HIF-independent hypoxic transcripts described by Shen et al. (2005). As expected, control animals responded robustly to hypoxic challenge through upregulation of transcript. Additionally, in each case, mutation of *hif-1* failed to extinguish this response (Figure 4.7.b); however, while *icl-1* and *F44E5.5* were entirely unaffected in this background, *mnk-1*, *mod-5*, and *zip-1*, exhibited somewhat muted, though still significant, response profiles, suggesting that the hypoxic regulation of these transcripts may entail a combination of HIF- and non-HIF-mediated events. Of the three clones that were effective at mitigating GFP signal in both cobalt chloride and unaffected in this background, *mnk-1*, *mod-5*, and *zip-1*, exhibited somewhat muted, though still significant, response profiles, suggesting that the hypoxic regulation of these transcripts may entail a combination of HIF- and non-HIF-mediated events. These results differed from those obtained in the *blmp-1* mutant background, where all hypoxic responsiveness was eliminated (Figure 4.7.b).
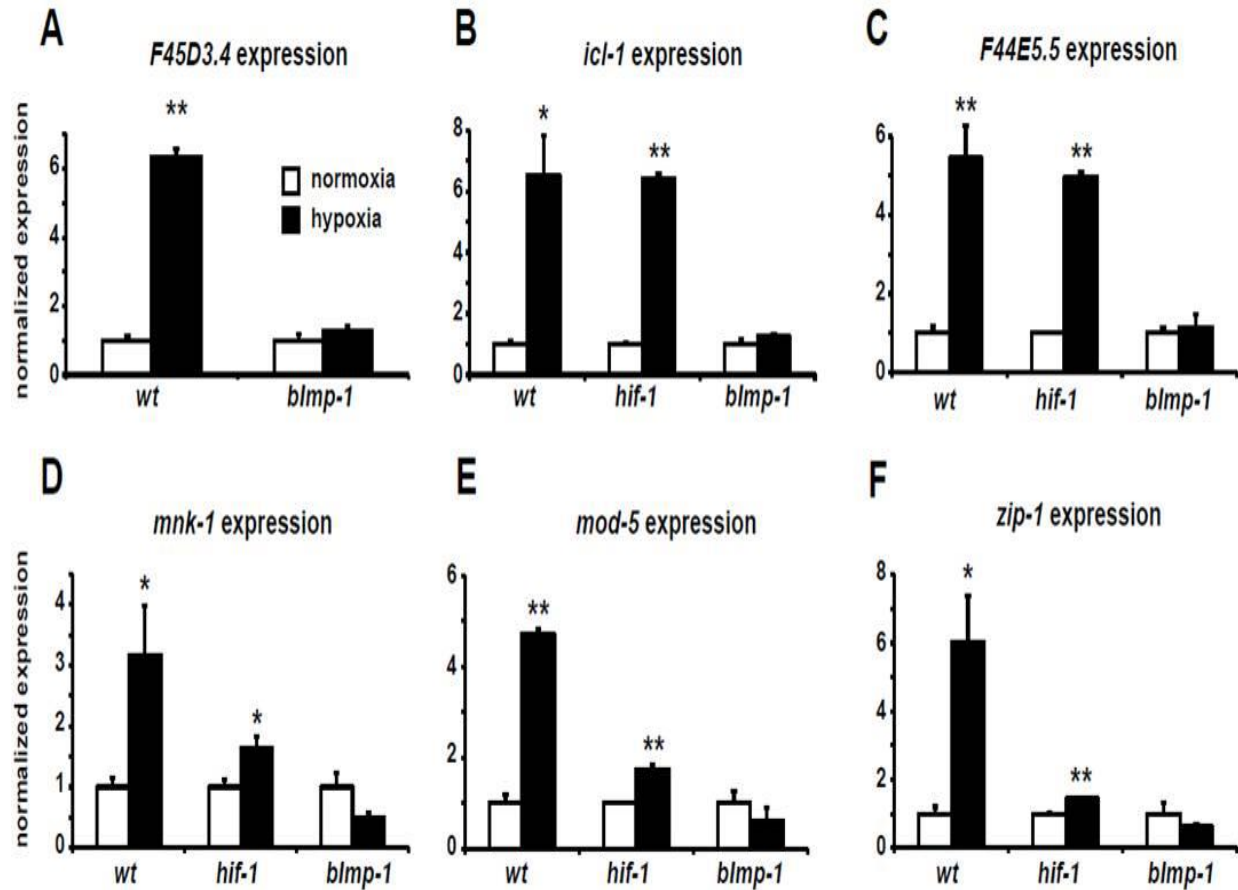
Figure 4.7.b. Hypoxic regulation of transcripts by BLMP-1 is HIF-independent

A – I Total mRNA was collected from wild type animals and *blmp-1*(*tm548*) mutants (A) or from wild type animals, *hif-1*(*ia4*) mutants, and *blmp-1*(*tm548*) mutants (B-I) after incubation in normoxia or hypoxia (0.1% O2) for 12 hours. The expression levels of HIFindependent genes *F45D3.4* (A), *icl-1* (B), *F44E5.5* (C), *mnk-1* (D), *mod-5* (E), and *zip-1* (F) were analyzed by qRT-PCR. Normalized values are the average of at least three biological replicates. Error bars are SEM. ** indicates a p-value < 0.01 and * indicates a p-value < 0.05. A student's t-test was used to determine significance.

4.8: *blmp-1* does not mediate expression of a distinct subset of HIF-1-dependent hypoxia response genes

We assayed known HIF-dependent hypoxic transcripts, and found that the hypoxic expression of *nhr-57*, *egl-9*, and *fmo-12* was greatly affected (Figure 4.8), if not eliminated, in the *hif-1* mutant, similar to published data (C. Shen et al., 2005). In contrast—and in line with BLMP-1 acting independently from HIF-1—the effect of *blmp-1* mutation on the hypoxic expression of these same transcripts was significantly less impactful, and in the case of *fmo-12* had no effect on hypoxic expression. These collective results demonstrate that BLMP-1 is essential for the hypoxic regulation of a unique subset of hypoxic response genes that differs from those regulated by HIF-1.
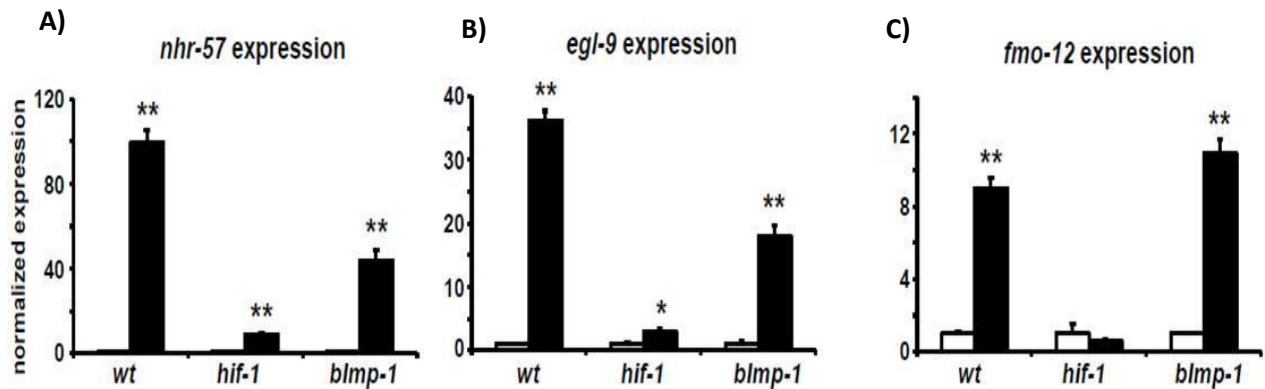
Figure 4.8. BLMP-1 does not regulate HIF-dependent genes Total mRNA was collected from wild type animals, *hif-1*(*ia4*) mutants, and *blmp-1*(*tm548*) mutants (A-C) after incubation in normoxia or hypoxia (0.1% O2) for 12 hours. The expression levels HIF-dependent genes *nhr-57* (A), *egl-9* (B), and *fmo-12* (C) were analyzed by qRT-PCR. Normalized values are the average of at least three biological replicates. Error bars are SEM. ** indicates a p-value < 0.01 and * indicates a p-value < 0.05. A student's t-test was used to determine significance.

4.9. *blmp-1* mutants are sensitive to hypoxia.

To further investigate the effects of BLMP-1 in HIF-independent hypoxic responses, we evaluated if *blmp-1* mutants are sensitive to hypoxia treatment. Two different types of hypoxia lethality assays were conducted.

In the severe hypoxia treatment, wild type, *hif-1* mutants, *blmp-1* mutants, and *hif-1; blmp-1* double-mutant embryos are treated with 0.1% hypoxia and scored for embryos that made it to adulthood. The number of adults that make it to adulthood is counted and a percentage value of number surviving compared to number treated (hypoxia/ normoxia) is plotted. As seen in Figure 4.9, only 60% of *blmp-1* mutants make it to adulthood compared to about 95% of the wild type. The *hif-1* mutants and the *hif-1; blmp-1* double mutants do not survive this treatment.
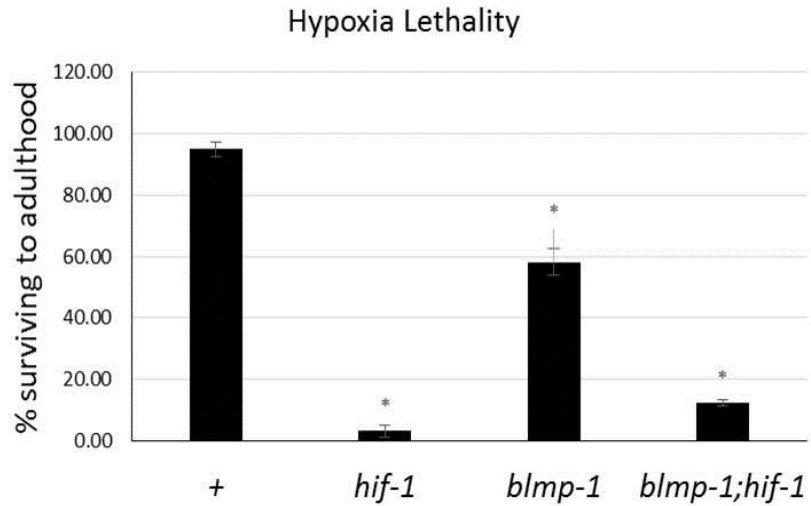
Figure 4.9.a. *blmp-1* **mutants are sensitive to severe hypoxia** Wild type*, hif-1(ia4)*, *blmp-1*, and *hif-1;blmp-1* double mutant embryos were counted and treated to 0.1% hypoxia for 24 hours. Embryos were allowed to proceed to adulthood in normoxia. The number of adults that make it to adulthood is counted and a percentage value of number surviving compared to number treated (hypoxia/ normoxia) is plotted

