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Mechanisms of Oxygen Sensing and Hypoxic Increase in Replicative Lifespan

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Abstract

Mechanisms of Oxygen Sensing and Hypoxic Increase in Replicative Lifespan

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The response to reduced levels of oxygen (hypoxia) is essential for embryonic development by promoting the proper formation of vascular networks. Conversely, hypoxia also promotes the development of pathophysiological processes such as pulmonary hypertension and cancer. The main mediator of responses to hypoxia within the cell is the transcription factor hypoxia-inducible factor (HIF). Defining the mechanism(s) that regulate HIF activation, and understanding the consequences of activating HIF, are essential to understand how hypoxia mediates proper development, as well as the promotion of pulmonary hypertension and cancer.

Mitochondrial electron transport is required for cellular oxygen sensing. However, it is unknown which mitochondrial function, respiration or the generation of reactive oxygen species (ROS), necessary for mitochondrial oxygen sensing. This thesis demonstrates that cells deficient in the mitochondrial complex III subunit cytochrome b, which are respiratory incompetent, increase ROS and activate HIF. RNAi of the complex III subunit Rieske iron sulfur protein in the cytochrome b null cells abolished ROS generation at the Q_o site of complex III and hypoxic activation of HIF. Treatment with antioxidants maintained hydroxylation of HIF-1α protein thereby preventing its activation during hypoxia. These results provide genetic evidence that the Q_o site of complex III is

required for oxygen sensing by releasing ROS that inhibit HIF-1α hydroxylation, thereby stabilizing and activating the HIF-1α protein.

Physiological hypoxia extends the replicative lifespan of human cells in culture. Increased replicative lifespan contributes to the progression of cancer and it may influence the development of pulmonary hypertension. The hypoxic extension of replicative lifespan is ablated by a dominant negative HIF. HIF is sufficient to induce telomerase reverse transcriptase mRNA, telomerase activity, and extend replicative lifespan. Furthermore, the down-regulation of the von-Hippel Lindau (pVHL) tumor suppressor protein by RNA interference increases HIF and extends replicative lifespan under normoxia. These findings provide genetic evidence that hypoxia utilizes mitochondrial ROS as signaling molecules to activate HIF dependent extension of replicative lifespan, further linking HIF activation to diseases associated with increased replicative lifespan. Furthermore, they provide the first association of an increase in ROS with an increase in replicative lifespan

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Chapter 1: Introduction

Oxygen is necessary for survival of higher eukaryotes. Just as carbon sources are used by glycolysis to generate energy, cells utilize oxygen to generate the majority of ATP required for normal cellular processes through oxidative phosphorylation (OXPHOS) (Saraste, 1999). In the complete absence of oxygen (anoxia 0-0.3% O_2), most organisms and cells cannot survive for long periods of time (Graeber et al., 1996; Schroedl et al., 2002). However, in conditions of limited oxygen (Hypoxia 0.3-5% O_2), cells activate an adaptive response that is mediated by the transcription factor Hypoxia Inducible Factor (HIF) (Semenza, 1998). This response facilitates survival through the upregulation of a number of genes involved in various physiological processes such as glycolysis and angiogenesis that promote survival in hypoxic conditions by increasing the capacity for generating ATP and inhibiting the depletion of oxygen. This adaptation response is not due to compromised bioenergetics during hypoxia. The activity of the terminal complex in OXPHOS, cytochrome c oxidase, is not significantly decreased, and therefore cellular ATP levels do not change in hypoxic conditions.   The K_m of cytochrome c oxidase is less than 0.3% O_2 (Chandel et al., 1996).   In contrast to hypoxia, cytochrome c oxidase activity is substantially decreased during anoxia resulting in a decrease in the generation of ATP (Chandel et al., 1996).

Oxygen Homeostasis

The transcriptional activation of HIF is a stress response developed through evolution to allow cells to avoid a bioenergetic crisis in low oxygen levels, making HIF a master regulator of oxygen homeostasis. An example of organismal maintenance of

oxygen homeostasis in higher organisms is their ability to increase their capacity for systemic oxygen delivery. This requires erythropoiesis, the process of increased generation of erythrocytes. Erythropoiesis is increased under conditions of decreased oxygen availability through an increase in expression of the protein erythropoietin, a HIF target gene (Semenza, 1994). In fact, early examination of the regulation of erythropoietin in response to oxygen led to the discovery of the transcription factor HIF.

Oxygen homeostasis is critical for proper development, making HIF mediated transcription necessary for normal organismal development. The fact that HIF knockout mice are embryonic lethal at E9.5 highlights the importance of HIF mediated transcription in development (Maltepe et al., 1997; Ryan et al., 1998). The lethality displayed by HIF knockout mice is due to impaired vascular development and abnormal placental development (Adelman et al., 2000). An important HIF target gene is VEGF, which promotes neovascularization and is required for development (Breier et al., 1992; Forsythe et al., 1996). Unfortunately, genes such as VEGF and other HIF target genes that are required for normal maintenance of oxygen homeostasis also promote pathophysiological processes such as pulmonary hypertension and cancer. Studies have indicated that preventing HIF activation can suppress tumorigenesis and hypoxia induced pulmonary hypertension (Kondo et al., 2002; Kung et al., 2000; Maranchie et al., 2002b; Yu et al., 1999). Therefore, defining how a cell senses decreased levels of oxygen to regulate the activity of HIF has broad implications for normal physiology as well as numerous diseases associated with hypoxia.

Hypoxia-Inducible Factor

HIF is a heterodimer of two basic helix loop-helix/PAS proteins, HIFα and the aryl hydrocarbon nuclear trans-locator (ARNT or HIF-1β) (Wang et al., 1995). Both subunits are ubiquitously expressed, however the alpha subunit is labile in conditions of normal oxygen (5-21% O_2). Under hypoxic (5-0.3% O_2) conditions, the alpha subunit is stabilized and then dimerizes with ARNT, and translocates to the nucleus to initiate gene transcription (Jiang et al., 1996b). Recent studies indicate that oxygen levels regulate the hydroxylation of two proline residues, 402 and 564, within the oxygen dependent degradation domain (ODDD) of HIFα (Masson et al., 2001). This hydroxylation reaction is catalyzed by a family of proline hydroxylation enzymes (PHDs) (Bruick and McKnight, 2001; Epstein et al., 2001). The PHDs require Fe^{++}, oxygen, and 2-oxoglutarate to catalyze the hydroxylation reaction. Hydroxylated proline serves as a binding site for the von Hippel-Lindau protein (pVHL), the substrate recognition component of the VBC-CUL-2 E3 ubiquitin ligase complex (Ivan et al., 2001; Iwai et al., 1999; Jaakkola et al., 2001). Once bound, pVHL tags HIFα with ubiquitin thereby targeting it for proteasomal degradation (Maxwell et al., 1999). When oxygen levels decrease below 5% O_2, HIFα protein is stabilized due to a lack of proline hydroxylation (Figure 1.1). In the absence of proline hydroxylation, pVHL cannot bind HIFα to initiate ubiquitin-proteasomal degradation. When stabilized, HIFα translocates to the nucleus and dimerizes with ARNT. Once in the nucleus, the HIF dimer binds to HIF response elements (HRE) located throughout the genome (Jiang et al., 1996a). Understanding how cells decreased oxygen levels to inhibit the hydroxylation enzymes would provide the next step in elucidating the hypoxic response. Furthermore determining the

necessary components of the cellular oxygen sensor would provide drug discovery targets for the treatment of pathologies associated with HIF activation, such as pulmonary hypertension and cancer.
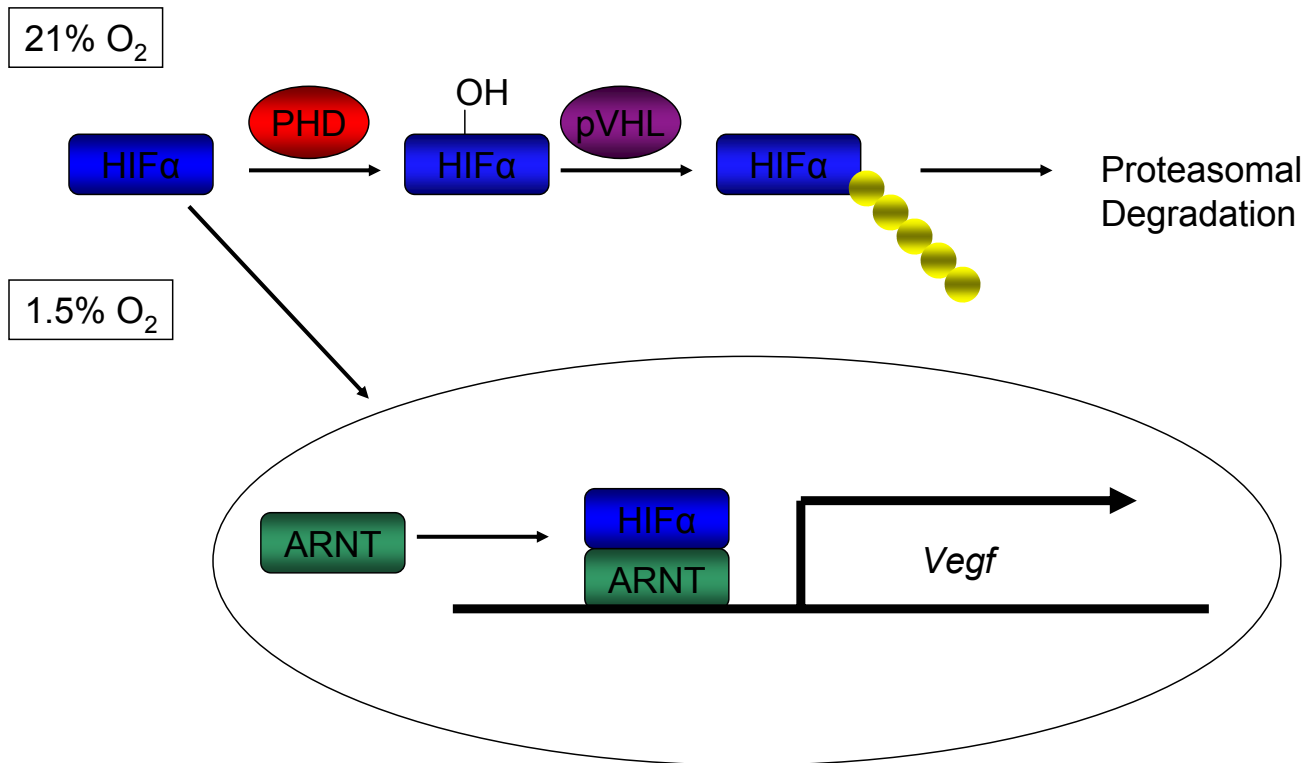
Figure 1.1 Oxygen regulation of HIF. In normal oxygen conditions (21% O_2) HIF is hydroxylated by the PHDs. This allows for pVHL to target HIFα for proteasomal degradation. In hypoxic conditions (1.5% O_2) HIFα is not hydroxylated and therefore stabilized. When stabilized HIFα translocates to the nucleus, dimerizes with ARNT and induces transcription of target genes such as *Vegf*.

Oxygen Sensing

In order to regulate the hypoxic activation of HIF for therapeutic treatments, the absolute upstream regulator of HIF, i.e. the oxygen sensor, needs to be defined. Initial studies focused on identifying the cellular oxygen sensor were based on the assumption that it was an oxygen binding protein. The most notable protein that binds oxygen is hemoglobin. Oxygen directly binds to hemoglobin at the center of the porphyrin ring of heme moieties. Using carbon monoxide, which competes with oxygen for binding to heme groups, Goldberg and colleagues demonstrated that a heme protein is necessary for the hypoxic induction of the HIF target gene erythropoietin. Furthermore, application of the heme synthesis inhibitor desferrioxamine also inhibited hypoxic induction of erythropoietin, indicating that cellular oxygen sensing required heme containing proteins (Goldberg et al., 1988; Ho and Bunn, 1996). However, this data was challenged by a study showing that both carbon monoxide and heme synthesis inhibitors do not reduce HIF mediated transcription (Srinivas et al., 1998). Thus, further investigation was required to explore the role of heme containing proteins in cellular oxygen sensing. Additional studies pointed to the mitochondria as having a prominent role in cellular oxygen sensing.

Oxygen Sensing by the Mitochondria

A functional mitochondrial electron transport chain is necessary for hypoxic stabilization and therefore activation of HIF (Chandel et al., 1998; Chandel et al., 2000). In the absence of mitochondrial electron transport, cells do not consume oxygen or generate ROS from the mitochondria. These functions of mitochondria have been

independently proposed as potential mechanisms by which mitochondrial electron transport chain activates HIF during hypoxia (Figure 1.2).

Molecular oxygen is used as the terminal electron acceptor in the mitochondrial electron transport chain when cytochrome c oxidase (COX) converts oxygen to water (Saraste, 1999). This property of mitochondria combined with the requirement of the PHDs for molecular oxygen as a co-substrate is the basis for a model in which mitochondrial oxygen consumption is the regulator of HIF activation via PHD regulation. This model hypothesizes that during conditions of limited oxygen, mitochondria create an oxygen gradient within the cells as a result of their ability to consume oxygen (Doege et al., 2005; Hagen et al., 2003). This gradient would effectively sequester molecular oxygen away from the cytosolic PHDs, thus inhibiting their ability to hydroxylate HIFα. However, cells that are respiratory deficient and are not ρ^0 cells can still stabilize HIFα protein during hypoxia (Brunelle et al., 2005a). Unfortunately, this study did not look at the ability of these cells to generate ROS.

A second model of mitochondrial oxygen sensing is based on ROS generation by mitochondria. It has been demonstrated that cytosolic ROS levels paradoxically increase in hypoxic conditions (Chandel et al., 1998). The increase in ROS during hypoxia is reversible since reoxygenation to normoxia decreases the ROS signal. The increase in cytosolic ROS during hypoxia is required to stabilize HIFα protein (Chandel et al., 2000). Cells deficient in cytochrome c or Rieske iron-sulfur protein are unable to stabilize HIF during hypoxia and do not display an increase in cytosolic ROS during hypoxia (Guzy et al., 2005; Mansfield et al., 2005b). However, these cells would also be deficient in oxygen consumption therefore, these studies do not differentiate between

the ability of mitochondria to generate ROS or consume oxygen. Incubating cells with

pharmacological antioxidants such as ebselen and MitoQ attenuates HIF activation in

hypoxic conditions (Guzy et al., 2005; Sanjuan-Pla et al., 2005). Recently it has been

reported that MitoQ treatment affects respiration, thus, again, the effect of MitoQ cannot

be solely attributed to its antioxidant properties (Pan et al., 2007). However, studies

using protein antioxidants support the involvement of ROS. Expression of protein

antioxidants, such as glutathione peroxidase (GPX) and catalase, also attenuates HIFα

protein stabilization and activation, but expression of superoxide dismutase (SOD) has

no effect (Brunelle et al., 2005a). SOD converts superoxide to H_2O_2, while GPX and

catalase convert H_2O_2 to water. The specificities of these antioxidants for different

forms of ROS lends toward the conclusion that H_2O_2 is the ROS moiety required for

stabilization of HIFα protein. In fact, HIFα protein is stabilized when cells are pulsed

with 25µM t-butyl H_2O_2, a more stable form of H_2O_2, in normal oxygen conditions,

indicating that H_2O_2 is sufficient to activate HIF mediated transcription (Chandel et al.,

2000). ROS are a normal by product of electron transport within the mitochondria, so

the ability of cells to increase cytosolic ROS in hypoxic conditions provides a possible

link between mitochondrial electron transport and HIF activation. The mitochondrial

electron transport chain generates ROS at five sties (Figure 1.3). However, the

question of where within the electron transport chain these ROS originate and how

hypoxia increases cytosolic ROS remains.

The work presented in Chapter 2 of this dissertation uncouples ROS generation

from oxygen consumption in the hypoxic activation of HIF, addressing which function of

mitochondrial electron transport chain is the major regulator of HIF during hypoxia.

Genetic interventions are used to identify the site within the electron transport chain that generates the ROS that activate HIF. For the first time, it is demonstrated that these ROS inhibit the PHDs ability to hydroxylate HIF in hypoxic conditions. Furthermore, data is presented that indicate how hypoxia increases cytosolic ROS.

A ↑ROS ⊥ PHD ⊥ HIFα Protein stability

B ↓respiration ↓cytosolic O_2 ⊥ PHD ⊥ HIFα Protein stability
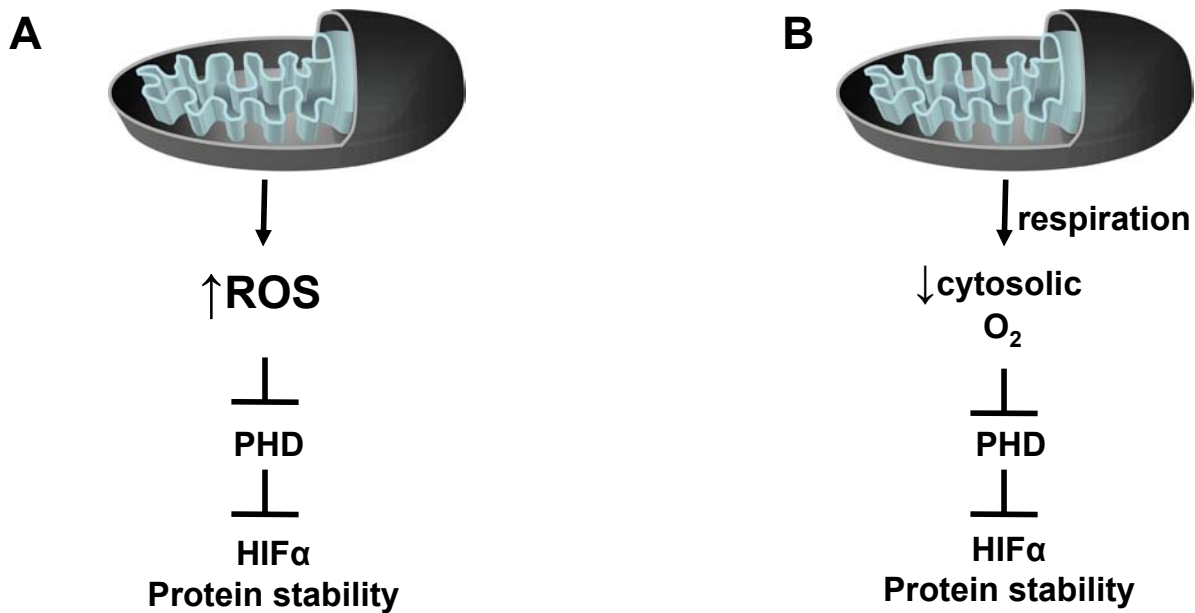
Figure 1.2 Mitochondrial Oxygen Sensing Models. **(A)** First model postulates that mitochondria release ROS during hypoxia to inhibit hydroxylation of HIFα protein thereby causing the protein to escape proteasomal degradation. **(B)** The second model hypotheses that mitochondrial respiration limits oxygen availability to the hydroxylases thereby not allow the hydroxylation reaction to occur. This results in accumulation of HIFα protein during hypoxia.
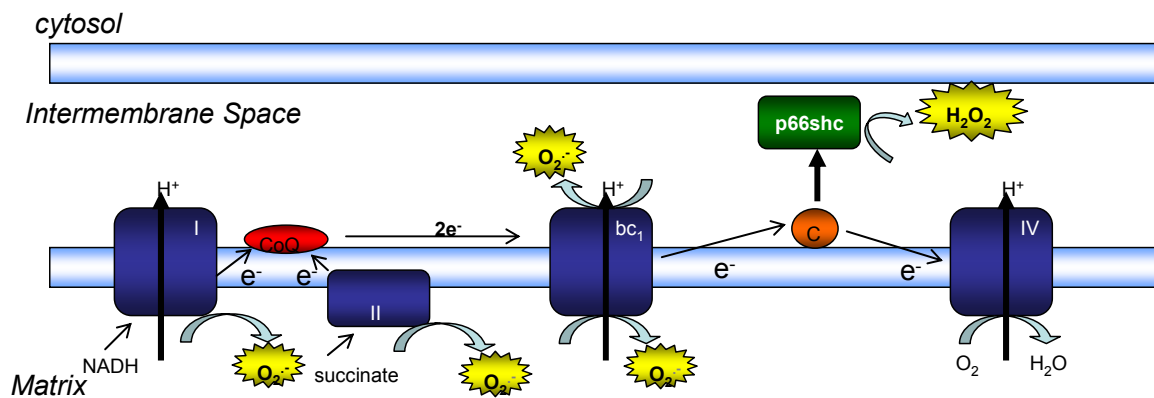
Figure 1.3 Sites of ROS generation by the mitochondrial electron transport chain. ROS are generated by Complex I and Complex II. The bc_1 (Complex III) generates ROS at two sites, one in the matrix and one in the intermembrane space. It has recently been described that cytochrome c can generate ROS by transferring electrons to p66Shc.

Hypoxia, HIF, and cancer.

As a tumor increases in size, it outgrows the existing vasculature, creating nutrient deprivation that ultimately limits tumor growth. At this point the tumor will survive, but with no net gain in growth because as the outside of the tumor grows, the center will die of necrosis and/or apoptosis due to nutrient and oxygen deprivation. In order for there to be a net gain in tumor size, the number of cells that proliferate must outnumber the amount of cells that die. Thus, tumors must acquire the ability to obtain nutrients in order to promote survival and supply cells with the energy needed to proliferate at the abnormal rate observed in tumor cells (Dang and Semenza, 1999). Tumors achieve this by modifying the surrounding microenvironment in order to provide an adequate supply pipeline for nutrient delivery at the site of initial growth (angiogenesis), or by disseminating to different areas of the organism where nutrients are more readily available (metastasis) (Harris, 2002). Furthermore, as tumor cells await angiogenesis they must adapt to the hypoxic environment. HIF activity is important in the tumor cells adaptive response. The importance of HIF mediated transcription in tumorigenesis is highlighted by studies indicating that preventing HIF activation can suppress tumorigenesis (Kondo et al., 2002; Kung et al., 2000; Maranchie et al., 2002b). Moreover, deletions or mutations of genes involved in suppressing HIF activity, such as the von Hippel-Lindau tumor suppressor protein (pVHL), promote the onset of various types of cancers (Kondo et al., 2002; Maranchie et al., 2002a; Seizinger et al., 1988).

Deregulation of genes involved in intrinsic cellular processes such as cellular proliferation, apoptosis and replicative lifespan promote aberrant and unregulated

cellular growth leading to the initiation of cancers (Green and Evan, 2002). Some of the genes induced by HIF control intrinsic cellular processes, therefore deregulation of HIF can promote the progression of tumorigenesis (Figure 1.4). Genes like insulin2-like growth factor-2 (IGF-2) and glycoytic enzymes act as intrinsic signals to promote tumorigenesis by modulating cellular proliferation and survival, thereby providing cells autonomy from extrinsic factors. Vascular Endothelial Growth Factor (VEGF) is a direct target of HIF and promotes the recruitment of endothelial cells to regions of hypoxia in order to promote the formation of new vascular network (Forsythe et al., 1996). This allows for the mass of tumor cells to eventually have a net gain in growth via increased supply of necessary nutrients. Other target genes, such as matrix metalloproteinase-2 (MMP-2), promote invasion of tumor cells, as well as the migration of the cells away from the primary tumor (c-MET) (Krishnamachary et al., 2003; Pennacchietti et al., 2003). Chapter 3 of this dissertation demonstrates that HIF is sufficient to induce the transcription of another gene known to promote tumorigenesis. This target, human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, increases replicative lifespan of cells, allowing cancer cells to divide infinitely. This HIF mediated increase in hTERT is sufficient to increase telomerase activity and more importantly increase replicative lifespan. These data provide a novel function of HIF that positively regulates tumorigenesis, further highlighting the importance HIF in tumor progression.
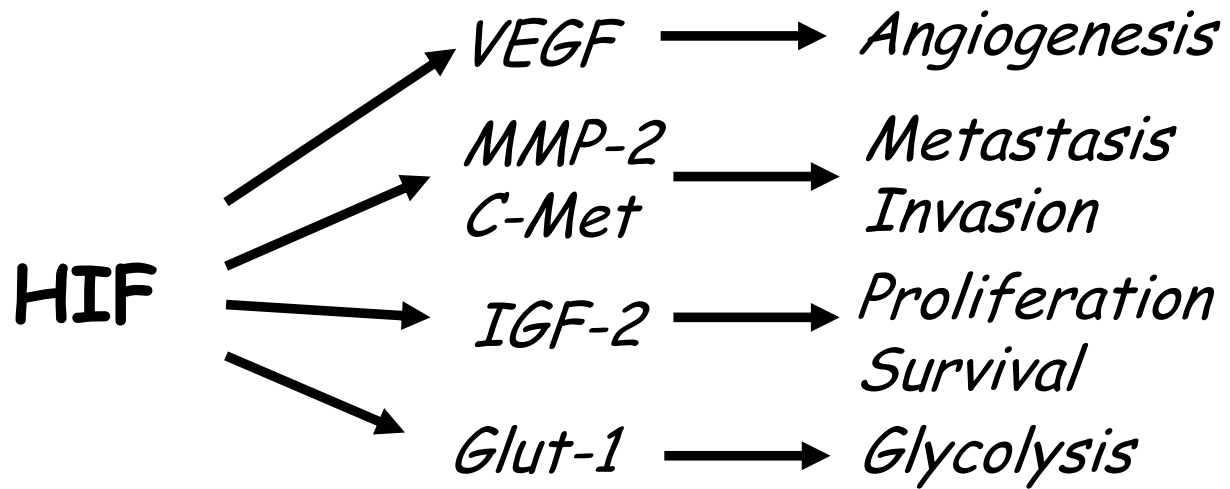
Figure 1.4 HIF targets multiple genes involved in tumorigenesis. HIF target genes promote angiogenesis, metastasis, invasion, proliferation, survival and aerobic glycolysis.

Chapter 2: The Q_o site of the mitochondrial complex III is required for oxygen sensing via ROS production.