We also wanted to evaluate the sensitivity of *blmp-1* mutants in continuous hypoxia and for these experiments, we collaborated with Dr Pamela Padilla for conducting hypoxia sensitivity assays under continuous hypoxia conditions. We challenged embryos of control animals, *hif-1* mutants, *blmp-1* mutants, and *hif-1; blmp-1* double-mutants, which were allowed to develop for four days in normoxia or hypoxia. As expected, the control and *hif-1* mutants developed equally well under normoxic conditions, while *blmp-1* mutants and *hif-1; blmp-1* double-mutants displayed a mildly penetrant lethal phenotype (Figure 4.9.b). In contrast, only 62.5% of control animals survived in 0.5% O_2; however, among surviving animals, the vast majority (~75%) were able to progress to adulthood, which was 48% of all the embryos surveyed. This was different from *hif-1* mutants and *hif-1; blmp-1* double-mutants, which showed no progression and no survivability in hypoxia. Importantly, *blmp-1* mutants were also severely affected by hypoxia; no animals matured to adulthood over the 4-day challenge. Further, even amongst the reduced number of surviving *blmp-1* mutants, less than half progressed out of the L1/L2 stage. Hence, *blmp-1* mediates transcriptional responses in hypoxia and is required for proper survival in hypoxia. Next, seeing that the *hif-1* and *hif-1; blmp-1* double-mutants show no survivability in hypoxia, we wanted to look at a less severe hypoxia concentration at which HIF-1 is not known to be stabilized. Indeed, we saw an increased sensitivity phenotype for *blmp-1* mutants and *hif-1; blmp-1* double-mutants under the less severe hypoxic treatment regimen of 2% oxygen (Figure 4.9.c). These results demonstrate that BLMP-1 is essential for normal developmental progression in hypoxia and that loss of both HIF-1 and BLMP-1 renders the animal more sensitive to hypoxic insult than the single mutants.
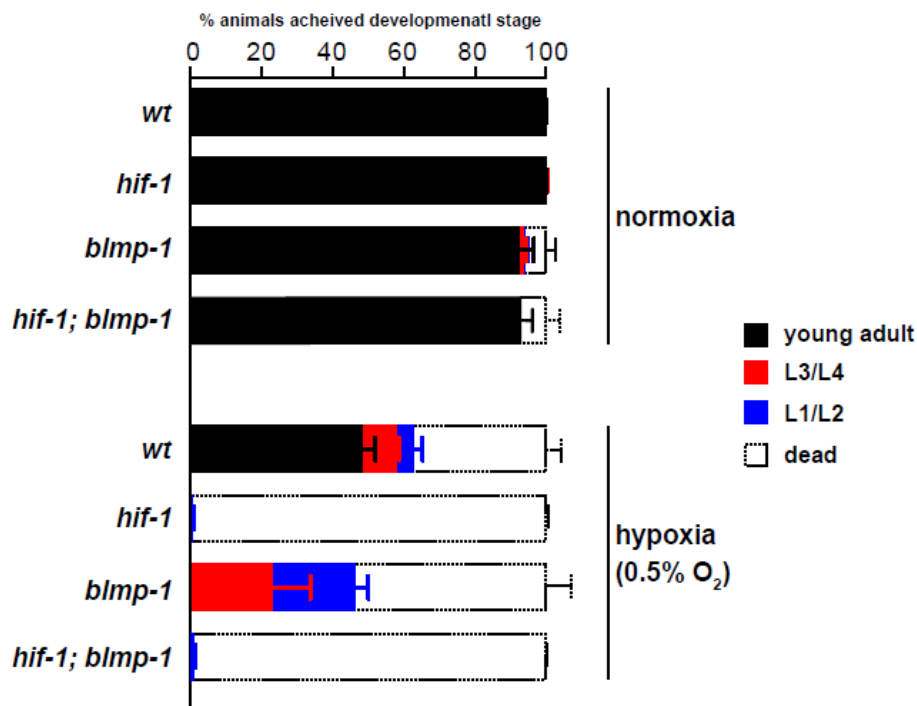
Figure 4.9.b. *blmp-1* **mutants are sensitive to continuous hypoxic exposure**

15-20 adult animals (wild type, *hif-1* mutants, *blmp-1* mutants, or *hif-1; blmp-1* double-mutants) per trial were allowed to lay eggs on an OP50-seeded NGM plate for 1-2 hours. The embryos were placed in either normoxia or hypoxia (0.5% O2) at 20°. After 4 days, surviving animals were scored for developmental progression as adults, L3/L4 larvae, or L1/L2 larvae. Note that hypoxia slows development, resulting in young adults, whereas in normoxia adults are 1 day old and gravid. For each genotype and condition, 4 independent experiments were performed with a total of 200-350 worms. Error bars are SEM.
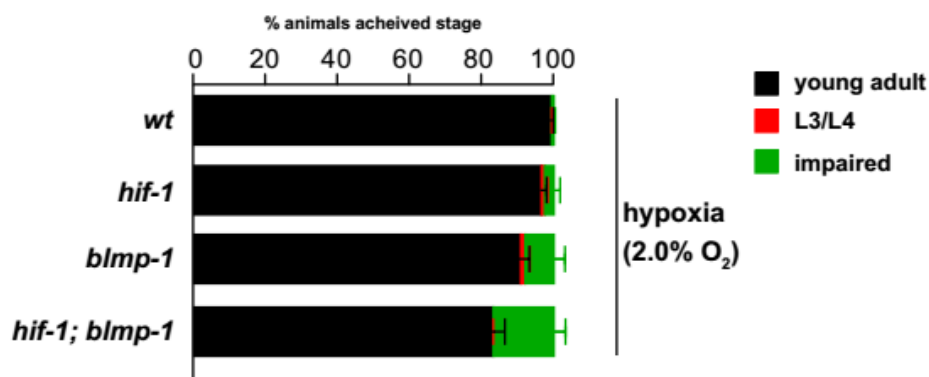
Figure 4.9.c. *blmp-1; hif-1* **double mutants are sensitive to less harsh hypoxic exposure** The experiment was conducted exactly as Figure 3.9.b, but at 2% hypoxia

4.10: *blmp-1* mRNA and protein levels do not change in hypoxia

Since BLMP-1 plays a critical role in hypoxia, I wondered whether *blmp-1* expression is modified in hypoxia. Wild type and *hif-1* mutants were treated to hypoxia and the *blmp-1* expression level was measured by qRT-PCR. As seen in Figure, there is no apparent change in the levels of *blmp-1* upon hypoxia treatment. Since we did not have access to *Ce*-BLMP-1 antibody, I used a *blmp-1* overexpression system with an integrated *blmp-1::gfp* transgene and looked for changes in *blmp-1::GFP* levels following hypoxia treatment. The *blmp-1::GFP* transgene is expressed in the nucleus in neurons, tail, and vulva (Niu et al., 2011). Hypoxia treatment did not alter the localization of the transgene, although more detailed studies may be necessary considering this is an overexpression system and BLMP::GFP may not regulated like endogenous BLMP-1. In conclusion, it appears that the message and protein levels of BLMP-1 are not altered in hypoxia.

A)

blmp-1 expression

B)

anti-GFP
anti-tubulin
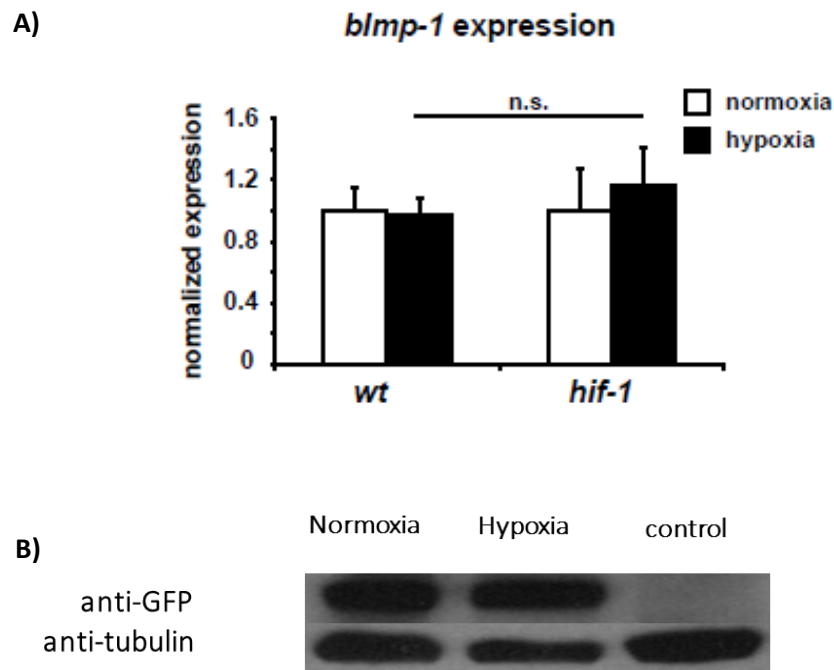
Figure 4.10. BLMP-1 levels do not change in hypoxia The potential hypoxic regulation of *blmp-1* transcript was assayed by qRT-PCR from total RNA collected from adult wild type or *hif-1*(*ia4*) mutant animals treated in normoxia or hypoxia (0.1% O2) for 6hours (**A**). The hypoxic regulation of BLMP-1 protein was assayed using an integrated *blmp-1::gfp* transgene (*wgIs109,* Sarov et al 2012) that was treated to 6 hours of hypoxia. Wild type with no transgene was used as a control (**B**).

4.11. BLMP-1 localizes to promoter of HIF-independent gene

Apart from its non-dependency on HIF-1 and lack of EGL-9 regulation, the mechanisms supporting *blmp-1*-dependent control of hypoxic transcription are unknown. As an initial step toward uncovering a possible mechanism, we looked at several aspects of BLMP-1 biology in hypoxia. As mentioned earlier, we determined that *blmp-1* expression remains unchanged in hypoxia; this is the case in control animals and *hif-1* mutants (Figure 5A). Hence, transcriptional upregulation cannot account for a change in BLMP-1 activity in hypoxia. Further, chromatin immunoprecipitation (ChIP) was used to assess if hypoxic treatment altered recruitment of a GFP-tagged version of BLMP-1 (NIU *et al.* 2011) to the *F45D3.4* promoter. These results were not a product of non-specific antibody binding, since no enrichment was observed in the wild type background (Figure 4.11). Although we found a significant enrichment of BLMP-1-GFP at the proximal *F45D3.4* promoter sequence, our results indicate the localization is unaffected by hypoxia treatment (Figure 4.11). The previous data suggested that changes in oxygen status are relayed to pre-localized BLMP-1 at respective loci, which subsequently trigger increased transcriptional activity. Consistent with this idea, additional ChIP experiments demonstrated a surge in the acetylation of histone H3 following hypoxia treatment (Figure 4.11), indicating an oxygen-sensitive increase in chromatin decondensation in the same region bound by BLMP-1-GFP. Importantly, similar increases were not observed in the *blmp-1* mutant (Figure 4.11), a result that aligns with a failure to upregulate transcription.

A) enrichment of GFP-BLIMP at *F45D3.4* promoter

B) lack of enrichment of GFP signal at *F45D3.4* promoter

C) enrichment of acetylated histone H3 at *F45D3.4* promoter

D) enrichment of acetylated histone H3 at *F45D3.4* promoter
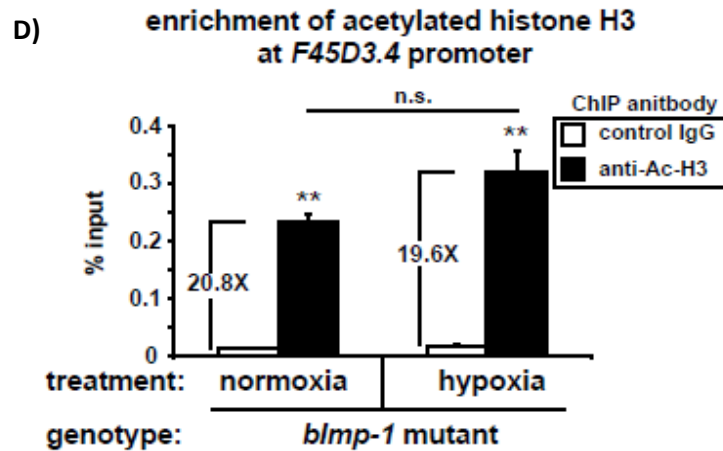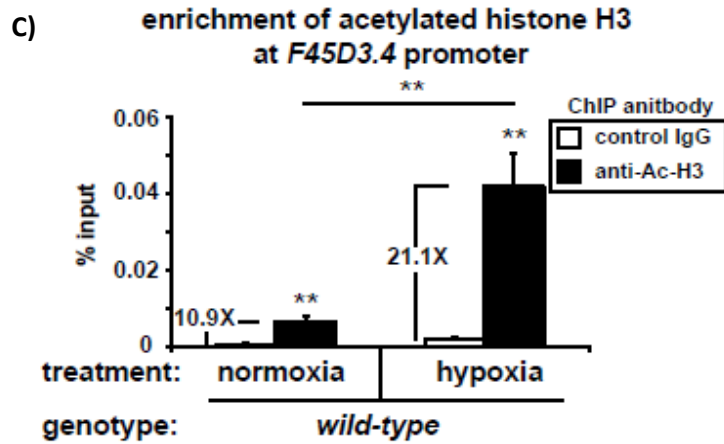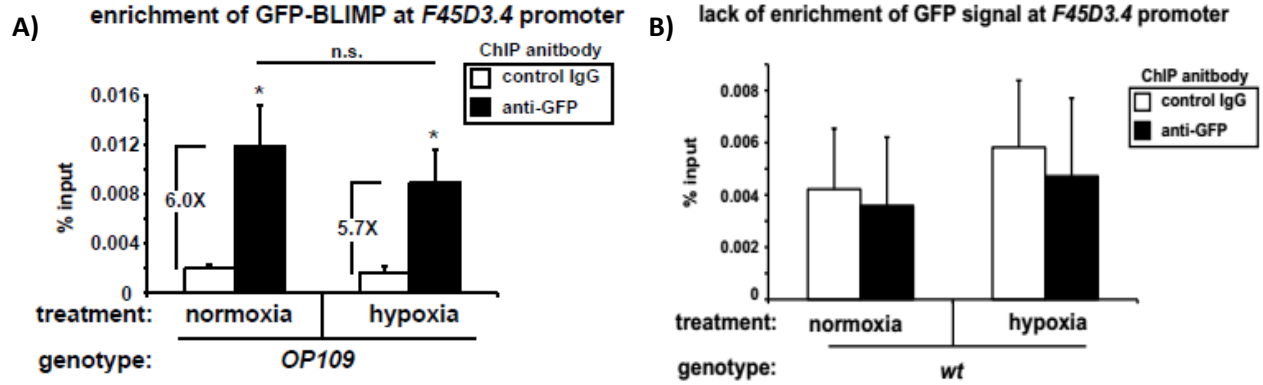
Figure 4.11. BLMP-1 mediates hypoxic-induced histone acetylation Chromatin immunoprecipitation was used to monitor the effects of 0.1%O2 hypoxia for 6 hours on BLMP-1 localization (**A&B**) or histone H3 acetylation (**C&D**) at the *F45D3.4* promoter. *F45D3.4* promoter sequences were enriched in the *OP109* background by IP of BLMP-1-GFP with anti-GFP antibodies compared to control IgG antibodies, but results were unaffected by hypoxic treatment (**A**). No enrichment of signal is seen with the anti-GFP antibody over IgG alone in the wild type in normoxia or hypoxia (**B**) Acetylation of the *F45D3.4* promoter increased in a hypoxia-dependent manner in the wild type background (**C**), but no such increase was apparent in *blmp-1*(*tm548*) mutants (**D**), by ChIP using anti-acetylated histone H3 antibodies versus IgG control antibodies. Values are the average of at least 3 biological replicates and are plotted as a factor of percent input. Fold-change increases between control IgG antibody and anti-AcH3 or anti-GFP signals are shown. Error bars are SEM. ** indicates a p-value < 0.01 and * indicates a p-value < 0.05. Statistical insignificance between measurements is noted by n.s. One-way and two-way ANOVA was used to compare results within and between groups with a post-hoc Tukey HSD test (**A-D**).