Introduction

Oxygen homeostasis is important for normal cellular function (Semenza, 2000). As oxygen levels decrease in the surrounding environment (hypoxia), cells respond by activating hypoxia-inducible factor (HIF) dependent gene transcription to facilitate cellular adaptation to hypoxia. HIF is a heterodimer of two basic helix loop-helix/PAS proteins, HIF-α and the aryl hydrocarbon nuclear trans-locator (ARNT or HIF-β) (Wang et al., 1995). Under normal oxygen conditions, ARNT is constitutively stable while the alpha subunit is labile. In normal oxygen conditions, the alpha subunit is hydroxylated at proline residues by a family of prolyl hydroxylase enzymes (PHDs) (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). Proline hydroxylation targets the protein for ubiquitination by the von Hippel-Lindau protein (pVHL)/E3 ubiquitin ligase and for subsequent proteasomal degradation (Ivan et al., 2001; Jaakkola et al., 2001; Maxwell et al., 1999). The alpha subunit is also hydroxylated at an asparagine residue by the enzyme Factor Inhibiting HIF-1 (FIH-1) under normal oxygen conditions (Lando et al., 2002a; Lando et al., 2002b; Mahon et al., 2001). Asparagine hydroxylation blocks the binding of the transcriptional co-activators p300 and CBP to HIF-1 (Dames et al., 2002; Freedman et al., 2002). Under hypoxic conditions, the alpha subunit is not hydroxylated by the PHDs or FIH, resulting in the stabilization of the HIF-α protein, dimerization with ARNT, and association with p300/CBP to initiate gene transcription.

The mechanism(s) by which cells sense oxygen to transduce the hypoxic signal to activate HIF is a subject of ongoing research. Previous studies indicate that mitochondria are involved in oxygen sensing; however, the mechanism is not fully understood. One model proposes that mitochondria regulate the ability of the PHDs to hydroxylate HIF-1α protein due to their capacity to consume oxygen (Hagen et al., 2003). Mitochondrial oxygen consumption would generate a gradient of oxygen within the cytosol of the cell, thereby limiting the availability of oxygen, a necessary co-substrate for PHD activity. Another model proposes that mitochondria increase the levels of cytosolic reactive oxygen species (ROS) during hypoxia to activate HIF (Bell et al., 2005). Initial evidence to support this model came from observations that cells treated with mitochondrial electron transport inhibitors, and cells devoid of mitochondrial DNA (ρ^0 cells), fail to activate HIF during hypoxia due to a lack of mitochondrially generated ROS (Chandel et al., 1998; Chandel et al., 2000). Recently, three independent studies genetically confirmed the initial finding that mitochondria generated ROS are required for hypoxic activation of HIF. Cells devoid of the cytochrome c gene do not increase cytosolic ROS or stabilize HIF-1α in hypoxic conditions (Mansfield et al., 2005). Cells with either transient or stable RNAi of the Rieske Fe-S protein, a component of the mitochondrial Complex III (the bc_1 complex), inhibits hypoxic increase of cytosolic ROS and stabilization of HIF-1α protein (Guzy et al., 2005; Brunelle et al., 2005).

Although these studies indicate genetic evidence that ROS generated within the mitochondrial electron transport chain are required for oxygen sensing, they do not indicate which complex of the electron transport chain is the site of ROS generation.

Moreover, they do not differentiate between the ability of mitochondria to respire and its ability to generate ROS as the necessary function in mitochondrial oxygen sensing. The mitochondrial electron transport chain generates superoxide at complexes I, II, and III (Turrens, 2003). Complexes I and II generate superoxide within the mitochondrial matrix (Genova et al., 2001; Kushnareva et al., 2002; Lenaz, 2001; Paddenberg et al., 2003; Turrens and Boveris, 1980; Turrens et al., 1982; Zhang et al., 1998). Complex III generates superoxide at the Q_o site resulting in the release of superoxide into either the inter-membrane space or the matrix (Boveris et al., 1976; Cadenas et al., 1977; Han et al., 2001; Muller et al., 2004; Starkov and Fiskum, 2001; Turrens et al., 1985). Complex IV has not been reported to generate ROS; however, recently cytochrome c was demonstrated to participate in the generation of hydrogen peroxide by providing electrons to p66 Shc (Giorgio et al., 2005). The observations that loss of cytochrome c gene or RNAi of the Rieske Fe-S protein prevent hypoxic stabilization of the HIF-1α protein implicate either complex III or cytochrome c as the source of ROS generation required for hypoxic stabilization of the HIF-1α protein. In this chapter, we examined which site within the mitochondrial electron transport chain is required for the generation of ROS and hypoxic stabilization of the HIF-1α protein independently of respiration. Moreover, we present data that suggests the mechanism cells utilize to increase cytosolic ROS in hypoxic conditions.

Results

Hypoxic stabilization of HIF-1α protein is independent of respiration and cytochrome b.

Mitochondrial complex III consists of 11 subunits, three of which have known electron transport activity (the Rieske Fe-S protein, cytochrome b, and cytochrome c_1). The electron flux from ubiquinol (QH_2) to cytochrome c occurs through the ubiquinone (Q) cycle within complex III (Hunte et al., 2003). QH_2 carries two electrons and cytochrome c is a one electron acceptor; thus, the first electron from QH_2 is transferred down the Rieske Fe-S/cytochrome c_1/cytochrome c axis, while the other electron is cycled through cytochrome b. In between the transfer of the first and second electron from QH_2, the highly reactive intermediate ubisemiquinone ($Q^.$) is formed at the Q_o site of complex III. This allows for the generation of ROS at the Q_o site of complex III through the interaction between $Q^.$ and molecular oxygen within the bc_1 complex. Theoretically, $Q^.$ is also generated at the Q_i site of complex III, thereby also allowing for the generation of ROS at the Q_i site (Figure 2.1). In order to explore the role of complex III in the stabilization of the HIF-1α protein, we utilized cells that are deficient in cytochrome b and thus the Q_i site. These cells are cybrids that were generated by repopulating 143B ρ^0 cells with mitochondria that contain either a wild-type mitochondria DNA or a 4-base pair deletion of the cytochrome b gene (Rana et al., 2000). The cytochrome b deficient cells (Δcyt b) do not consume oxygen, similar to ρ^0 cells (Figure 2.2). However, the Δcyt b cybrid cells retain the ability to stabilize HIF-1α protein under hypoxia or in the presence of dimethyloxyglycine (DMOG), an inhibitor of PHD mediated

hydroxylation (Figure 2.3). These data indicate that the ability of cells to consume

oxygen is not related to their ability to stabilize HIF-1α protein.

Moreover, under hypoxia, the Δcyt b cybrids increase H_2O_2 levels measured in

the cytosol using Amplex Red (Figure 2.4). ρ^0 cells did not display an increase in ROS

in the cytosol during hypoxia, indicating mitochondria as the major source of ROS

production during hypoxia. These data indicate that that the ability of mitochondria to

increase cytosolic ROS and stabilize HIF protein in hypoxia is independent of both

cytochrome b and oxygen consumption. Additionally, the levels of cytosolic antioxidants

proteins Cu/Zn SOD and catalase did not change drastically in hypoxic conditions

(Figure 2.5).
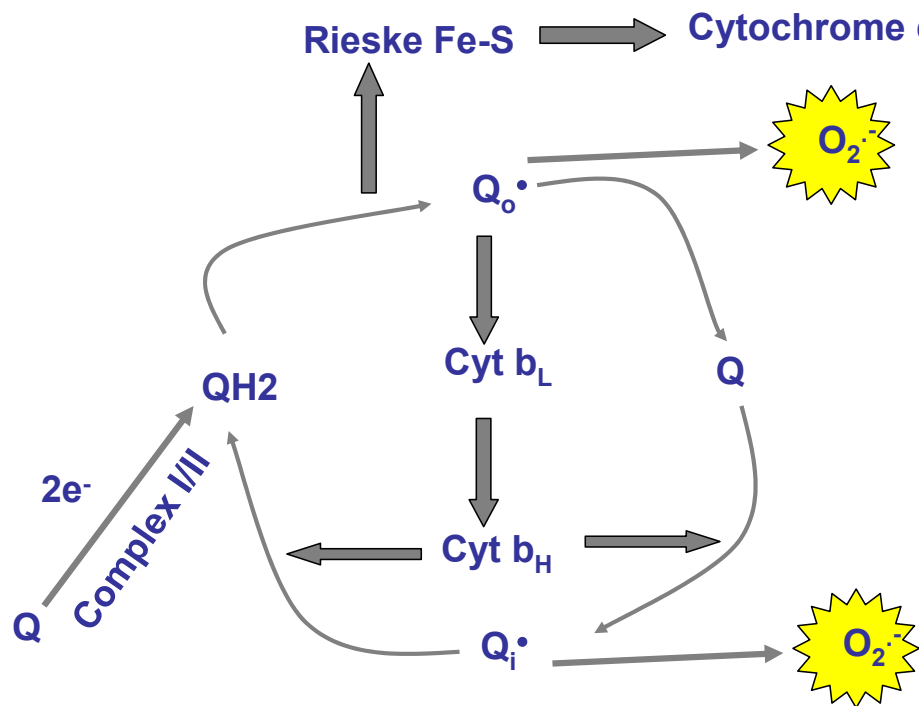
Figure 2.1 Mitochondrial complex III generates ROS through the ubiquinone (Q) cycle. The Q cycle generates ROS at the Q_o and Q_i site. The Rieske Fe-S protein is required for ROS production at the Q_o site. The loss of complex III subunit cytochrome b abolishes the Q_i site.
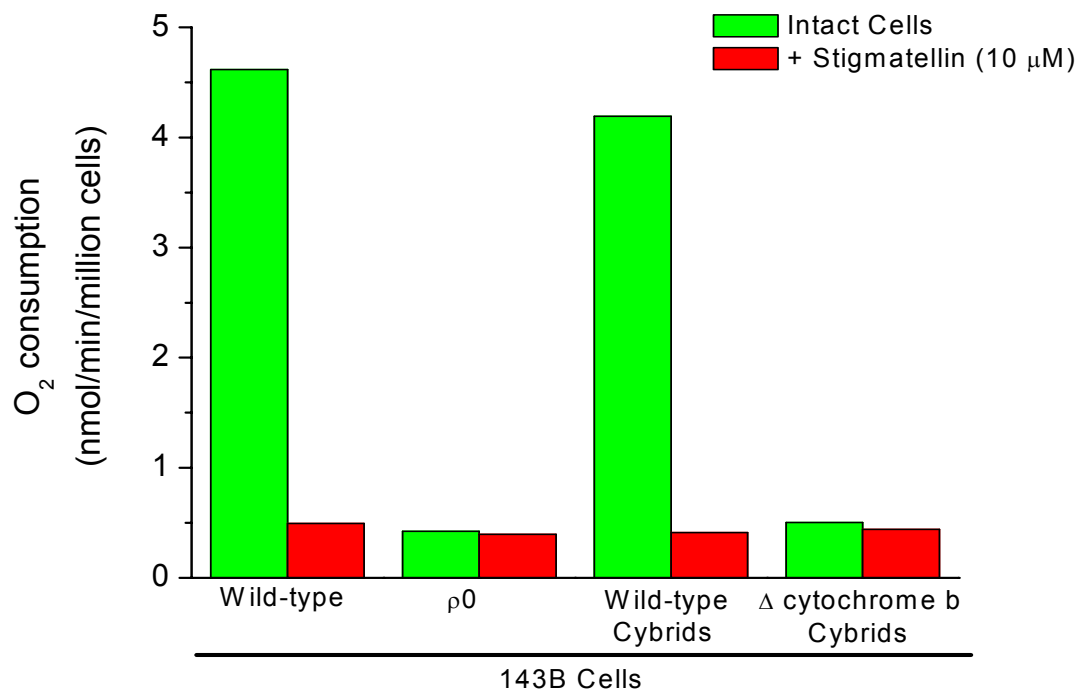
Figure 2.2 143B Cybrid cells lacking cytochrome b are deficient in oxygen consumption. Oxygen consumption of 143B cells, 143B ρ^0 cells, wt cybrids, and Δ Cytb cybrids with and without the mitochondrial electron transport inhibitor stigmatellin (1uM).
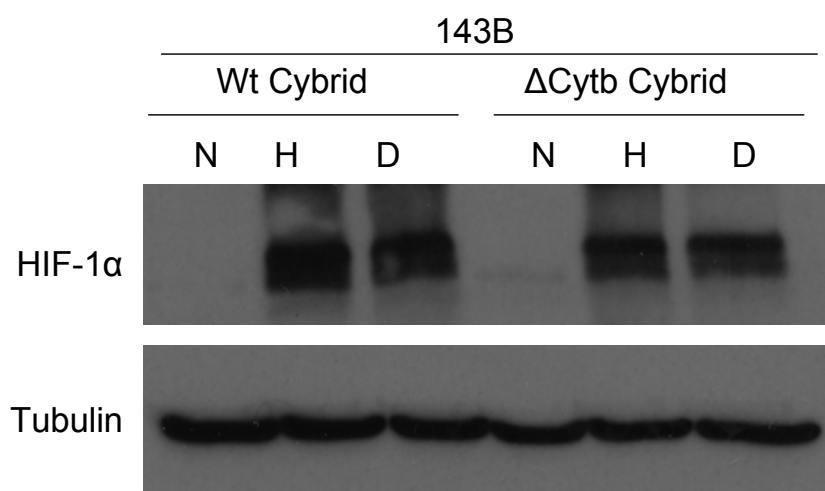
Figure 2.3 Wt and Δcytochrome b cybrids stabilize HIF-1α protein in hypoxic conditions. HIF-1α protein levels in wt and Δ Cytb cybrids subjected to 21%O_2 (N), 1.5%O_2 (H), or 1mM DMOG (D) for 4 hours. Representative blot from four independent experiments.
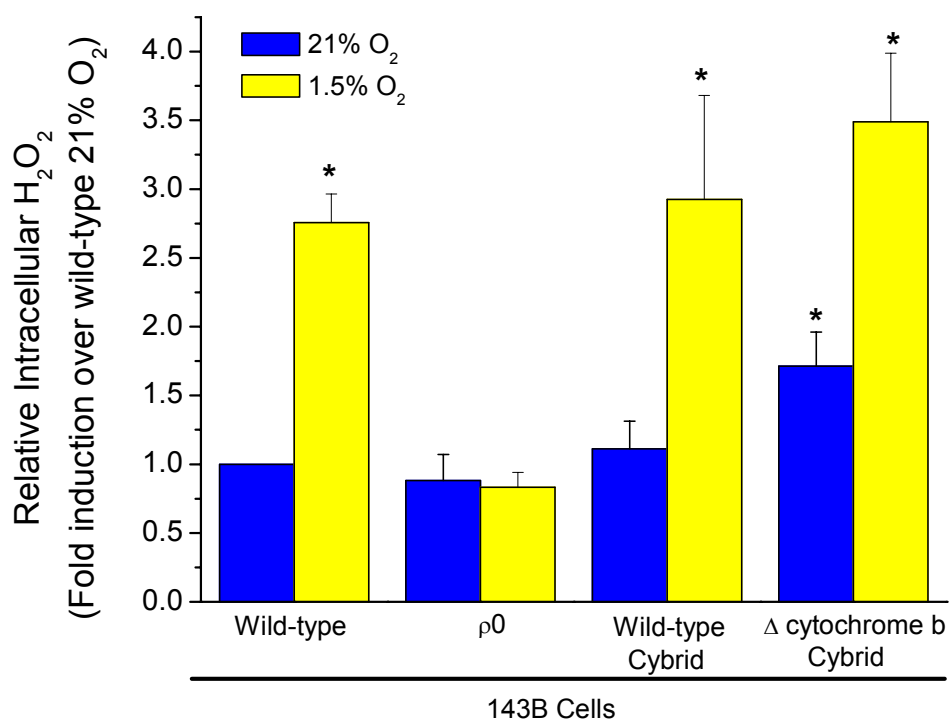
Figure 2.4 Δcytochrome b cybrids increase cytosolic ROS in hypoxic conditions. Intracellular H_2O_2 levels were measured by Amplex Red in 143B cells, 143B ρ^0 cells, wt cybrids, and Δ Cytb cybrids exposed to 1.5%O_2 or 21%O_2 for 4 hours. n=4 (mean ±S.E.M) *p<0.05 (all groups were compared to the normoxia sample of wild-type cells)