4.12: Upstream modulators of *blmp-1*-dependent hypoxia response

We also conducted a candidate screen of other known regulators of the HIF-1 pathway in hypoxia, including *vhl-1*, *rhy-1*, and *swan-1*, as well as HIF-1α dimeric partner arnt-1 or HIFβ. To determine if the *3kb-F45D3.4::GFP* reporter was regulated similarly to HIF-1 in normoxia by oxygen-dependent hydroxylation via the HIF prolyl hydroxylase EGL-9 (Epstein et al., 2001), we further assayed the reporter in *egl-9; hif-1* double-mutants (Figure 4.12). We found that the behavior of the reporter was unchanged in normoxia and indistinguishable from the hypoxic activity observed in the control background. This suggests that the oxygen sensor in the HIF-1-independent pathway is different from that of the canonical pathway.

We also looked at the effects of knockdown of chromatin remodeling factors involved in stress responses. Barring *lin-40* (described later) and *hda-1* the other candidate genes did not mediate the HIF-independent response. The fact that *hda-1* was isolated by a candidate screen is interesting, because a recently published study independently isolated *hda-1* to be important in the regulation of HIF-independent gene *hsp16.1* (J. Lee & Lee, 2013). *hda-1* is responsible for the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4) and is an essential gene. The design of my screen, which looked at mediators of the hypoxia response from L1 to adult, permitted me to explore the role of *hda-1.* This result coupled with the identification of *lin-40* strongly suggest that chromatin remodeling factors are involved in the regulation of HIF-independent genes in hypoxia.

Next, I looked at effects of knockdown of guanylyl cyclases that sense changes in oxygen concentration in *C.elegans* (Gray et al., 2004). Knockdown of *gcy-31* but not

gyc-33 and *gcy-35* resulted in a 48% reduction in *3kb-F45D3.4:GFP* levels. Only genes

whose knockdown resulted in at least 75% reduction in *3kb-F45D3.4:GFP* levels were

considered positive hits in my screen; however, further studies exploring the role of *gcy-*

31 in the HIF-independent hypoxia response may be necessary. In conclusion, the

BLMP-1 dependent hypoxia response does not seem to be regulated by some of the

classic players of the HIF pathway; however, this candidate screen helped identify some

potential regulators that merit further study.

A)

RNAi	% of hif-/- reporters showing fluorescence upon hypoxia treatment
L4440	87
blmp-1	20.19
lin-40	24.48
Molecules involved in HIF-dependent hypoxia response	
egl-9	82.07
vhl-1	72.56
swan-1	81.25
rhy-1	86.76
Chromatin Remodeling Complexes involved in stress responses	
hda-1	8.88
hda-2/3/4/6/11	88.42
swi/snf	84.9
egl-27	75.92
Soluble guanyl cyclases	
gcy-31	48.14
gcy-33	78.57
gcy-35	84.78

B)

-

egl-9; hif-1

normoxia   hypoxia

Figure 4.12. Candidate screen of potential regulators of the *hif-1*-independent hypoxia response: **A)** The 3kb-F45D3.4:GFP reporters were fed RNAi of genes involved in the HIF-1 pathway, chromatin remodeling factors involved in stress responses, and guanylyl cyclases. Percentage of reporters showing GFP expression upon hypoxia treatment was scored. n = 80 and the study was in duplicate. The red boxes indicate potentially interesting hits. **B)** Expression of GFP in wild type, *hif-1*(*ia4*) mutant, and *egl-9*(*sa307*); *hif-1(ia4)* double mutant backgrounds carrying the 3kb-45D3.4::GFP construct after treatment in normoxia and hypoxia for 12 hours. Scale bars indicate 10 μm.

4.13: *blmp-1* and ER stress

As mentioned earlier, hypoxia can induce ER stress and activate the UPR through 3 signaling pathways mediated by ER sensors IRE1-XBP-1, PERK-eIF2-ATF4 and ATF6 (Ron & Walter, 2007). The transgenic transcriptional reporter *Phsp-4::GFP(zcIs4)* has been shown to be a reliable reporter of activation of the UPR in *C. elegans* (Calfon et al., 2002). The *hsp-4* gene encodes a homologue of mammalian BiP/GRP78, a hypoxia response gene, and its induction reflects the activation of the IRE-1–XBP-1 branch of the UPR (Mao & Crowder, 2010; Roll et al., 1991). The activation of HSP-4/ BiP by XBP-1 in hypoxia is HIF-independent and I hypothesized that BLMP-1 may play a role in the HIF-independent ER stress pathway in hypoxia.

Phsp-4::GFP reporters were fed control RNAi and *blmp-1* RNAi and then treated with hypoxia. I found that hypoxia induced the expression of *Phsp-4::GFP* fed control RNAi, consistent with the expectation that hypoxia would elicit the UPR in *C. elegans*. Interestingly, the reporters fed *blmp-1* RNAi showed greater induction of *Phsp-4::GFP* upon hypoxia treatment than control RNAi worms (Figure 4.13.a), suggesting that loss of BLMP-1 causes significant induction of the ER stress pathway in hypoxia. We confirmed the hypoxia-induced activation of the endogenous *hsp-4* mRNA and spliced *xbp-1* levels by qRT–PCR and saw that the expression levels of both *hsp-4* and spliced *xbp-1* were significantly increased in hypoxia.

A)

Phsp-4::GFP reporter fed control RNAi

HYPOXIA

Phsp-4::GFP reporter fed *blmp-1* RNAi

B)

C)

hsp-4

xbp-1 spliced
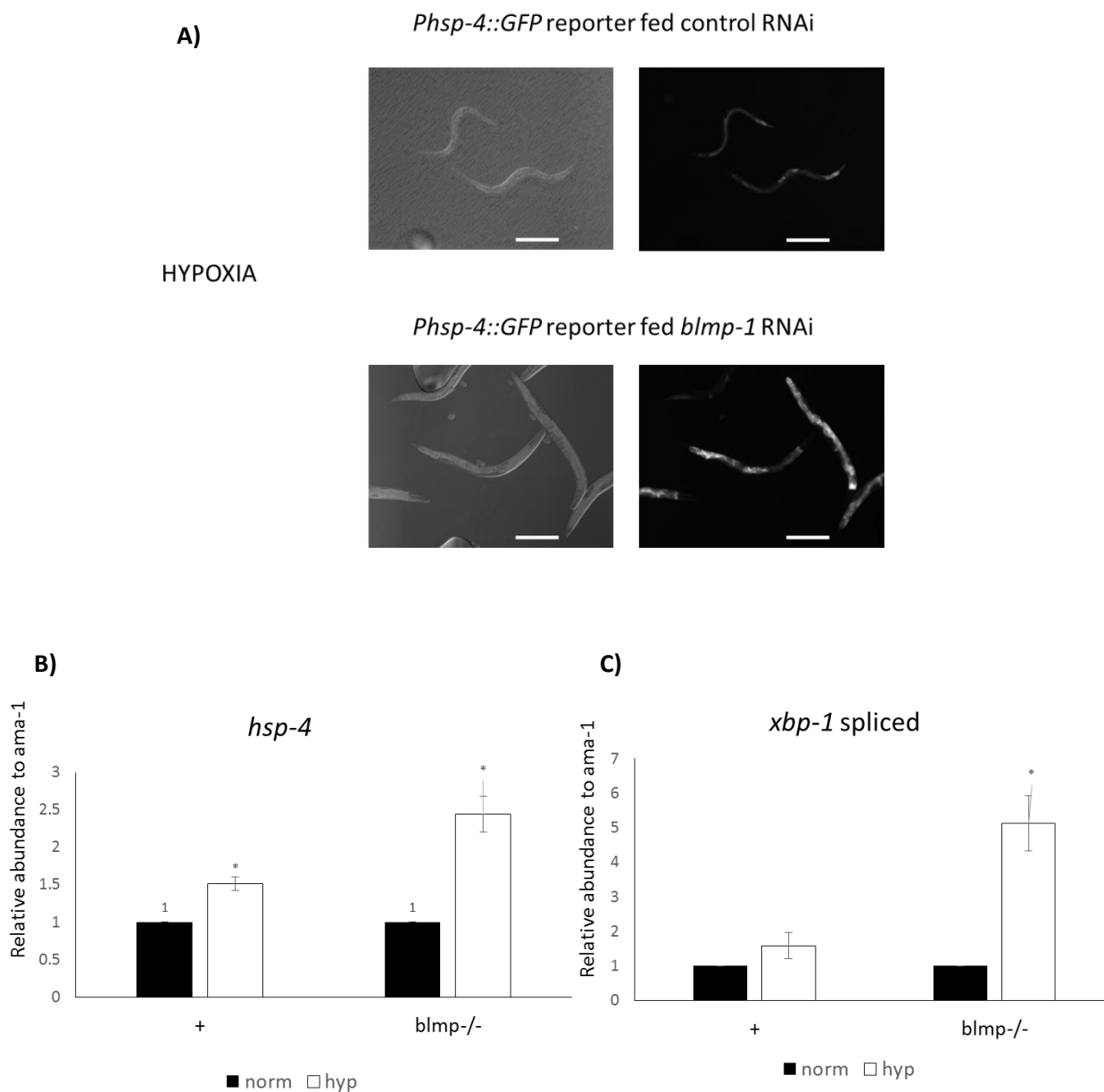
Figure 4.13.a. Loss of BLMP-1 enhances ER stress in hypoxia ER stress reporter *Phsp-4::GFP* that was fed *blmp-1* RNAi showed marked increase in GFP levels compared to reporters fed control RNAi (**A**). Total mRNA was collected from wild type animals and *blmp-1*(*tm548*) mutants after incubation in normoxia or hypoxia (0.1% O2) for 4 hours. The expression levels of *hsp-4* (**B**) and spliced *xbp-1* (**C**) were analyzed by qRT-PCR. Normalized values are the average of at least three biological replicates. Error bars are SEM. ** indicates a p-value < 0.01 and * indicates a p-value < 0.05. A student's t-test was used to determine significance.

We next asked if BLMP-1 is required for resistance to tunicamycin, an antibiotic that inhibits the N-glycosylation of proteins and thereby increases the load of misfolded proteins transiting the endoplasmic reticulum (Merksamer et al. 2009). In *C. elegans*, tunicamycin reliably induces the UPR, presumably through its known activity to increase protein misfolding (Calfon et al., 2002).

 I tested two concentrations of tunicamycin on wild type and *blmp-1* mutants (Figure 4.13.b). Following treatment with 1 µg/ml of tunicamycin, nearly 85% of the first larval stage (L1) of the wild-type strain developed to at least the fourth larval stage (L4) in this assay, compared to their growth and development on the non-treated plates. In contrast, the *blmp-1* mutant on tunicamycin exhibited severely attenuated larval development and growth, as measured by the rate of progression between molts, size of larvae, and ability of larvae to reach the L4 stage by 72 h. Following a harsher treatment of tunicamycin at 5 ug/ml, 60% of the wild type developed to at least the L4 stage, but almost 70% of the *blmp-1* mutants did not survive the treatment. These data implicate a specific and protective role for BLMP-1 during ER stress.

A)

B)
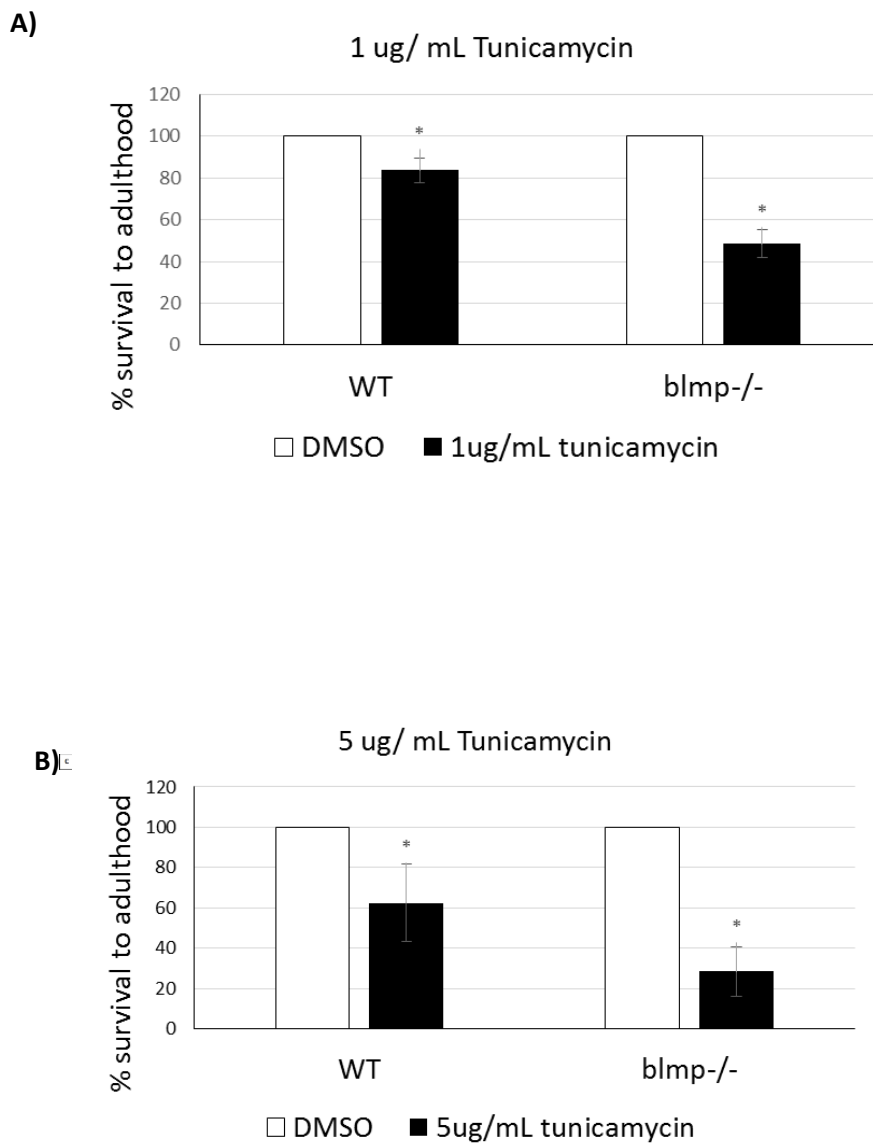
Figure 4.13.b. Loss of BLMP-1 increases susceptibility to ER stress by tunicamycin. Wild type and *blmp-1* mutant worms of the L1 larval stage were treated to either control DMSO or tunicamycin at 1 ug/mL **(A)** or 5 ug/mL (**B).** L4/ adults that survived the treatment 72 hours later were counted. * indicates a p-value < 0.05. A student's t-test was used to determine significance. Experiments were conducted in triplicate.

4.14: *blmp-1* and collagen

The cuticle is a collagenous extracellular matrix that is synthesized by the hypodermis, an underlying ectodermal cell layer that surrounds the body of the animal. Cuticle synthesis occurs five times during development, once at the end of embryogenesis prior to hatching, and then prior to molting at the end of each larval stage. Collagen biosynthesis is a complex process, and genes vital for cuticle formation are induced prior to every molting cycle.

A previous microarray study reported that genes governing collagen and extracellular matrice processes were misregulated in hypoxia (C. Shen et al., 2005). Of the genes induced in hypoxia were the *phy-2* prolyl 4-hydroxylase α subunit gene and the *bli-3* NADPH oxidase, which have central roles in collagen synthesis (Edens et al., 2001; Myllyharju, 2003; C. Shen et al., 2005). Moreover, genes encoding 4 predicted extracellular molecules with lectin domains (*ptr-8, C31G12.2, F35D11.10, and F47C12.2*) and one predicted chitin-binding protein (*K04H4.2*) were also differentially regulated in hypoxia.

Reduced expression of *blmp-1* leads to morphological defects like a dumpy phenotype and formation of a weak cuticle (Zhang et al. 2012). DiI staining of the cuticle (Schultz & Gumienny, 2012) showed that compared to wild type, *blmp-1* mutants have a defect in their annuli, the circumferential structure imparting ridges in outer layers of the cuticle. The *bmlp-1* cuticle appears fractured and unorganized compared to the wild type. This

phenotype is typical of mutants of other collagen encoding genes like *dpy-2* and *dpy-3* (Myllyharju & Kivirikko, 2004).

I hypothesized that BLMP-1 targets a set of molting and cuticle-related genes in *C. elegans* in hypoxia. I focused on two HIF-1-independent genes from the previous study that are involved in collagen formation: *bli-3* and *ptr-8* (C. Shen et al., 2005). BLI-3 is a major cuticle enzyme and is required for dityrosine cross-linking of collagen. Loss of *bli-3* is not lethal, but results in loss of cuticular integrity (Edens et al., 2001). *ptr-8* is a member of the patched related family of transmembrane glycoproteins and is important for collagen formation and molting (Fielenbach & Antebi, 2008).

As seen in Figure, *bli-3* and *ptr-8* are induced in hypoxia in wild type and *hif-1* mutants, while loss of *blmp-1* abrogates this induction, suggesting that *bli-3* and *ptr-8* are downstream of *blmp-1.* If *blmp-1* does regulate these genes in hypoxia, loss of function mutants of *bli-3* and *ptr-8* should phenocopy *blmp-1's* phenotype in hypoxia. Indeed, *bli-3* mutants are sensitive to hypoxia treatment: a known number of wild type and *bli-3* mutant embryos were treated with hypoxia and the number of embryos that made it to adulthood were counted. As seen in Figure 4.14, similar to *blmp-1* mutants, about 60% of *bli-3* mutants survive hypoxia treatment. These results strongly imply that collagen formation is important in hypoxia and that BLMP-1 may be regulating the expression of critical collagen formation genes like *bli-3.* Future studies looking at hypoxia phenotypes of *blmp-1; bli-3* double mutants are warranted.

A) Wild type   *blmp-1*$^{-/-}$

B) bli-3

C) ptr-8

D) *bli-/-* hypoxia survival
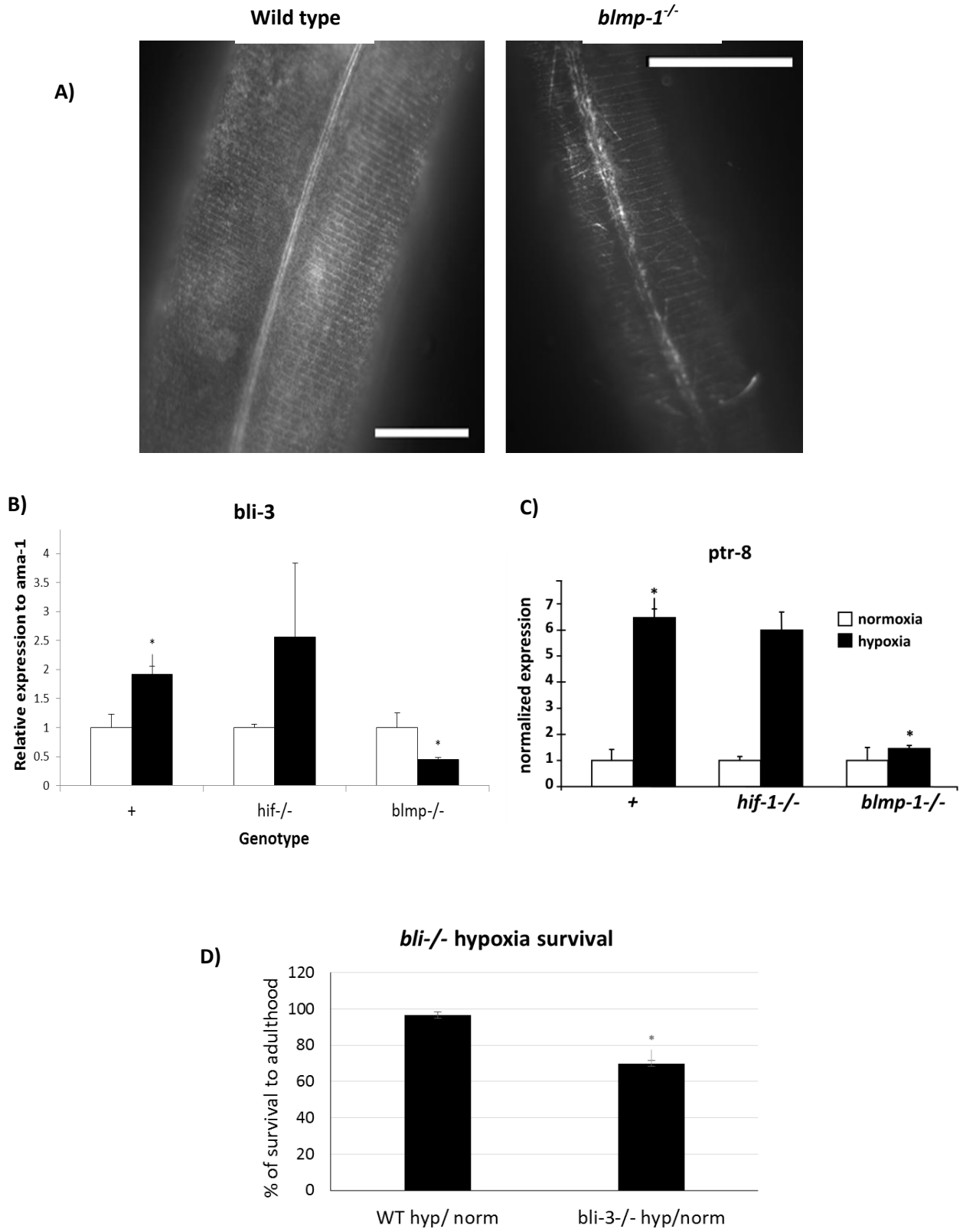
Figure 4.14. BLMP-1 mediates collagen formation genes in hypoxia A) DiI staining of wild type and *blmp-1* mutants shows marked changes in the cuticular structure **B, C)** qRT-PCR analysis of wild type, *hif-1,* and *blmp-1* mutants for HIF-independent genes *bli-3* **(B)** and ***ptr-8* (C) D)** Hypoxia lethality assays of wild type and *bli3-/-* mutant eggs treated to 0.1% hypoxia for 24 hours, followed by incubation in normoxia. The percentage of number of adults relative to the number of embryos treated is plotted. * indicates a p-value < 0.05. A student's t-test was used to determine significance. Experiments were conducted in triplicate.