A

Wt cybrid

N   H

-16Kd   CuZn SOD

-55Kd   catalase

-50kD   α-tubulin

B

Δcytb cybrid

N   H

-16Kd

-55Kd

-50kD
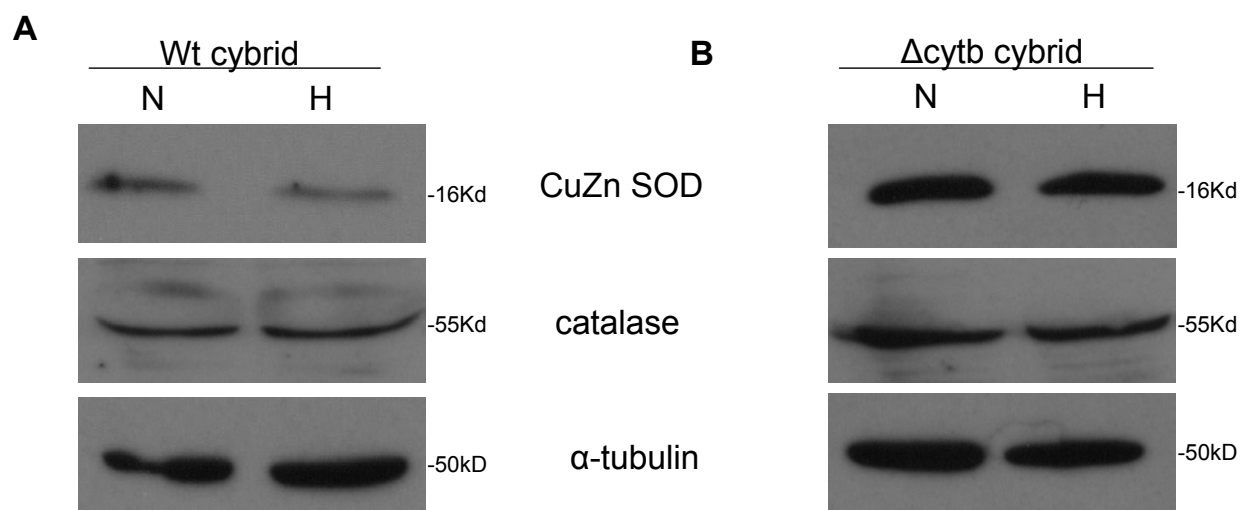
Figure 2.5 Hypoxia does not increase endogenous antioxidant proteins.
Antioxidant protein profile of whole cell lysates from **(A)** WT cybrids and **(B)** ΔCytb
cybrids subjected to either 21% O_2 (N) or 1.5% O_2 (H), for 4 hrs.

To determine whether mitochondrial ROS generation was responsible for the increase in cytosolic ROS and stabilization of the HIF-1α protein in the Δ cytb cybrids, these cells were treated with the mitochondrial targeted antioxidant MitoQ. MitoQ abolished the increase in cytosolic ROS during hypoxia in the Δ cytb cybrids (Figure 2.6). Hypoxic stabilization of the HIF-1α protein was diminished in both wt and Δ cytb cybrid cell lines treated with MitoQ but not in the presence of DMOG (Figure 2.7). The decrease in HIF-1α protein stabilization in the presence of MitoQ occurs in a dose dependent manner in both cell types (Figure 2.8). These results are corroborated with the observation that EUK-134, a mimetic of both catalase and superoxide dismutase, inhibits hypoxic stabilization of HIF-1α protein in both the wild type and the Δ cytb cybrids (Figure 2.9).
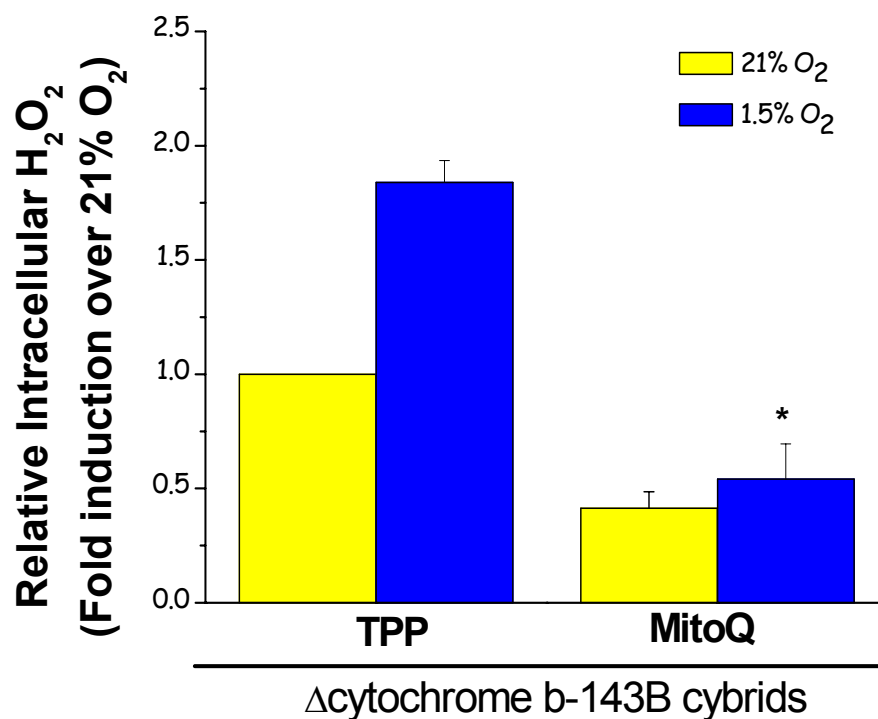
Figure 2.6 Mitochondrial targeted antioxidant MitoQ prevents hypoxic increase in intracellular H_2O_2. **(A)** Intracellular H_2O_2 levels were measured in Δ Cytb cybrids using Amplex Red in the presence of MitoQ (1uM) or control compound TPMP (1uM) for 4 hrs. . n=4 (mean $\pm$S.E.M) *$p<0.05$ (TPMP hypoxia samples compared to MitoQ hypoxia samples).

A

ΔCytb Cybrid

TPMP   MitoQ

N   H   D   N   H   D

HIF-1α

-120Kd

Tubulin

-50Kd

B

WT Cybrids

TPMP   MitoQ

N   H   D   N   H   D

HIF-1α

-120Kd

Tubulin

-50Kd
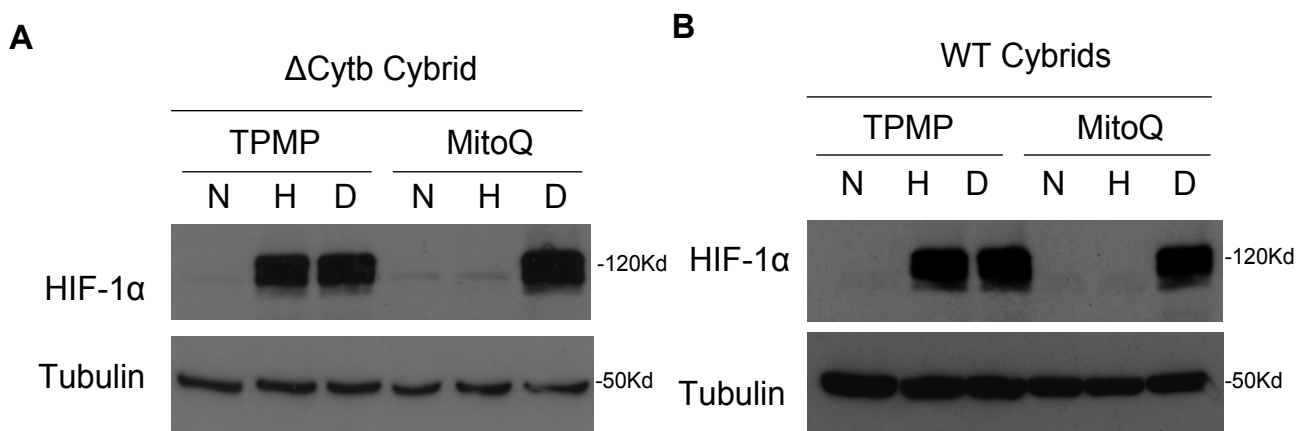
Figure 2.7 Mitochondrial targeted antioxidant MitoQ prevents hypoxic stabilization of HIF-1α protein. HIF-1α protein levels of whole cell lysates from **(A)** Wt cybrids and **(B)** ΔCytb cybrids pre-incubated with MitoQ (1uM) or control compound TPMP (1uM) for 4 hrs and then subjected to either 21% O_2 (N), 1.5% O_2 (H), or 1mM DMOG (D) for 4 hrs. Representative blot from four independent experiments.
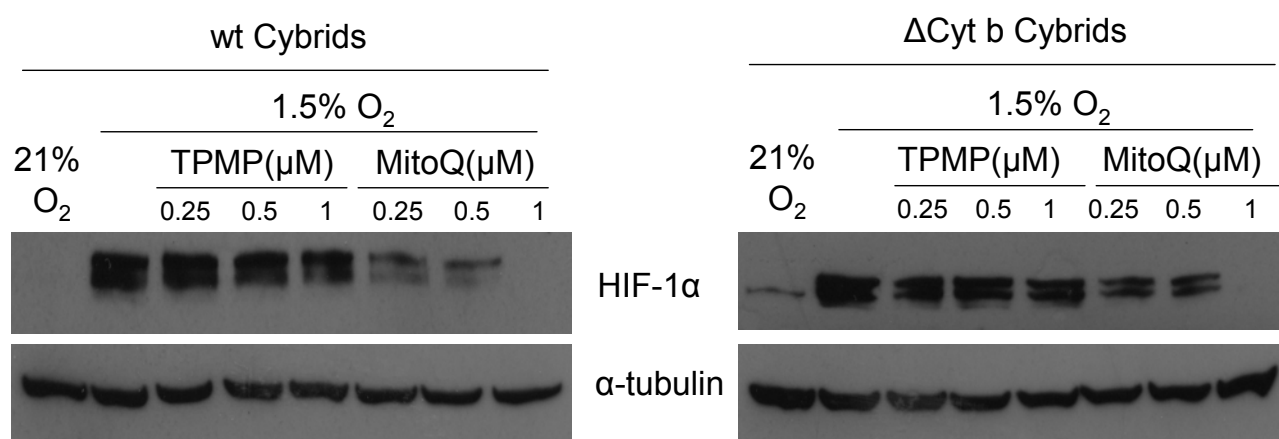
Figure 2.8 HIF-1α protein levels in **(A)** wt and **(B)** Δ Cytb cybrids subjected to 21%O_2 (N), 1.5%O_2 (H), or 1mM DMOG (D) for 4 hours at various concentrations of MitoQ. Representative blot from two independent experiments.