4.15: LIN-40 and the HIF-independent hypoxia response

The chromatin remodeling factor *lin-40* was isolated by my screen as a HIF-independent mediator of the hypoxia response. Knockdown of *lin-40* abrogated GFP expression in the HIF-independent reporter, both in hypoxia as well as cobalt chloride treatments. The *lin-40* gene encodes a protein that is homologous to MTA1 and MTA2 in mammals, which were identified as components of the NuRD complex (Solari & Ahringer, 2000). MTA1 levels are increased in breast and ovarian cancer cells (Murakami, Kaul, & Robertson, 2014). In hypoxia, MTA1 recruits HDACs to stabilize HIF-1α to promote tumor growth and metastasis (Yoo, Kong, & Lee, 2006). In *C.elegans* LIN-40 was recently shown to promote stress resistance and longevity in a circuit that involves the germline and the insulin pathway (Zimmerman and Kim 2014).

lin-40 is an essential gene and is required for proper vulval cell fate specification and morphogenesis (Solari & Ahringer, 2000; Zimmerman & Kim, 2014). Worms fed *lin-40* RNAi are healthy suggesting that the knockdown of *lin-40* is not complete. Wild type and *hif-1(ia4)* worms were fed *lin-40* RNAi and qRT-PCR was conducted to determine the level of *lin-40* knockdown. The RNAi treatment reduced *lin-40* RNA levels only by 40% in both wild type and *hif-1(ia4)* mutants. To verify that *lin-40* does regulate the expression of HIF-independent gene F45D3.4, I conducted a qRT-PCR analysis of wild type and *hif-1* mutant worms fed *lin-40* RNAi from larval stage L1 and treated with hypoxia in adulthood. Indeed, qRT-PCR analysis showed that *lin-40* knockdown abrogates the induction of *F45D3.4* in both wild type and *hif-1* mutants. To further explore if *lin-40* contributes to the hypoxia response, we conducted a hypoxia lethality assay on wild type and *hif-1* mutants that were fed *lin-40* RNAi for one generation.

Embryos, collected from progeny whose mothers were fed *lin-40* RNAi, were counted and treated with normoxia and hypoxia for 24 hours. The number of embryos that made it to adulthood were counted and the percentage of embryos treated that reached adulthood was plotted. As seen in Figure 4.15, in hypoxia, knockdown of *lin-40* in the wild type resulted in 40% reduction in embryo viability compared to wild type fed control RNAi (L4440), which is considerable since *lin-40* RNAi treatment reduced *lin-40* mRNA levels to about 50%. In conclusion, these results show that the regulation of HIF-independent gene *F45D3.4* involves chromatin remodeling factor LIN-40 and that *lin-40* may play a role in hypoxia survival.

A)

lin-40 knockdown levels

B)

F45D3.4

C)

lin-40 knockdown hypoxia lethality
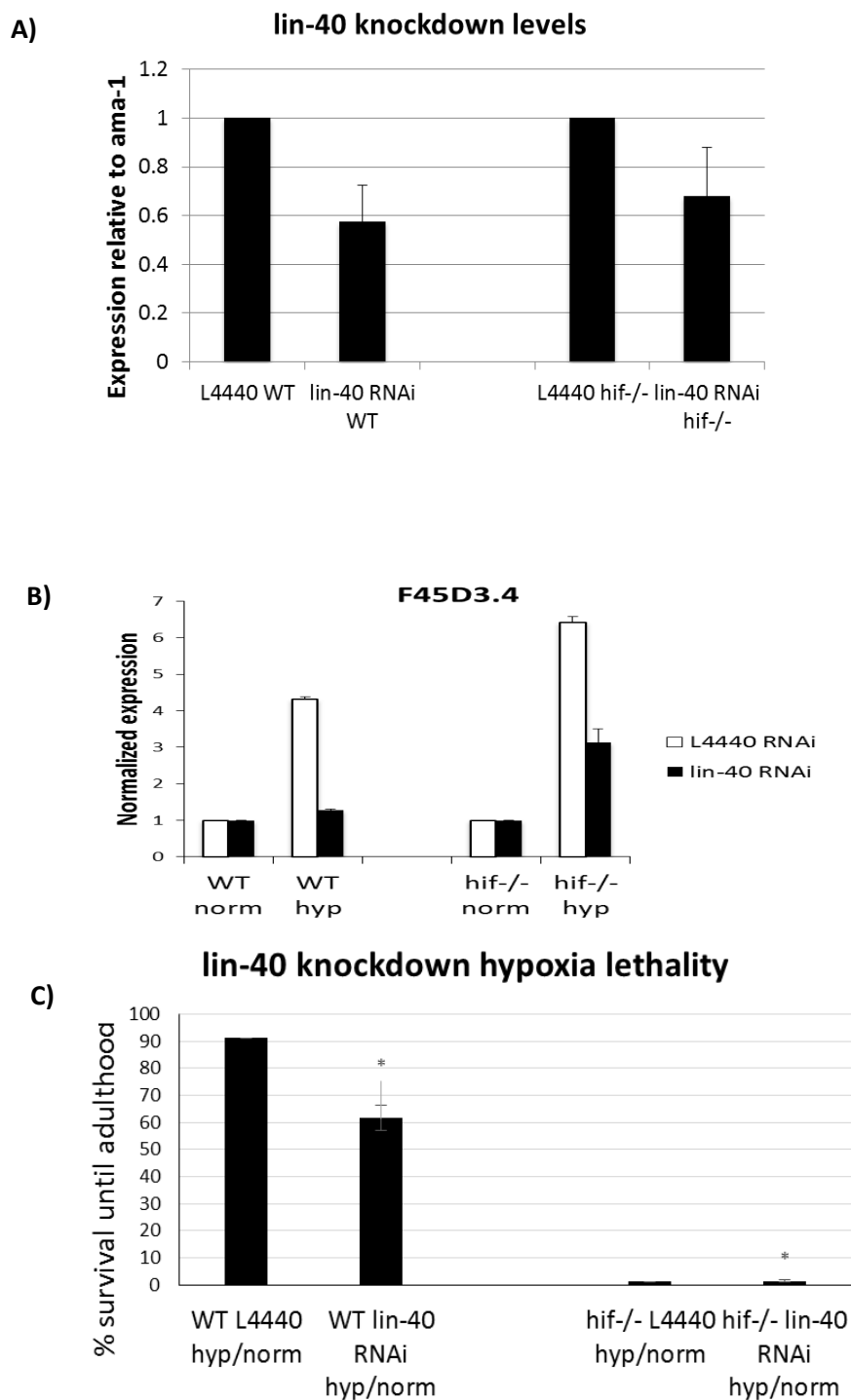
Figure 4.15: LIN-40 plays a role in the HIF-independent response A) qRT-PCR showing *lin-40* mRNA knockdown in wild type and *hif-1* mutants fed *lin-40* RNAi. **B)** qRT-PCR analysis showing *F45D3.4* expression levels in wild type and *hif-1* mutant worms fed *lin-40* RNAi. **C)** Embryos from wild type and *hif-1* mutants fed control RNAi and *lin-40* RNAi treated to 0.1% hypoxia for 24 hours * indicates a p-value < 0.05.

5. Discussion

All large organisms face the fundamental physiological challenge of matching oxygen supplies to the needs of respiring tissues. In higher animals, specialized organs facilitate oxygen delivery, and the lungs, heart, blood and vascular system are all devoted to this purpose. The coordination of these complex homeostatic systems requires robust mechanisms for detecting and responding to imbalances in oxygen homeostasis. The conserved HIF-1 complex regulates the expression of a broad set of genes that are important for metabolism, vascularization, and cell survival across species (Semenza 2011). HIF, expressed very widely in mammalian cells (Wang & Semenza, 1993; Firth *et al.* 1994), is conserved in simpler animal species that lack erythropoietin, red blood cells, and specialized oxygen-delivery organs (Nagao *et al.* 1996; Loenarz *et al.* 2011). A recent study on HIF binding to DNA has now revealed that more than 500 genes are directly regulated by HIF in a given cell line (Mole *et al.* 2009; Xia *et al.* 2009; Schödel *et al.* 2012*b*). These primary targets will initiate secondary targets, so that the overall complexity of activating the HIF response and hence, the hypoxia response is enormous.

The total hypoxic response, however, is not entirely dependent on the HIF pathway. Although HIF-independent hypoxia-induced activities have also been identified in other organisms and HIF-1α is dispensable for hypoxic upregulation of a host of transcripts in mammalian cells, these pathways remain poorly understood. These results suggest that HIF-independent hypoxic signaling mechanisms may act in concert with, or even

supplant, the HIF response pathway in a context-dependent manner. Further support of this idea is evident in the expression of Alas2, the rate-limiting enzyme for heme production. *Alas2* has been identified as a HIF-dependent and a HIF-independent hypoxia-regulated gene in mammals. In *C. elegans,* there is clear evidence of HIF-independent responses in hypoxia. Shen et al. (2005) showed that out of 110 hypoxia-induced genes, 47 were induced in a HIF-independent manner. Additionally, *C. elegans* can survive for about 24 hours in anoxic conditions and this response does not require *hif-1;* instead, this 'suspended animation' response is mediated by the spindle checkpoint protein SAN-1 in embryos and the ceramide synthase HYL-2 in adults (Padilla et al., 2002; Nystul et al., 2003; Miller and Roth 2009). Further, Lee and Lee (2013) described the role of the chromatin-remodeling factor NURF-1 in regulating the expression of a novel HIF-1-independent protein, heat shock protein HSP-16.1 (Lee and Lee 2013).

5.1: HIF-independent screen reveals interesting candidates

In this study, through a targeted RNAi approach, we screened for transcription factors that mitigated hypoxic induction. Eight factors were identified in the cobalt chloride-based screen, and three of these were found to be regulated by both hypoxia and cobalt chloride. BLMP-1, which has been discussed in depth in this study, regulates several HIF-independent transcripts in hypoxia and loss of *blmp-1* makes worms sensitive to hypoxia insult. LIN-40, also discussed previously, is an interesting target because of the known functions of its homolog MTA-1 in hypoxia.

The other positive hit was *tbx-38,* a T-box transcription factor that functions with Notch to mediate early cell fate decisions; at the 12-cell stage, Notch and *tbx-38* interact to render the mesodermal fate to AB.a descendant cells (Good et al., 2004). Interestingly, tbx-38 was also identified as a HIF-independent gene in an independent microarray study, thereby validating the robustness of the screen I conducted (C. Shen et al., 2005). The isolation of a mediator of differentiation is important, considering that some of the defined developmental roles of hypoxic response in low oxygen are stimulation of the proliferation of CNS precursor cells (Studer et al., 2000) and neural crest stem cells (Morrison et al., 2000) and inhibition of adipocyte differentiation (Yun et al., 2002). The molecular processes underlying these effects are less understood; however, recent studies have highlighted the importance of the synergy between hypoxia and Notch pathways for inhibiting differentiation of precursor cells during early stages of embryogenesis (Gustafsson et al., 2005). Hypoxia activates Notch, which physically interacts with HIF-1 and HIF-independent gene FIH-1 to activate downstream target genes to maintain the undifferentiated cell state (Cejudo-Martin & Johnson, 2005). Moreover, given that the PRDM mediates the activation of Notch target genes, it will be interesting to explore the role of the Notch pathway in HIF-independent mediated cell differentiation, particularly with respect to *tbx-38* and *blmp-1* (Hohenauer & Moore, 2012).

The 4 other hits were only isolated from the cobalt chloride screen and although the phenotype could not be confirmed in the hypoxia chamber, the potential role of these hits in hypoxia is interesting. To faithfully understand the difference in the regulation of

these genes in cobalt chloride as opposed to hypoxia, it may be necessary to modify the

treatment protocol to include other stages of the worm (Vengellur & LaPres, 2004).