A Wt Cybrid

– Euk-134

N H D N H D

B ΔCytb Cybrid

– Euk-134

N H D N H D

HIF-1α

α-tubulin
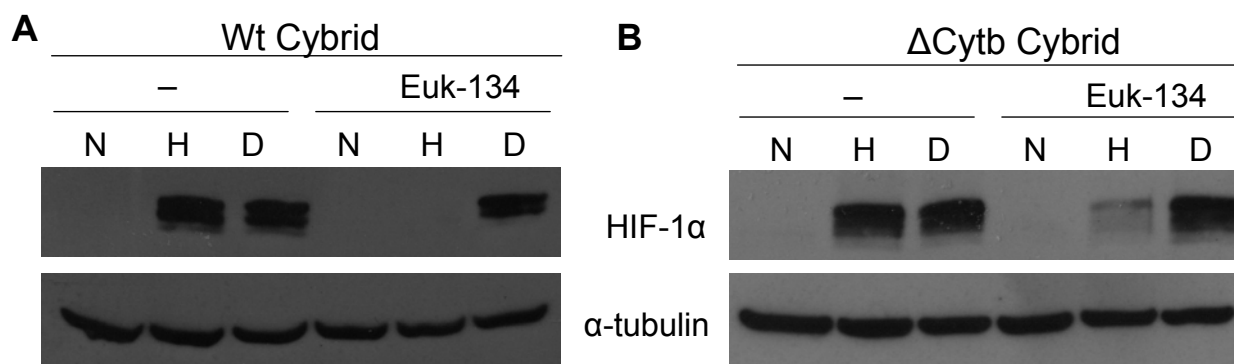
Figure 2.9 Cytosolic antioxidant EUK-143 prevents hypoxic stabilization of HIF-1α protein. HIF-1α protein levels of whole cell lysates from **(A)** Wt cybrids and **(B)** ΔCytb cybrids pre-incubated with EUK-134 (10 uM) for 2 hrs and then subjected to either 21% O_2 (N), 1.5% O_2 (H), or 1mM DMOG (D) for 4 hrs. Representative blot from three independent experiments.

Hypoxic stabilization of HIF-1α protein requires generation of ROS from the Q_o site of the mitochondrial complex III.

To further validate that the mitochondrial electron transport chain is required for hypoxic stabilization of the HIF-1α protein in the Δ cytb cybrids, we utilized shRNA against the mitochondrial transcription factor A (TFAM). The mitochondrial transcription factor A is required for the proper transcription and replication of mitochondrial DNA (Ekstrand et al., 2004; Larsson et al., 1998). In the absence of TFAM, cells become depleted of their mitochondrial DNA (ρ^0 cells) (Larsson et al., 1998). Expression of TFAM shRNA in the Δ cytb cybrids lowered TFAM mRNA expression and mitochondrial copy number by 75% compared to cells expressing the control shRNA against *D. melonogaster* HIF (dHIF) (Figure 2.10). The cell containing TFAM shRNA were unable to increase cytosolic ROS in hypoxic conditions (Figure 2.11). Furthermore, their ability to stabilize HIF-1α protein under hypoxia was attenuated (Figure 2.12). These data indicate that mitochondrial electron transport having an important role in hypoxic stabilization of HIF-1α protein.
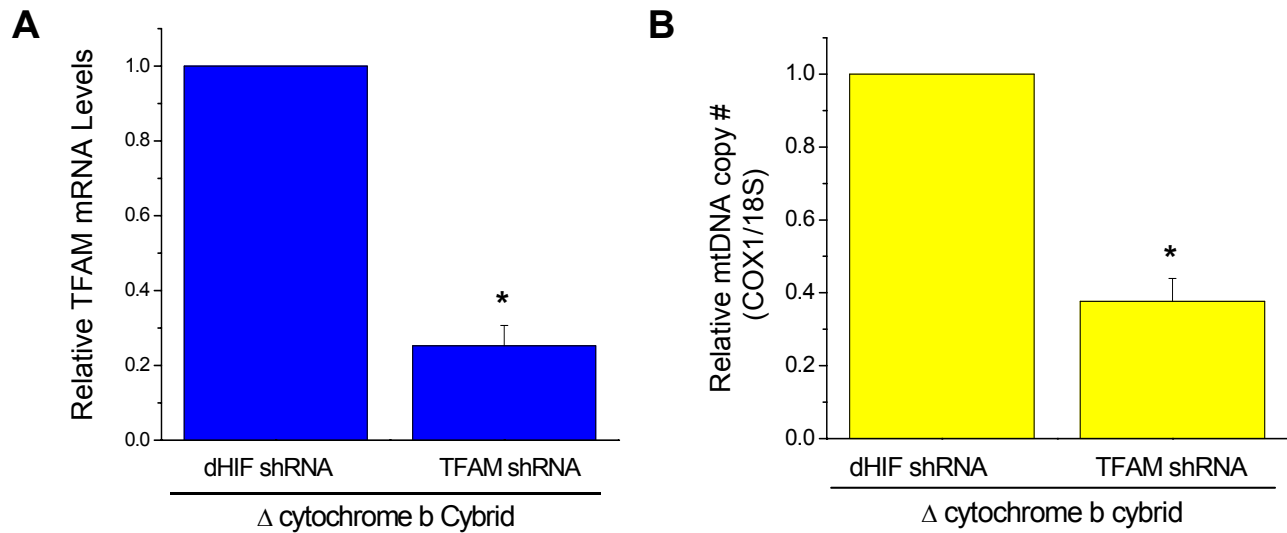
Figure 2.10 shRNA targeting TFAM decreases TFAM mRNA and mitochondrial copy number. Quantitative real-time PCR of cDNA **(A)** or total DNA **(B)** generated from ΔCytb cybrids stably expressing shRNA for *D. Melanogaster* HIF (dHIF) or TFAM. n=3 (mean ±S.E.M) *p<0.05 (dHIF shRNA compared with TFMA shRNA).
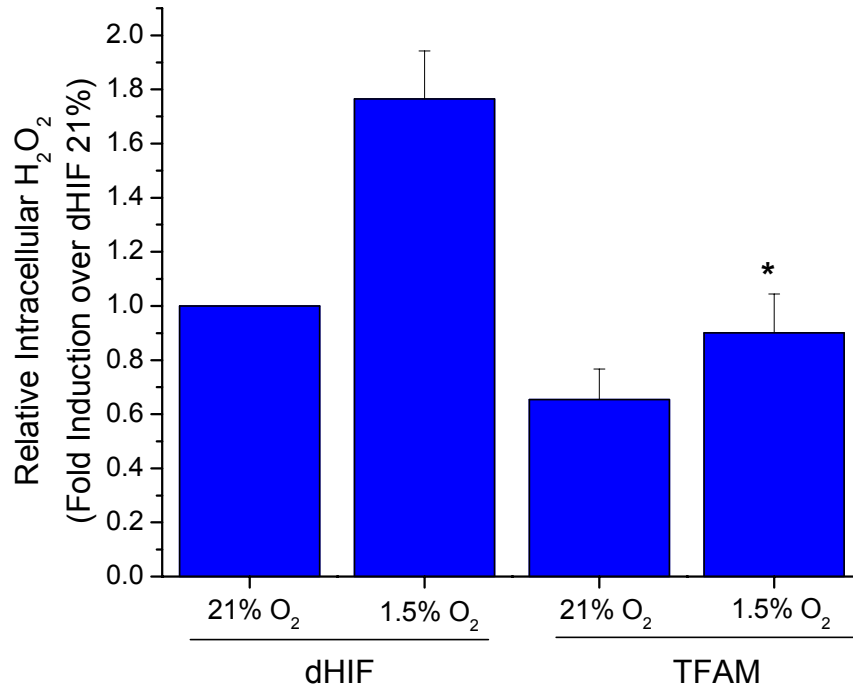
Figure 2.11 Hypoxic increase of cytosolic H_2O_2 is attenuated in cells expressing a shRNA targeting TFAM. Intracellular H_2O_2 levels measured with Amplex Red in ΔCytb cybrids stably expressing shRNA for either *D. melanogaster* HIF or TFAM exposed to either $21\%O_2$ or $1.5\%O_2$ for 4 hours. n=4 (mean ±S.E.M) *p<0.05 (hypoxic dHIF shRNA compared with hypoxic TFMA shRNA).
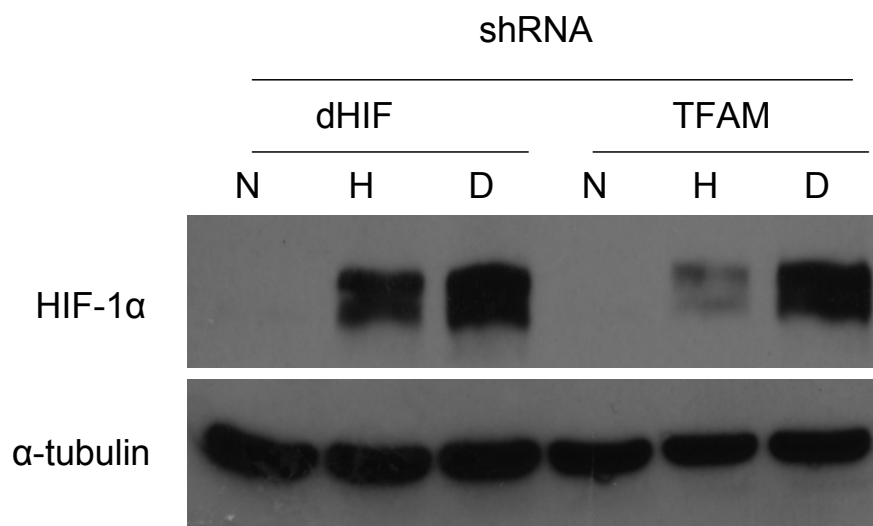
Figure 2.12 RNAi of TFAM diminishes hypoxic stabilization of HIF-1α protein. HIF-1α protein levels from whole cell lysates of ΔCytb cybrids stably expressing shRNA for either *D. Melanogaster* HIF (dHIF) or TFAM, exposed to 21% O2 (N), 1.5% O2 (H), or 1mM DMOG (D) for 4 hours. Representative blot from three independent experiments.

To determine whether ROS generation from the Q_o site is responsible for the increase in cytosolic ROS and stabilization of the HIF-1α protein, the Δ cytb cybrid cells were stably infected with retrovirus containing shRNA against the Rieske Fe-S protein. In the absence of the Rieske Fe-S protein, the Q-cycle is not initiated and ROS are not generated at the Q_o site. It is theoretically possible that the Q_i site might generate ROS. However, activity of the Q_i site is abolished in cells deficient in the cytochrome b protein (Figure 2.1). Therefore, the data presented in Figure 2.3 and 2.4 indicate that the Q_i site is dispensable for hypoxic increase in cytosolic ROS and stabilization of HIF-1α. Stably expressing a shRNA against the Rieske Fe-S protein in the Δ cytb cybrid cells decreases expression of the Rieske Fe-S protein (Figure 2.13A). These cells were not able to increase cytosolic ROS under hypoxic conditions (Figure 2.13B). Furthermore, shRNA Fe-S cells do not stabilize the HIF-1α protein when exposed to hypoxia, but they do retain HIF-1α protein stabilization in the presence of DMOG (Figure 2.14).

A

B
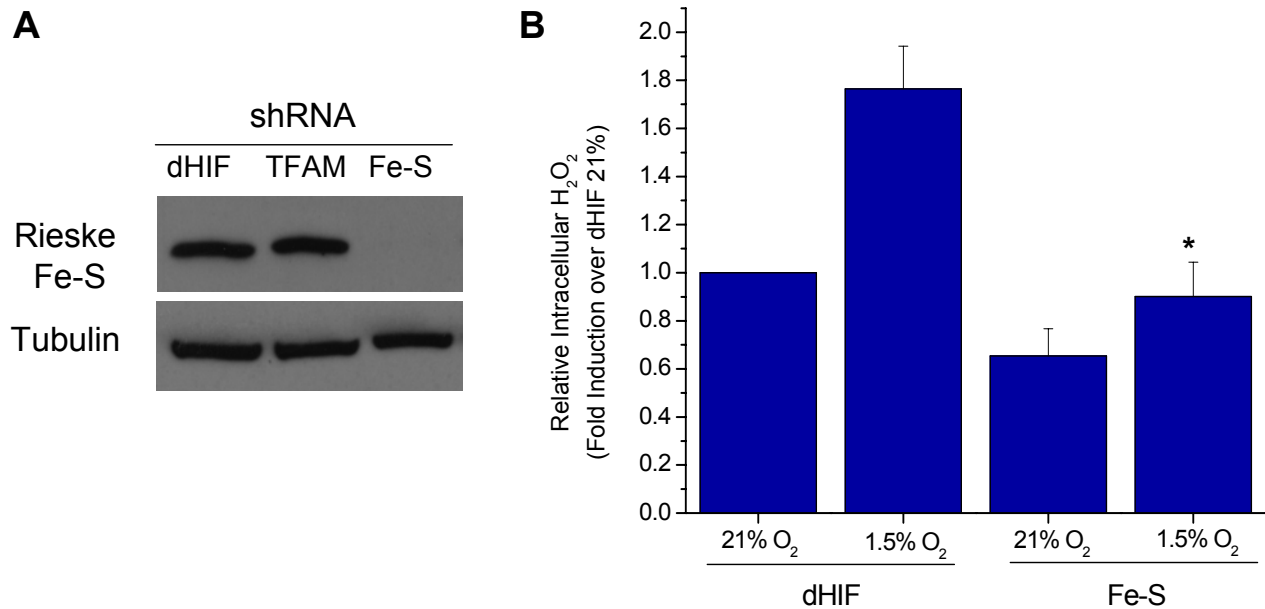
Figure 2.13 Electron transfer at the Q_o site of the bc1 complex is necessary for hypoxic generation of ROS. **(A)** Immunoblot analysis for Rieske Fe-S protein of whole cell lysates from ΔCytb cybrids stably expressing shRNA for either *D. Melanogaster* HIF (dHIF), TFAM, or Rieske Fe-S protein (FE-S). **(B)** Intracellular H_2O_2 levels measured with Amplex Red in ΔCytb cybrids stably expressing shRNA for either *D. melanogaster* HIF, TFAM, or Rieske Fe-S protein exposed to either $21\%O_2$ or $1.5\%O_2$ for 4 hours. n=4 (mean ±S.E.M) *$p<0.05$ (hypoxic dHIF shRNA compared with hypoxic Rieske Fe-S shRNA).
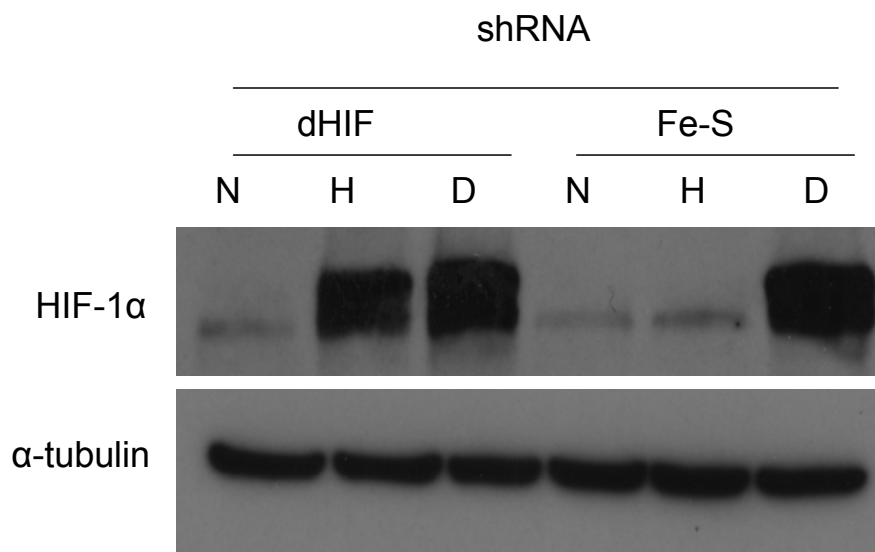
Figure 2.14 Electron transfer at the Q_o site of the bc_1 complex is necessary for hypoxic stabilization of HIF-1α protein. HIF-1α protein levels from whole cell lysates of ΔCytb cybrids stably expressing shRNA against either *D. Melanogaster* HIF (dHIF) or Rieske Fe-S protein (FE-S) exposed to 21% O_2 (N), 1.5% O_2 (H), or 1mM DMOG (D) for 4 hours. Representative blot from four independent experiments.

To ensure that our results were not due to any adaptation to the loss of cytochrome b protein or shRNA against Rieske Fe-S protein, we corroborated our genetic findings in wild-type cells using well established pharmacological inhibitors of complex III. Incubating wild-type cells with the complex III inhibitor stigmatellin, which binds to the Q_o site, inhibits hypoxic stabilization of HIF-1α protein (Figure 2.15). By contrast, the complex III inhibitor Antimycin A, which preserves the ROS generation at Q_o site of complex III, did not decrease hypoxic stabilization of HIF-1α in the wild type cybrids (Figure 2.16). Addition of antimycin does increase ROS produced by the Q_o site, however these levels are not sufficient to stabilize HIF-1α protein. Collectively these data implicate the Q_o site of complex III as the primary site of ROS generation for hypoxic stabilization of HIF-1α protein.
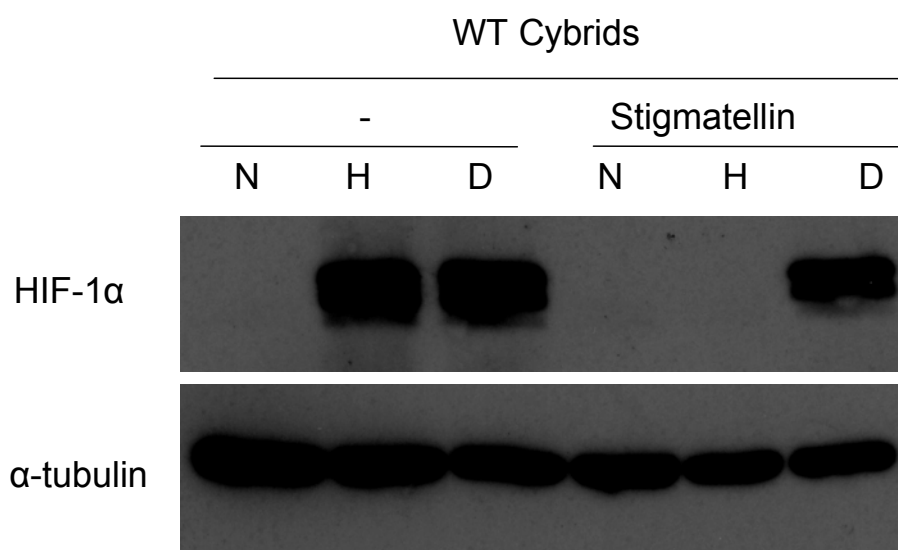
Figure 2.15 Hypoxic HIF-1α protein stability is attenuated by pharmacological inhibitor of the Q_o site in wild-type cells. HIF-1α protein levels of whole cell lysates from WT cybrids incubated with stigmatellin (1uM) and subjected to either 21% O_2 (N), 1.5% O_2 (H), or 1mM DMOG (D) for 4 hrs. Representative blot from three independent experiments.
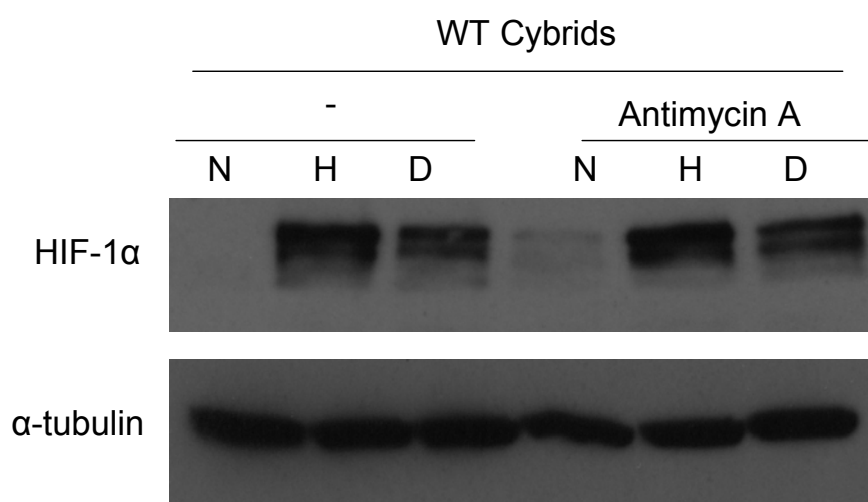
Figure 2.16 Hypoxic HIF-1α protein stability is not attenuated by pharmacological inhibitor of the Q_i site in wild-type cells. HIF-1α protein levels of whole cell lysates from WT cybrids incubated with antimycin A (1 uM) and subjected to either 21% O_2 (N), 1.5% O_2 (H), or 1 mM DMOG (D) for 4 hrs. Representative blot from three independent experiments.

ROS released from the Q_o site is partitioned to the cytosol by hypoxia.

The data presented above demonstrate that the Q_i site is not involved in the generation of ROS in hypoxic conditions and that the Q_o site is the source of ROS. However, this data does not demonstrate how cytosolic ROS are increased. It is possible that the observed increase in cytosolic ROS is a result of increased generation of $Q^.$ at the Q_o site. $Q^.$ passes its electron to molecular oxygen to generate ROS in the intermembrane space of the mitochondria. Therefore, an increase in the generation of $Q^.$ would result in an increase in ROS generated at the Q_o site. The ROS made at complex III in normal oxygen conditions is equally distributed to the intermembrane space and the mitochondrial matrix (Muller et al., 2004). An alternative explanation to the increase in cytosolic ROS observed in hypoxic conditions is that hypoxia induces a shift in the distribution of ROS made by the Q_o site to the intermembrane space. To test this hypothesis, we incubated cells with dihydroethidium (DHE) or MitoSOX Red at 21% O_2 or 1.5% O_2 with or without antimycin a. DHE detects ROS in the cytosol of cells and MitoSOX Red is the mitochondrial targeted version of DHE, therefore we can determine the localization of ROS in hypoxic conditions. Cytosolic ROS are increased by 1.5% O_2 or antimycin a alone (Figure 2.17A). Interestingly, cells exposed to hypoxia in the presence of antimycin a display increased cytosolic ROS compared to 1.5% O_2 or antimycin a alone. ROS localized to the matrix, as measured by MitoSOX Red, is increased in the presence of antimycin a (Figure 2.17B). However, cells exposed to 1.5% O_2 alone or in combination with antimycin a, have decreased levels of ROS in the matrix. These data imply that hypoxia shifts the localization of ROS generated by the Q_o site from the matrix to the intermembrane space (Figure 2.18)

A

Cytosolic ROS

B

Matrix ROS
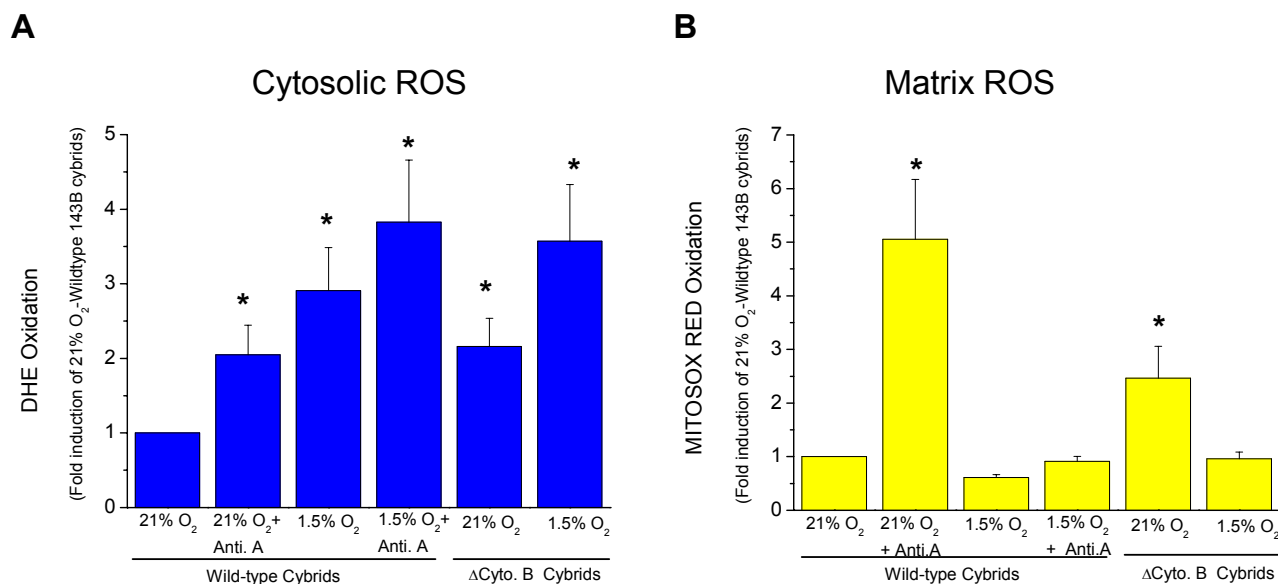
Figure 2.17 Hypoxia induces a shift in the localization of ROS generated by the Q_o from the matrix to the cytosol. **(A)** Cytosolic ROS measured in live cells with DHE exposed to 21% O_2 or 1.5% O_2 with or without antimycin a. N=3 one-way ANOVA analysis followed by paired t test. **(B)** ROS localized to the matrix measured in live cells with MitoSOX Red exposed to 21% O2 or 1.5% O2 with or without antimycin a. N=3 one-way ANOVA analysis followed by paired t test.
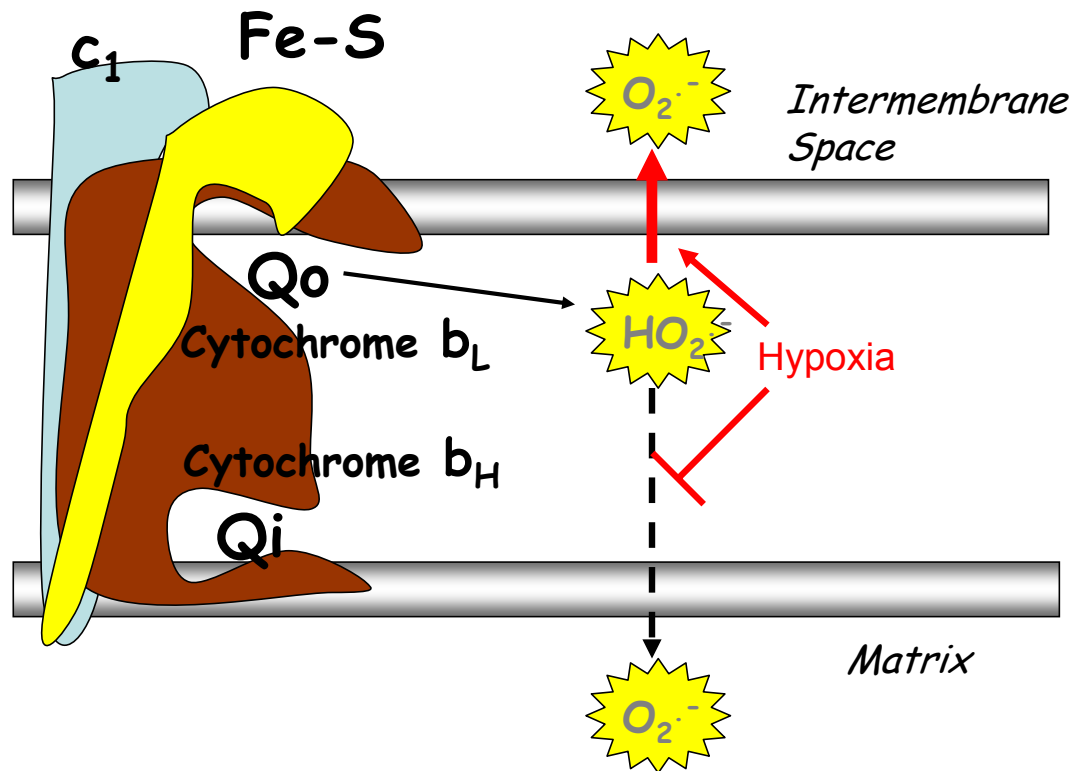
Figure 2.18 Hypoxia changes the localization of ROS generated by the Q_o site. ROS generated by the Q_o site is normally equally distributed to the intermembrane space and the matrix. Hypoxia induces a shift in the localization of ROS to be partitioned to the intermembrane space.