For example, one of the hits isolated from the cobalt chloride treatment not for hypoxia

is *npp-12*, which encodes a conserved membrane protein gp120 of the nuclear pore

complex. In *C.elegans*, NPP-12 is phosphorylated by CDK-1/cyclin B and is important

for the depolymerization of lamin and required for nuclear envelope break down (NEBD)

(Padilla et al., 2004). Embryos treated with 0.1% hypoxia go into a HIF-independent

state called suspended animation, in which the rate of energetically expensive

processes is reduced and the occurrence of damaging events like aneuploidy is

prevented (Nystul & Roth, 2004). In hypoxic embryos, the blastomeres arrest in

prophase and nuclear envelope break down is halted to prevent the transition to

prometaphase; however, embryos mutant for nucleoporin genes are unable to halt and

are unable to survive the hypoxia insult (Padilla et al., 2004). Hence, hypoxia influences

the cell cycle machinery in *C. elegans* and it will be interesting to see how *npp-12*

mutant embryos fare in severe hypoxia.

The other positive hit from the cobalt chloride related screen is *taf-11.2,* which encodes

a subunit of TFIID, which comprises the TATA-binding protein (TBP) and TBP-

associated factors (TAFs). This is interesting because it has been previously shown that

HIF1α recruits TFIID to the promoters of hypoxia responsive genes *glut-1* and

phosphoglycerate kinase 1 (Okino, Chichester, & Whitlock, 1998).

The roles of a GATA transcription factor *elt-3*, which regulates everal aging genes

(Zimmerman & Kim, 2014), and nuclear hormone receptors *nhr-31* and *nhr-89* in

hypoxia signaling need further exploration.

5.2: BLMP-1 and the hypoxia response

We identified the zinc finger protein BLMP-1 as a mediator of the HIF-independent

response. Until very recently, the role of nematode BLMP-1 was not clearly defined. In a

previously published genome-wide RNAi screen in *C. elegans*, *blmp-1* was found to

affect male tail tip morphogenesis and loss of *blmp-1* was found to result in precocious

retraction of the male tail tip (Nelson et al., 2011). Moreover, *blmp-1* was shown to

modulate adult lifespan and cuticle morphogenesis (Samuelson et al., 2007; Greer et

al., 2010; Zhang et al., 2012). Recently, it was shown that *blmp-1* is a heterochronic

gene controlling epidermal and gonadal maturation and that it is targeted by an E3

ubiquitin ligase DRE-1. This BLMP-1/ DRE-1 module controls dauer formation, molting,

and longevity in *C. elegans* (Horn et al., 2014).

Mammalian BLIMP-1 is extensively studied in different cellular contexts: BLIMP1 is

necessary and sufficient to drive terminal differentiation of B cells into antibody-

secreting plasma cells and also governs various cell fate decisions, including primordial

germ cell specification and skin differentiation, generally working as a transcriptional

repressor (Horsley et al., 2006; Ohinata et al., 2005; Turner et al., 1994; Shapiro-Shelef

et al., 2003). Moreover, PRDM1/BLIMP-1 is a well-known regulator of plasma cell

differentiation and is an important regulator of T cells, which can be dramatically

influenced by the dynamic and sometimes hypoxic microenvironments that are known to affect T cell mediated cytokine production and inflammatory responses. Given that *C. elegans* BLMP-1 and mammalian BLIMP-1 show striking domain conservation (Tunyaplin et al., 2000) and the conserved nature of HIF- and non-HIF-mediated hypoxic signaling pathways in general, it will be interesting to determine if the mammalian equivalent of *C. elegans* BLMP-1 fulfills similarly important roles in mammalian hypoxic responses.

5.3: BLMP-1 and downstream effectors in hypoxia

Because BLMP-1 is a transcriptional regulator, a natural question arising from our studies is, what are the BLMP-1-regulated targets important for hypoxia adaptation? Through qRT-PCR, I identified a few targets of BLMP-1. The regulation of icl-1 and *F44E5.5* in hypoxia seem to be entirely dependent on BLMP-1. *icl-1* encodes an isocitrate lyase that is important in the glyoxylate pathway for catalyzing the cleavage of isocitrate to succinate and glyoxylate. Although a role for *icl-1* in mammals has not been extensively studied, the glyoxylate pathway is of importance to plants and bacteria as it enables the utilization of lipid for generating glucose. Interestingly, *icl-1* is preferentially uregulated in hypoxia and essential for the survival of Mycobacterium in hypoxia (Eoh & Rhee, 2013). Further, *F44E5.5* is a chaperone protein and a member of the hsp70 family of heat shock proteins. The precise role of *F44E5.5* is not known, but the hsp70 family of proteins are crucial for protecting cells from ER stress-induced apoptosis. *mnk-1*, *mod-5*, and *zip-1*, exhibited somewhat muted, though still significant, response profiles. *mnk-1* encodes the *C. elegans* ortholog of the Mnk MAP kinase-interacting

kinase, while MOD-5 is a serotonin transporter. Moreover, a CHIP-SEQ study conducted in the first larval stage of worms in normoxia showed that BLMP-1 binds to the promoters of *F45D3.4, F44E5.4, bli-3,* and *zip-1* (Niu et al., 2011)*.*

However, to identify the whole range of transcripts that are regulated in hypoxia, RNA-seq and ChIP-seq experiments should be conducted in wild type and *blmp-1* mutants treated with hypoxia. This would be interesting considering a recent RNA-seq conducted on *blmp-1* mutants in normoxia showed BLMP-1 to target a set of molting and cuticle-related genes in *C.elegans*, consistent with my observation of misregulation of collagen formation genes in *blmp-1* mutants (discussed later).

5.4: BLMP-1 and HIF

BLMP-1 actions in hypoxia do not require HIF-1, but our data does not exclude that it acts in concert with it. For example, loss of *hif-1* can dampen, but not extinguish hypoxic induction of *mod-5* and *zip-1,* while loss of *blmp-1* eliminates it altogether. This suggests that BLMP-1 acts as a hypoxic competence factor in certain contexts that provides a path for further response by other factors, including HIF-1. This is not surprising, considering we have previously shown in *Drosophila* that ERR mediates HIF-dependent hypoxic responses in conjunction with HIF-1 as well as independently of HIF-1. In this respect, the ability of a BLMP-1-mediated non-HIF-dependent pathway to work apart from and in conjunction with the HIF pathway in *C. elegans* should be explored further (Lı *et al.* 2013). HIF-1 transcripts levels were not altered in *blmp-1* mutants nor were *blmp-1* transcript levels in *hif-1* mutants.

blmp; hif-1 double mutant eggs are unable to survive severe hypoxia insult, similar to

hif-1 mutants. Interestingly, *blmp; hif-1* double mutant eggs treated to a more permissive

hypoxia treatment of 2% show a synthetic phenotype and are more sensitive to the

treatment than the single mutants alone. It is likely that the mutations in two critical

genes overburdened the worm and affected its ability to survive stress. However, it is

also possible that both *blmp-1* and *hif-1* function redundantly or are required for the

same hypoxia survival pathway. Indeed it will be interesting to determine the

transcriptional profile of the *blmp-1*; *hif-1* double mutants, especially in temperature-

controlled hypoxia chambers. I attempted to conduct qRT-PCR experiments on the

double mutants, but the response varied greatly in response to temperature fluctuations.

5.5. BLMP-1 and epigenetic modifications

In mammals, a variety of BLIMP-1 targets have been identified across tissues. For

example BLIMP-1 resides at the *c-myc* promoter to block its transcription, thereby

preventing proliferation during B cell maturation (Lin et al., 1997). BLIMP-1 is thought to

switch-off whole gene expression programs such as mature B cell programs during B

cell differentiation on the one hand and somatic programs during primordial germ cell

specification on the other hand (Vincent et al., 2005; Ohinata et al., 2005; Shaffer et al.,

2002). How BLIMP-1 fulfills these highly context-specific functions remains poorly

understood. In particular, germ cell development involves major epigenetic remodeling,

which strongly relies on BLIMP-1 function (Surani, Hayashi, & Hajkova, 2007). BLIMP-1

harbors a SET domain, but BLIMP-1 itself bears no intrinsic enzymatic activity (Ancelin

et al., 2006; Dillon et al., 2005). Instead, it cooperates with epigenetic regulators to control transcription and epigenetic remodeling (Surani et al., 2007)(Su et al., 2009; Ancelin et al., 2006; Yu et al., 2000; Gyory et al., 2004). Therefore, a distinct set of cofactors present in different tissues might allow BLIMP-1 context-specific action. Moreover, one could speculate that also in *C. elegans* BLMP-1, mediates epigenetic regulation for hypoxia adaptation. It is intriguing to note that BLMP-1-dependent increases in histone acetylation at the *F45D3.4* promoter during hypoxia may occur in conjunction with the pro-longevity factor LIN-40, one of the other two positive hits identified in our screen. As mentioned earlier, LIN-40 is an essential component of the nucleosome remodeling deacetylase (NuRD) complex (Johnsen and Baillie 1991; Solari *et al.* 1999) and is critical for coordinating the hypoxia response with HIF-1 in ovarian and breast cancer cells. Interestingly, unpublished results from our lab are consistent with LIN-40 and BLMP-1 acting together through direct interaction in stress conditions to regulate common targets for dauer formation (M. Hyun personal communication). Indeed, precisely how the cobalt chloride or hypoxia insult affects BLMP-1 to alter transcriptional output in *C. elegans* is interesting and will require more investigation.

5.6 Investigation of Upstream Regulators

Another key question is what are the critical upstream regulators of BLMP-1 protein stability? In particular my findings that BLMP-1 levels are constant upon hypoxia treatment, point to other cofactors that contribute to BLMP-1 regulation. Their identification may provide information on coordination of the hypoxia response across species. Interestingly mammalian BLIMP-1 has recently been shown to be sumoylated

by SUMO-1 at Lys816 (Shimshon et al., 2011; Ying et al., 2012). This modification is catalyzed by the E3 SUMO-protein ligase PIAS1 and has been shown to mediate opposite effects based on the context: on one hand sumoylation is reported to facilitate BLIMP-1's proteolytic degradation, and on the other hand, it promotes BLIMP-1 dependent transcriptional repression during B cell maturation (Shimshon et al., 2011; Ying et al., 2012). How and whether BLIMP-1 sumoylation affects the hypoxia-dependent BLIMP-1 action remains to be elucidated. Notably, the affected Lys residue is not conserved in nematodes, indicating that another Lys or another yet unidentified type of BLMP-1 modification might contribute to the regulation of BLMP-1 function and stability in *C. elegans* (Tunyaplin et al., 2000).

As already initiated in this study, one could screen all known mediators of the oxygen response using available RNAi libraries by looking at loss of *3kb-F45D3.4:GFP* levels as a readout. Alternatively, considering that HIF-1 acetylation in hypoxia increases its stability, one can test a panel of acetylase and deacetylase RNAi libraries for their potential to modify the BLIMP-1 dependent GFP induction of F45D3.4:GFP in hypoxia. I found that *hda-1* could potentially mediate the HIF-independent hypoxia response, although it will be necessary to expand the screen.

RNAi-based screens have some limitations such as targeting only coding sequences and incomplete knockdown rates resulting in many false negatives. In a more unbiased approach one could perform EMS mutagenesis in *3kb-F45D3.4::GFP* reporters in the *hif-1* mutant background that were treated with hypoxia and look for lack of GFP

induction (Jorgensen and Mango, 2002). In such a screen one would expect to find mutations in *blmp-1* and other unknown modulators of the HIF-independent response, including *lin-40* or perhaps other recently discovered regulators like *dre-1*. *dre-1* encodes a *C. elegans* F-box protein that functions in an SCF E3-ubiquitin ligase complex. DRE-1 promotes BLMP-1 ubiquitination to coordinate developmental progression through L3. I generated *dre-1*(*RNAi)* worms to check if the knockdown of *dre-1* would modify *3kb-F45D3.4:GFP* expression in hypoxia. Although the *3kb-F45D3.4:GFP* readout in hypoxia was not altered with *dre-1* knockdown, it may be important to evaluate the regulation of BLMP-1 at L3. *dre-1::GFP* is not expressed in the adult stages of the worm, and my screen was limited to the adult stages.