ROS regulate hydroxylation of HIF-1α protein.

Under normal oxygen conditions, HIF-1α protein is hydroxylated by the PHDs, thereby facilitating ubiquitination and subsequent proteasomal degradation. Exogenous ROS are sufficient to stabilize HIF-1α protein under normal oxygen conditions (Chandel et al., 2000). Using an antibody that specifically recognizes HIF-1α protein hydroxylated on proline 564, we demonstrate that ROS inhibit the ability of the PHDs to hydroxylate HIF-1α protein. Quenching the increase in cytosolic ROS under hypoxia with MitoQ recovers hydroxylation of HIF-1α protein in both the wild type (Figure 2.19) and Δ cytb cybrids (Figure 2.20). To test whether exogenous ROS are sufficient to prevent hydroxylation of the HIF-1α protein, cells were exposed to glucose oxidase, an enzyme that generates H_2O_2. Addition of glucose oxidase (10 μg/mL) increases intracellular ROS to levels that are similar to those measured in hypoxic conditions (Figure 2.21). These levels of ROS generated in normal oxygen conditions are sufficient to stabilize HIF-1α protein (Figure 2.22). Addition of the antioxidant protein catalase in this experiment inhibits stabilization of HIF-1α protein, indicating that H_2O_2 is responsible for the stabilization of the HIF-1α protein. The presence of glucose oxidase attenuates hydroxylation of HIF-1α protein as assessed by reactivity with the hydroxylation specific antibody (Figure 2.23). The addition of catalase in the presence of glucose oxidase under normal oxygen conditions recovers hydroxylation of HIF-1α protein indicating that the ability of the PHDs to hydroxylate HIF-1α protein is indeed regulated by ROS.
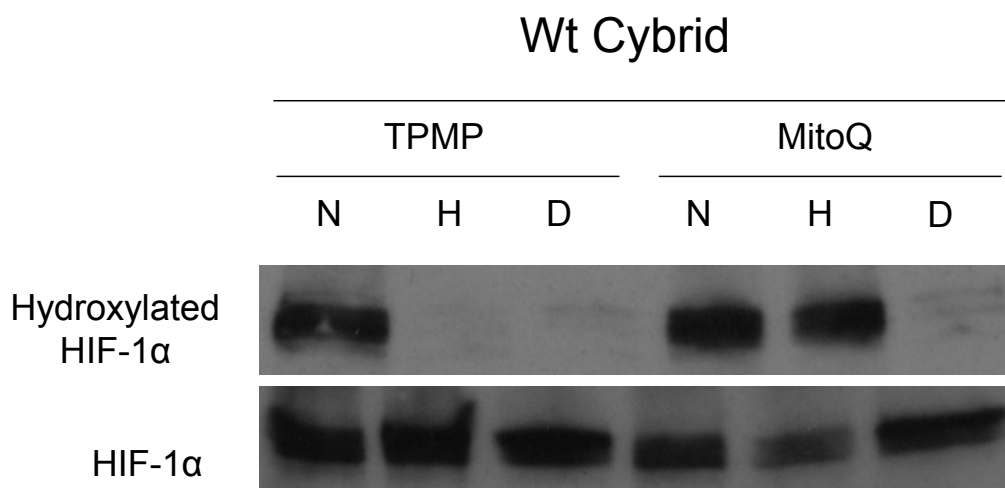
Wt Cybrid

Figure 2.19 Hypoxic increase in cytosolic ROS generated from the mitochondria inhibits hydroxylation of HIF-1α protein. Immunoblot analysis of whole cell lysates for hydroxylated HIF-1α protein from Wt cybrids treated with 20μM MG132 to stabilize HIF-1α protein pre-incubated with MitoQ (1uM) or control compound TPMP (1uM) for 4 hrs and then subjected to either 21% O_2 (N), 1.5% O_2 (H), or 1mM DMOG (D) for 4 hrs. Representative blot from three independent experiments.

ΔCytb Cybrid

	TPMP			MitoQ		
	N	H	D	N	H	D

Hydroxylated HIF-1α

HIF-1α
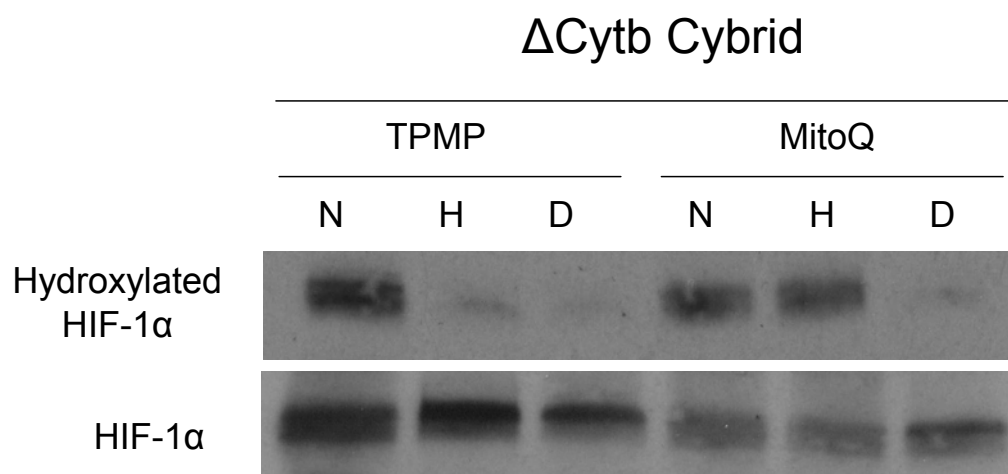
Figure 2.20 Hypoxic increase in cytosolic ROS generated from the Q_o site of complex III inhibits hydroxylation of HIF-1α protein. Immunoblot analysis of whole cell lysates for hydroxylated HIF-1α protein from ΔCytb cybrids treated with 20µM MG132 to stabilize HIF-1α protein pre-incubated with MitoQ (1uM) or control compound TPMP (1uM) for 4 hrs and then subjected to either 21% O_2 (N), 1.5% O_2 (H), or 1mM DMOG (D) for 4 hrs. Representative blot from three independent experiments.
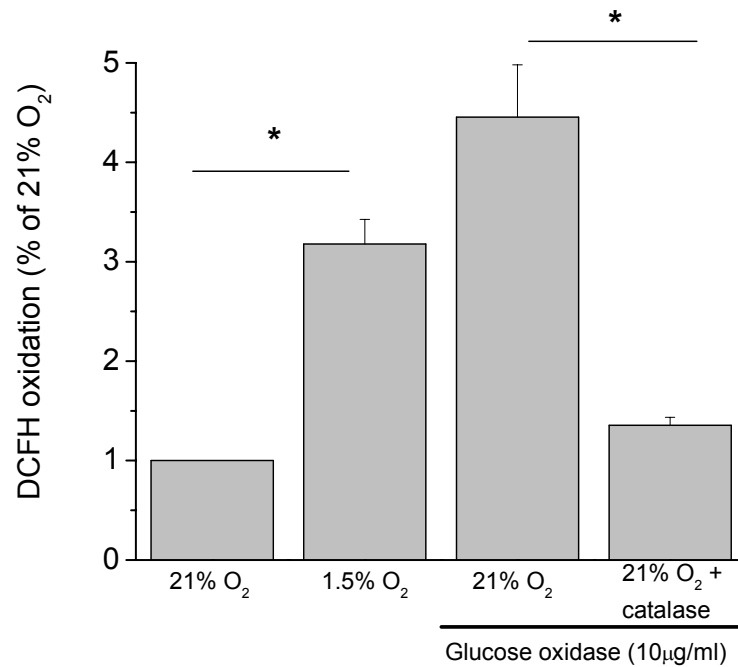
Figure 2.21 Glucose oxidase increase intracellular ROS. Intracellular ROS measured by DCFH in WT cybrids subjected to either, 21% O_2 (N), 1.5% O_2 (H), 21% O_2 plus glucose oxidase (10 μg/ml), or 21% O_2 plus glucose oxidase and catalase for 2 hrs. n=4 (mean $\pm$S.E.M) *p<0.05 (normoxia compared to hypoxia or normoxia+glucose oxidase compared to normoxia+glucose oxidase +catalase). n=4 (mean $\pm$S.E.M) *p<0.05 (normoxia compared to hypoxia or normoxia+glucose oxidase compared to normoxia+glucose oxidase +catalase).
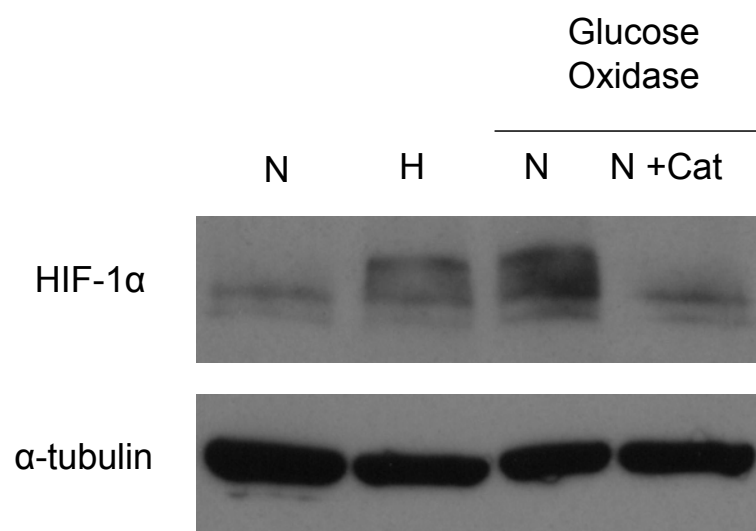
Figure 2.22 ROS are sufficient to stabilize HIF-1α protein. Immunoblot analysis of whole cell lysates for HIF-1α protein from WT cybrids subjected to either, 21% O_2 (N), 1.5% O_2 (H), 21% O_2 plus glucose oxidase, or 21% O_2 plus glucose oxidase and catalase for 2 hrs. Representative blot from three independent experiments.
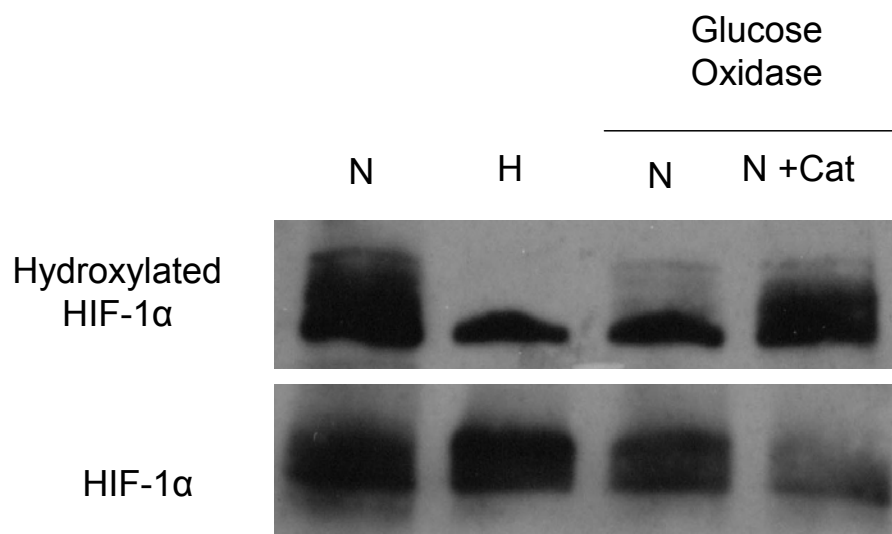
Figure 2.23 ROS are sufficient to prevent hydroxylation of HIF-1α protein. Immunoblot analysis for hydroxylated HIF-1α protein from whole cell lysates of wt cybrids treated with 20µM MG132 subjected to either, 21% O_2 (N), 1.5% O_2 (H), 21% O_2 plus glucose oxidase, or 21% O_2 plus glucose oxidase and catalase for 2 hrs. Representative blot from three independent experiments.