It will be particularly interesting to find an upstream regulator of the HIF-independent hypoxia response that also acts as an oxygen sensor. Owing to their ability to regulate HIF-1, prolyl hydroxylases have been considered to be oxygen sensors. Since *egl-9* mutation does not alter *3kb-F45D3.4::GFP* expression, it is likely that *egl-9* does not function as a sensor for the HIF-independent response. In *C. elegans*, changes in oxygen concentration are sensed by guanylate cyclases (GCs) *gcy-31*, *33* or *35*, that reside in either BAG or URX/AQR/PQR. URX, AQR and PQR neurons respond to oxygen shifts above the physiologic range and BAG neurons respond to oxygen shifts below the physiologic range. It is likely that a GC residing in one of these neurons, particularly *gcy-31* that resides in the BAG neurons, acts as the oxygen sensor that mediates the HIF-independent response. Indeed, RNAi knockdown of *gcy-31* resulted in a 48% reduction in *3kb-F45D3.4:GFP* levels (Figure 4.12). Knockdown of *gyc-33* and *gcy-35* did not modify the GFP readout. Only genes whose knockdown resulted in at

least 75% reduction in *3kb-F45D3.4:GFP* levels were considered positive hits in my screen, so *gcy-31* was not considered a positive hit. Nevertheless, it will be interesting to explore the role of *gcy-31* in the HIF-independent hypoxia response.

5.7. BLMP-1 and ER stress

A cell undergoing hypoxia must curtail oxygen consumption by energy expensive processes like protein synthesis. Through the lack of oxygen acting as the terminal electron acceptor in the redox reaction for disulphide bond formation, hypoxia impairs protein folding in the ER. The accumulation of unfolded proteins within the ER activates the Unfolded Protein Response (UPR), which mediates a global reduction in transcription and translation through IRE-1-XBP-1, PERK-eIF2A-ATF4, and ATF6 signaling pathways (X. Shen et al., 2001). UPR is activated by several stress conditions like starvation and pathogenesis (Richardson, Kinkel, & Kim, 2011). Importantly, the UPR is an important mediator of the hypoxia microenvironment and it functions independently of HIF-1 (Wouters & Koritzinsky, 2008). Given that BLMP-1 mediates the transcriptional response in hypoxia, including that of an HSP70 protein F44E5.5, I wondered if BLMP-1 contributes to the UPR response to promote hypoxia tolerance.

I focused on the IRE-1-XBP-1 pathway that is conserved from yeast to mammals (Cox & Walter, 1996; X. Shen et al., 2001; Yoshida, Matsui, Yamamoto, Okada, & Mori, 2001). Upon accumulation of unfolded proteins in the ER, IRE-1, an integral ER-membrane protein, activates XBP-1 by alternative splicing of the *xbp-1* mRNA, thereby changing the reading frame. The activated 'spliced form' of XBP-1 regulates expression

of genes involved in ER homeostasis, such as those encoding chaperones including heat shock proteins. In *C.elegans*, The IRE-1 and XBP-1 pathway has been studied with respect to aging (D. Chen et al., 2009), hypoxic preconditioning (Mao & Crowder, 2010) and larval development and immunity (Richardson et al., 2011).

The marked increase in the *hsp-4::GFP* transgene and expression levels of *hsp-4* mRNA and spliced *xbp-1* in hypoxia with the loss of *blmp-1* suggests that BLMP-1 functions in the IRE-1-XBP-1 pathway of UPR. To establish if BLMP-1 based *hsp-4::GFP* induction reflects canonical IRE-1-XBP-1 UPR signaling, it would be important to investigate if concurrent loss of *xbp-1* abrogates the *blmp-1* dependent increase in hypoxia (Calfon et al., 2002).

The sensitivity of *blmp-1* mutants to ER stressing agent tunicamycin suggests that BLMP-1 plays a protective role in hypoxia by limiting damage by UPR in hypoxia. Indeed prolonged or unresolved ER stress activates pro-apoptotic genes including CHOP/GADD153 to induce cell death (Rao, Ellerby, & Bredesen, 2004). To make any conclusive statements regarding BLMP-1's role in hypoxia, however, it will be important to test how the other UPR sensors, PERK-eIF2-ATF4 and ATF-6, contribute to BLMP-1 dependent hypoxia protection. For instance, the phosphorylation levels of eukaryotic translation initiation factor eIF2α could be tested in *blmp-1* mutants treated to hypoxia (Ron & Walter, 2007). If BLMP-1 indeed protects the cell from hypoxia stress, loss of BLMP-1 should show marked increases in eIF2α phosphorylation in hypoxia, which

would indicate an inability to shut down global protein synthesis. (Mao & Crowder, 2010).

The idea that BLMP-1 could contribute to UPR is intriguing, given mammalian BLIMP-1's role in B cell differentiation (Shaffer et al., 2004). BLIMP-1 is essential in the differentiation of quiescent B cells to plasma cells allowing them to become antibody producing cells, a process that is also mediated by components of the UPR, in particular XBP-1 (Yanjun and Hendershot, 2003). Moreover, a microarray study showed that mammalian BLMP-1 regulates a host of stress proteins, including hsp70 and ER-resident processing genes. BLIMP-1 has not been observed in the ER in normoxia, but does it translocate to the ER to mediate IRE-1 dependent *xbp-1* splicing in hypoxia? Does the loss of BLIMP-1 and prolonged hypoxia distort ER morphology in hypoxia? (Rao et al., 2004). Further studies exploring the role of BLMP-1 hypoxia mediated UPR are warranted.

5.8. BLMP-1 and collagen

Of the genes induced in hypoxia, several were involved in collagen synthesis and molting; Collagens and extracellular matrix proteins were found to be induced in hypoxia (C. Shen et al., 2005). Moreover, embryos mutant for *phy-2*, *a* prolyl 4-hydroxylase involved in collagen formation, were unable to survive hypoxia treatment (C. Shen et al., 2005). Interestingly, a recent ChIP-seq study for targets of BLMP-1 in *C. elegans*, showed that BLMP-1 bound to the promoters of collagen formation genes, including HIF-independent genes *bli-3* and *ptr-8* (Horn et al., 2014). I found that *bli-3* and *ptr-8*

were regulated in a BLMP-1 dependent, but HIF-1 independent manner in hypoxia. Moreover, *bli3-/-* mutants were sensitive to hypoxia treatment, in a manner similar to that of *blmp-1* mutants. Taken together, these data strongly suggest that BLMP-1 targets a set of molting and cuticle-related genes in *C.elegans*, consistent with a general role in the molt cycle and the synthesis of the adult cuticle. However, the relation between collagen formation and hypoxia has not been well explored. Interestingly, in mammals, hypoxia treatment increases the expression of prolyl-4-hydroxylases. Exposure of human dermal fibroblasts to hypoxia for 72 hours led to a threefold, dose-dependent increase in collagenous protein (Falanga, Zhou, & Yufit, 2002). My results open up some interesting questions regarding hypoxia and collagen formation. Why is hypoxia important for collagen formation? Is the ER stress phenotype of BLMP-1 related to its role in collagen synthesis? It will be interesting to explore the answers to these questions in the future.

6. Significance

The work presented in this thesis has helped uncover novel regulators of a hypoxia-sensitive pathway that works independent of HIF-1 in *C. elegans*. Through a targeted RNAi approach, I isolated a novel transcription factor and a chromatin remodeling factor that mediate the hypoxia response in *C.elegans*. Given the high similarity between the worm and human counterparts of both these entities, this study presents some exciting avenues for future research in the field of hypoxia. Moreover, I also identified some very interesting upstream regulators, including histone deacetylase 1 and oxygen sensor guanylyl cyclase, *gcy-31*, as potential regulators of the HIF-independent hypoxia response. Additionally, the robust HIF-independent hypoxia reporter *3kb-F45D3.4:GFP* can be used to screen for non-transcription factor regulators. I am very excited at the future possibilities from this work.

7. CPT1: A short story in lipid oxidation

Introduction:

In limiting nutrition conditions, the oxidative degradation of free fatty acids (FAs) liberates stored energy for ATP production. Several pathologies occur owing to mutations in key steps in lipid oxidation pathways. Some of these pathologies can be managed through diet; for example, carnitine palmitoyltransferase deficiency, in which the body cannot utilize fat owing to a deficiency in the CPT1 enzyme, can be managed through diet. For several other disorders, for instance, in Refsum disease or X-linked adrenoleukodystrophy, treatment options are not available.

Although lipid oxidation can proceed through several cellular pathways, the preferred route for fatty acids is through mitochondrial β-oxidation (mβ-ox), which occurs in the mitochondrial matrix. To enter the matrix, LCFAs must first be activated by CoA-ligation, which is then exchanged for carnitine in the inner membrane space. Hence, carnitine conjugation permits access to the matrix. This reaction is catalyzed We sought to determine the extent by the enzyme carnitine palmitoyltransferase I or CPT1. Since lipids are primarily stored in a long-chain form, the carnitine shuttle is an important regulatory barrier for most available FAs. In the absence of a working carnitine shuttle, as happens with CPTI mutation, LCFAs can be shortened by peroxisomal β-oxidation (pβ-ox). Though not capable of complete oxidation, pβ-ox truncates a variety of FAs to lengths that can enter the matrix without the need for carnitine, including dihydroxy acids (DHAs) and very (V) LCFAs, which must be metabolized in the peroxisome. Whereas VLCFAs are directly accepted by peroxisomes, LCFAs first require hydroxylation at the ω- or α-carbon in the endoplasmic reticulum (ER). Thus, α- and ω-

oxidation of LCFAs, coupled with pβ-ox, can be vital alternate routes for animals that cannot rely on the preferred route of FA oxidation (termed alt-ox animals).

We aimed to use *Drosophila melanogaster* as a model to investigate the oxidation of LCFAs. This system is genetically tractable and has a wealth of tools available with which to study the conserved regulatory aspects of metabolism in the context of an intact animal. As in mammals, flies rely on LCFA burn during starvation. In fact, adult emergence in *Drosophila* depends on metamorphic oxidation of LCFAs that are stored during the larval state (Palanker et al. 2010). Our previous studies had established that Estrogen-Related Receptor (dERR) mutants rely on alternative lipid oxidation pathways upon metamorphic-induced starvation for survival (Li et al., 2013). We sought to determine the extent to which alternative routes of lipid oxidation can be used as a rescue when the preferred route is unavailable. For this, we decided to use *Drosophila cpt-1$^{-/-}$* null mutants that lack the ability to oxidize lipids through the conventional fatty acid pathway.

Despite carnitine shuttle elimination, CPTI mutants survive to adulthood and are long-lived, indicating that they successfully employ the alternate lipid oxidation strategy. Surprisingly, 13 different alleles of CPTI have demonstrated that it is not an essential gene, despite it having the confirmed activity of a mitochondrial carnitine palmitoyltransferase (Strub et al., 2008). Given the central nature of CPTI in LCFA metabolism and the fact that there are not redundant paralogs in *Drosophila*, it is remarkable that the *cpt-1$^{-/-}$* mutants are healthy; they show mild phenotypes like withering of the wings and mild sensitivities to oxidative stress, starvation, and heavy metals (Strub et al., 2008). These attributes make the viable *cpt-1$^{-/-}$* null mutant the ideal

candidate to study alt-ox pathways. We decided to examine this by determining the

basal lipid metabolite and transcriptional profiles of animals reared on a standard diet

To handle the increased load of alternative LCFA metabolites, we reasoned that

transcripts encoding alt-ox enzymes will be correspondingly upregulated. Figure 1

describes the LCFA oxidation pathway. FA2H catalyzes the first step in α-oxidation,

while CYP4s3 does for ω-oxidation. Both CYP4s3 and FA2H act directly on free LCFAs

and are thought to be rate limiting for ω- and α-oxidation, respectively. Both of these

alternative pathways converge in peroxisomes where peroxisome-specific ACS

proteins, like the putative peroxisomal dicarboxylyl-CoA synthase CG6178, activate the

ω-and α-oxidation metabolites, thus allowing for pβ-ox to truncate the LC lipids to

shorter forms. The shorter form FAs eventually leave the peroxisome and gain entrance

into the mitochondria, bypassing the carnitine shuttle altogether, where they are

oxidized to completion by chain length-specific enzymes, including MCAD and SCAD.

Finally, Cyp6a8, a hydroxylase that putatively acts on the (ω -1)-carbon of FAs, may

allow for pβ-ox access for (ω -1)-modified LCFAs.

Aims:

In this short study, we hypothesized that enzymes mediating the alternative oxidation will be misregulated in *cpt-1^{-/-}* mutants and we were interested in knowing which of the pathways, ω- and α-oxidation, is preferentially used by these mutants to circumvent the block in LCFA oxidation. Additionally, we aimed to conduct a metabolic profile of the *cpt-1^{-/-}* mutants to establish an altered lipid profile for these mutants.
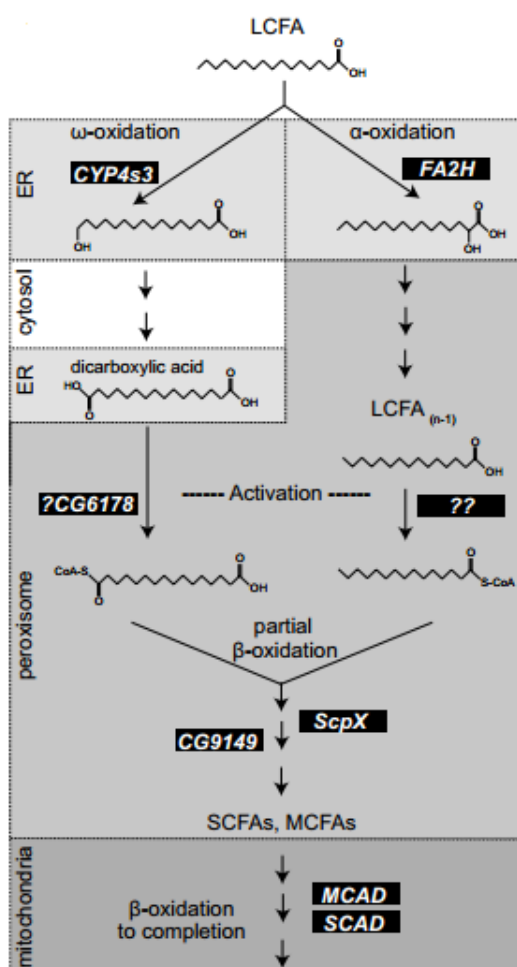
LCFA metabolism can proceed in a carnitine shuttle-independent manner through the oxidation pathways that converge in the peroxisome before moving into the mitochondria

Methods:

qRT-PCR analysis:

Total RNA samples were isolated using a TRIzol/RQ1 DNase hybrid extraction protocol (Promega, Madison, WI). RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) using the manufacturer's specifications. For real-time PCR, premixed primer-probe sets were purchased from Applied Biosystems. All amplifications were carried out on a CFX96 real-time PCR system (Bio-Rad, Hercules, CA). Experimental values were normalized to values obtained for the *Rp49* probe set. Data are reported as the mean±SEM. All values reported represent experiments performed on at least three biological replicates.

Sample collection for mass spectrometric analysis:

Sample preparation:

Wild type and CPT1 mutant flies of the white prepupa stage were washed thoroughly in PBS and frozen at -80C. After thawing the larvae were homogenized in 3 ml of 2:1 of methanol: chloroform in an all-glass homogenizer using an 'A' or 'tight' pestle. The sample was then centrifuged, the pellet washed once with the same solvent, and the wash was combined with the first extract. Mass matched triplicate samples were collected and the solvent evaporated in vacuum. The residue was taken up in 100ul methanol and 5 ul of this solution was injected for HPLC-MS analysis.

Mass spectrometric analysis:

HPLC-MS profiling was performed using the Agilent 1100 Series HPLC system equipped with an Agilent Eclipse XDB-C18 column (4.6 X 250 mm, 5 um particle diameter) connected to the Waters/Micromass Quattro Ultima triple-quad mass spectrometer using a 1:1 split. Chromatographic separation was performed with two different gradients. First, 0.1% acetic acid in water (solvent A) and acetonitrile (solvent B). Second, 0.1 % acetic acid in acetonitrile (A) and propanol-2 (solvent B). The first gradient was used for untargeted analysis of entire larvae metabolomes, while the second gradient for used for targeted analysis of fatty acids. In both cases the flow rate was 1 ml/min and the gradient started with 5% B for 5 min followed by a linear gradient from 5 to 100% B from 5 to 37 min. 100% B was then held for 8 min after which the column was reconditioned with 5% B for the next run.

Metabolic extracts were analyzed by HPLC-ESI-MS in a negative ion mode using a capillary voltage of 3.5 kV and a cone voltage of -60 V.

Results:

1) Gene expression analysis

We looked at the expression of few enzymes of the alternative oxidation pathway. These included: FA2H (Enzyme catalyzing the first step in α-oxidation); CYP4s3 (Enzyme catalyzing the first step in ω-oxidation); CG6178: Putative peroxisomal dicarboxylyl-CoA synthase that catalyzes the activation of ω-oxidation and α-oxidation metabolites to allow for peroxisomal beta oxidation; and Cyp6a8 (putative hydroxylase that acts on the (ω -1)-carbon of fatty acids). Interestingly, we saw a marked increase in *fa2h* levels in the *cpt-1$^{-/-}$* mutants, suggesting a marked increase in α-oxidation. Conversely, the levels of CYP4s3 and CG6178 was markedly reduced. These results suggests that the *cpt-1$^{-/-}$* mutants undertake alternative oxidation pathways, possibly α-oxidation, to utilize different lipids to bypass a block in LCFA burn in the mitochondria.
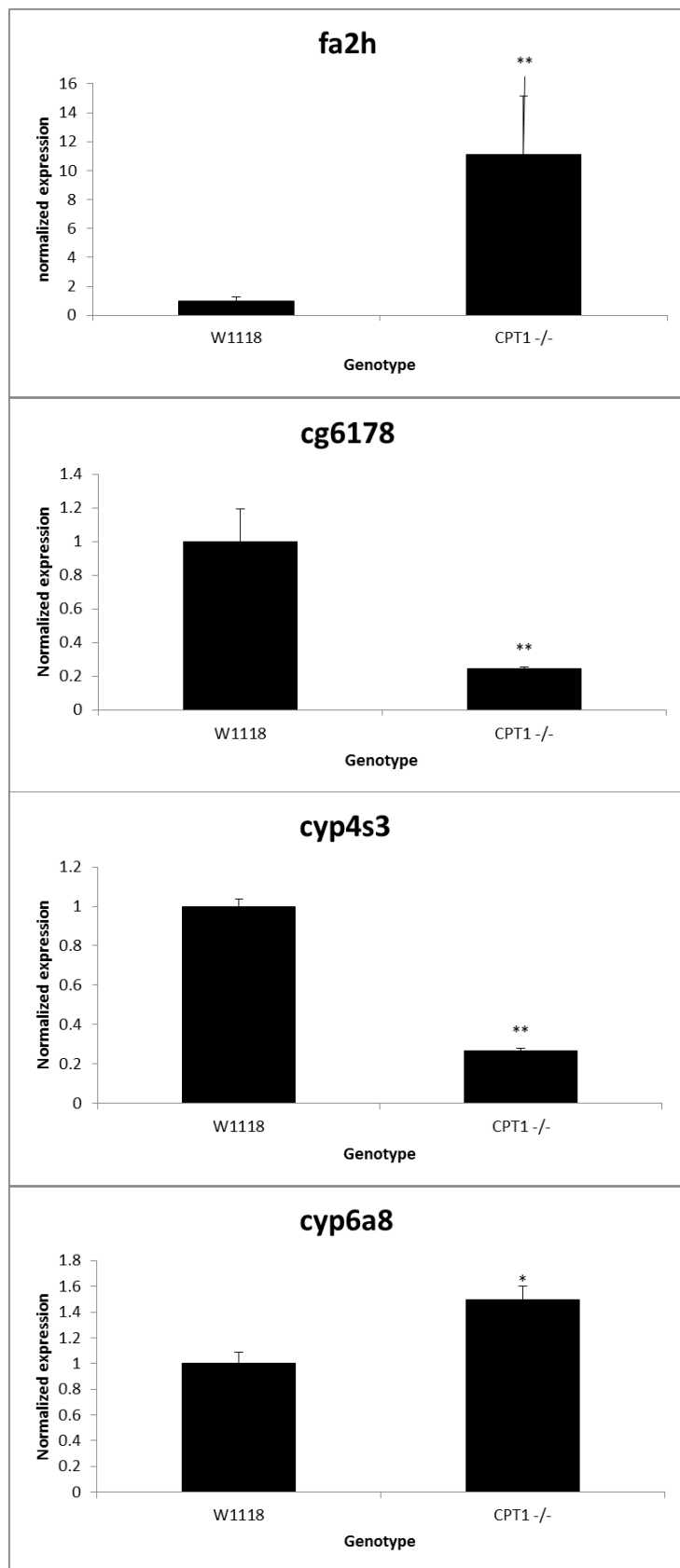
Figure 2: Alternative lipid oxidation pathway are altered in the *cpt-1^-/-* mutant qRT-

qRT-PCR results show that the expression of key enzymes in the alternative lipid

oxidation pathway are misregulated in the mutant. *p<0.05. **p<0.01. Data are

expressed as mean ± SEM

2) Metabolic analysis

The identity of each of the unknown metabolites was then queried via the Metlin repository comprising the masses and structures of known metabolites (Smith et al., 2005). Most of the unknown metabolites identified by my study could not be identified using the Metlin database, which is not surprising considering the database is not exhaustive and is not Drosophila specific. However, two of the unknown masses revealed potentially interesting results.

The unknown metabolite with m/z 160 with retention time of 11.2 min that was significantly up in the *cpt-1$^{-/-}$* mutant might be as L-carnitine *Formula:* $C_7H_{15}NO_3$. Although several further studies are needed to confirm this, the increase in carnitine levels in the *cpt-1$^{-/-}$* mutant provides reasonable validation to the importance of the data. The unknown metabolite with m/z 442 was identified as guanosine 5′-diphosphate (GDP) and that at mass 362 was identified as guanosine 3'-phosphate. Additionally, owing to the same retention time of 3.57 min and difference in masses of one sodium atom (22), the metabolite with m/z 447 is likely related to the metabolite with m/z 469.

Table 1: Unknown metabolites from mass spectrometric analysis

Metabolites that were increased in the *cpt-1*$^{-/-}$ mutant [M-H]$^-$			
m/z	Retention time (mins)	Fold induction	Standard error
437	7.11	116	±1.71
447	3.57	18	±2.99
160	11.12	16	±4.47
561	11.15	11	±2.16
469	3.57	8	±0.83
390	24.99	6	±0.48
Metabolites that were decreased in the *cpt-1*$^{-/-}$ mutant [M-H]$^-$			
m/z	Retention time (mins)	Fold reduction	Standard error
442	34.7	67	±5.59
344	34.7	49	±5.52
424	2.8	19	±3.75
308	2.7	15	1.45
342	34.34	10	1.29
175	2.73	9	±1.33
362	3.47	6	±0.27
357	8.95	6	0.24
155	18.49	5	0.09

Discussion and Future Directions:

This is the first study that has attempted to understand alternative lipid oxidation pathways. We conducted our study in *Drosophila cpt1-/-* mutants that are unable to oxidize fat in the conventional beta oxidation pathway, but yet, are relatively healthy. Our analysis of differences in the expression of key enzymes of the alt-oxidation pathway revealed interesting results. Our data suggests that $cpt-1^{-/-}$ mutants are able to route lipid oxidation to alternative pathways, and that it is likely that this alternative pathway allows them to survive even when the default pathway is inactive. We see marked upregulation of *fa2h* in *cpt-/-* mutants, which suggests that α-oxidation alone is sufficient for the succeeding steps in the peroxisome and that it is bypasses ω-oxidation. This brings up further interesting questions regarding $cpt-1^{-/-}$ mutants: How do they perform under starvation? Does diet promote or deter the effectiveness of the rescue?

We had previously used the services of the company Metabolon to conduct mass spectrometric analysis for us. To save on costs, we attempted to develop our own in-house protocol for conducting mass spectrometric analysis. We successfully generated extracts from pre-pupa larvae and were able to identify fifteen misregulated metabolites between the wild type and $cpt-1^{-/-}$ mutant.

One potential metabolite isolated is carnitine (m/z 160). Although the increase in carnitine levels in the $cpt-1^{-/-}$ mutant is plausible, several further studies are warranted. First, we need to compare the peak obtained from our fly extract with that generated by

a carnitine standard in order to verify the identity of our peak. Unfortunately, we were unable to find a cheap commercially available standard to compare our results with. Further, to confirm the elemental composition of the identified compounds, the metabolite masses can be obtained by high resolution LC/MS. Additional structural information can be derived from fragmentation analysis by MS/MS. For the unknown metabolites, NMR spectroscopic studies may be conducted.

To our knowledge, this is a first study of its kind and we hope this short story is a foundation for future experiments exploring alternative fatty acid oxidation pathways.

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