Cytochrome c reduction is not sufficient to stabilize HIF under hypoxia.

Previous results indicate that loss of cytochrome c prevents the hypoxic stabilization of the HIF-1α protein (Mansfield et al. 2005). To determine whether cytochrome c generated ROS are required for the hypoxic stabilization of the HIF-1α protein, we exposed ρ^0 cells to normoxia or hypoxia in the presence of *N,N,N*,*N*-tetramethyl-*p*-phenylenediamine (TMPD). ρ^0 cells lack a functional complex III and IV, resulting in a loss of electron flux through cytochrome c. TMPD donates electrons to cytochrome c, thereby fully reducing cytochrome c. The electrons from cytochrome c could then be donated to p66 Shc in the absence of a functional complex IV, resulting in ROS generation and HIF stabilization. Wild-type 143B or A549 cells stabilize the HIF-1α protein during hypoxia (1.5 % O_2) or in the presence of DMOG (Figure 2.24). By contrast, the 143Bρ^0 or A549ρ^0 cells do not stabilize HIF-1α protein during hypoxia. The addition of TMPD to either 143Bρ^0 or A549ρ^0 cells also did not stabilize the HIF-1α protein under normoxia (21% O_2) or hypoxic (1.5 % O_2) (Figure 2.25). This was not due to reduced levels of p66Shc or cytochrome c in the ρ^0 cells (Figure 2.26). TMPD did reduce cytochrome c levels and under these conditions it did not generate ROS (Figure 2.27). Previous results indicate that ROS due to electron transfer between cytochrome c and p66shc is observed only during DNA damage and may not be the normal physiological response in healthy cells (Giorgio et al. 2006). These results indicate that reduction of cytochrome c is not sufficient for hypoxic stabilization of HIF-1α protein.

A A549

N   H   D

HIF-1α

α-tubulin

B 143B

N   H   D

HIF-1α

α-tubulin
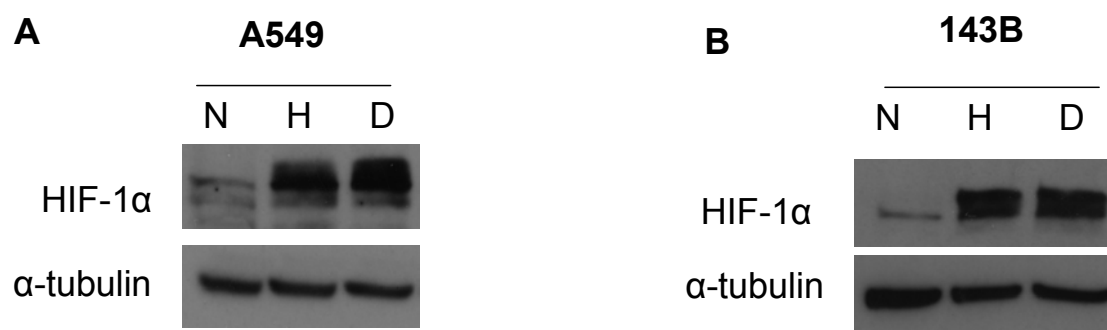
Figure 2.24 Reduced cytochrome c is not sufficient to stabilize the HIF-1α protein in A549 cells. **(A)** HIF-1α protein levels of A549 cells subjected to 21%O_2 (N), 1.5% O_2 (H) and 1mM DMOG (D) for 2 hours. Representative blot from three independent experiments. **(B)** HIF-1α protein levels of 143B cells subjected to 21%O2 (N), 1.5% O2 (H) and 1mM DMOG (D) for 2 hours. Representative blot from three independent experiments.
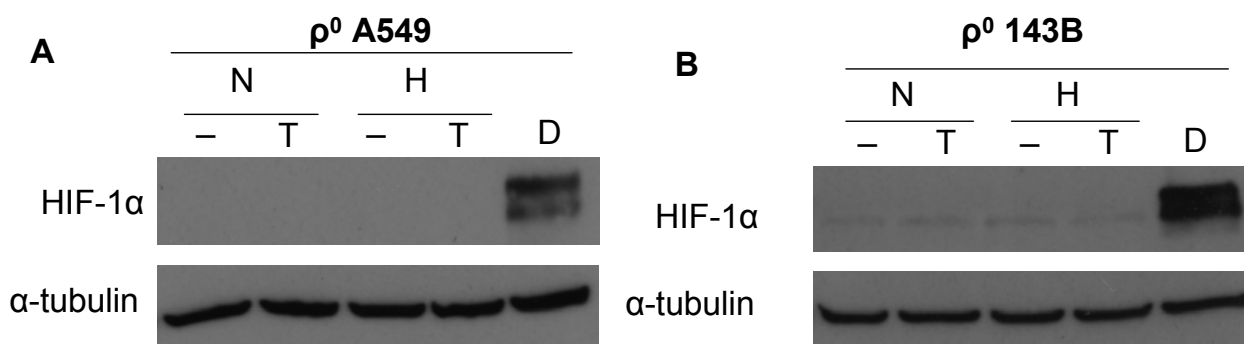
Figure 2.25 Reduced cytochrome c is not sufficient to stabilize the HIF-1α protein in 143B cells. **(A)** HIF-1α protein levels of A549 ρ⁰ whole cell lysates under 21% O_2 (N) or 1.5% O_2 (H) for 2 hours after a 15 minute pulse of the cytochrome c reducing agent TMPD (100 uM) and ascorbate (400 uM) (T) or DMOG (1mM, D). Representative blot from three independent experiments **(B)** HIF-1α protein levels of 143B ρ⁰ whole cell lysates under 21% O_2 (N) or 1.5% O_2 (H) for 2 hours after a 15 minute pulse of the cytochrome c reducing agent TMPD (100 uM) and ascorbate (400 uM) (T) or DMOG (1mM, D). Representative blot from three independent experiments

A A549

wt   ρ°

B 143B

wt   ρ°

Shc

Cytochrome c

α-tubulin
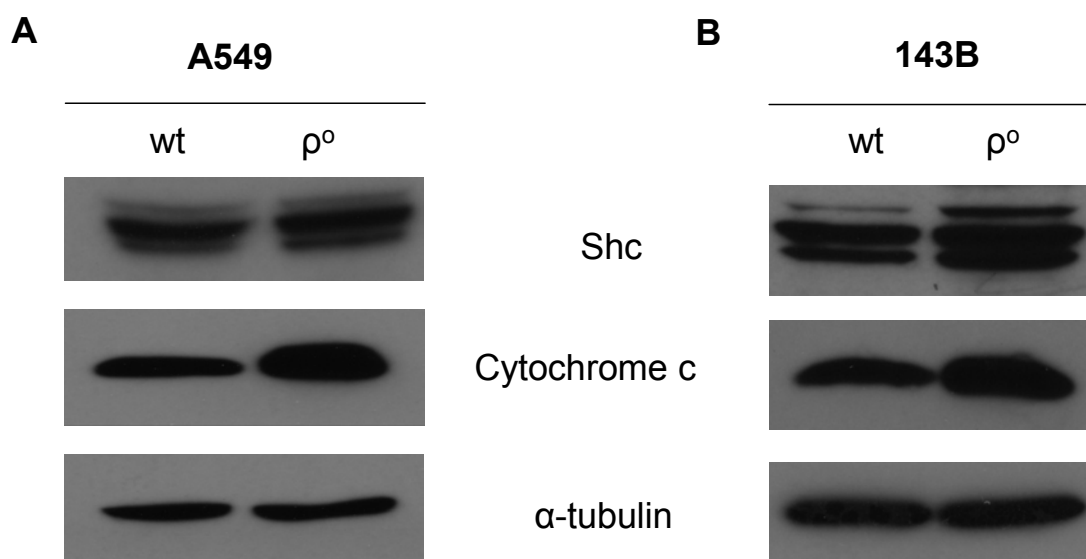
Figure 2.26 The inability of reduced cytochrome c to stabilize HIF-1α protein is not due to decreased p66 Shc protein. Cytochrome c and p66Shc protein levels in wt 143B, 143Bρ⁰ **(A)** and A549, A549 ρ⁰ **(B)** whole cell lysates. Representative blot from three independent experiments.

A

B
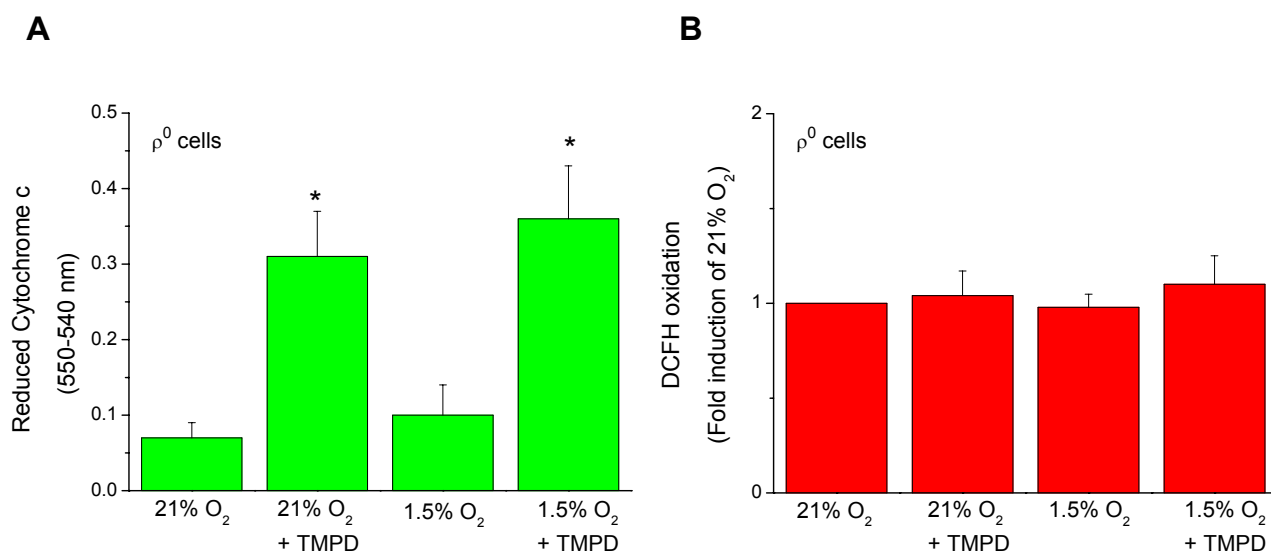
Figure 2.27 TMPD reduces cytochrome c but does not increase ROS. 143Bρ° cells were treated with TMPD/Ascorbate for 15 minutes. Subsequently, these cells were subjected to either 21% O_2 or 1.5% O_2. **(A)** Cytochrome redox state in isolated mitochondria was measured. N=3 **(B)** intracellular ROS were measured via DCFH oxidation in whole cells.

Discussion

Mammalian cells transduce signals that couple decreases in oxygen levels to initiate HIF dependent gene expression. The mechanism of how sense decreased oxygen is not fully understood. Mitochondrial electron transport chain has been proposed to be part of the cellular oxygen sensor. Indeed, previous genetic evidence indicates that loss of cytochrome c or the Rieske Fe-S protein prevents the hypoxic stabilization of HIF-1α, indicating that these proteins are involved in the increase in cytosolic ROS during hypoxia (Brunelle et al., 2005a; Guzy et al., 2005; Mansfield et al., 2005a). RNAi of the Rieske Fe-S protein prevents electron flux through complex III as well as cytochrome c, resulting in a loss of ROS generation at complex III and cytochrome c. Cells devoid of cytochrome c gene would also have loss of electron flux through cytochrome c as well as complex III, preventing ROS generation at both complex III and cytochrome c. In this Chapter, we demonstrate that cytochrome c is not the primary site of ROS generation in hypoxia. We demonstrate that fully reducing cytochrome c levels is not sufficient to stabilize the HIF-1α protein during hypoxia or normoxia. Therefore, cytochrome c does not contribute to hypoxic signal transduction through its ability to generate ROS via p66Shc.

Our data indicate that the Q_o site of complex III is part of the oxygen sensing machinery. Cells deficient in cytochrome b protein are able to generate ROS at the Q_o site of complex III. During hypoxia, these cells stabilize the HIF-1α protein. Preventing ROS generation at the Q_o site in the cytochrome b deficient cells with MitoQ or by shRNA against the Rieske Fe-S protein prevents the increase in cytosolic ROS and stabilization of the HIF-1α protein during hypoxia. The data presented in this Chapter

also demonstrate that ROS regulate the ability of the PHDs to hydroxylate HIF in both normoxic and hypoxic conditions. Quenching ROS with MitoQ in hypoxic conditions allows for continued hydroxylation of HIF-1α protein, while addition of exogenous ROS in normal oxygen conditions inhibits the ability of the PHDs to hydroxylate HIF-1α protein. These data demonstrate that the Q_o site of the bc_1 complex participates in oxygen sensing by producing ROS that initiate HIF mediated transcriptional responses that facilitate cellular adaptation to low oxygen. Interesting, the data presented here implies that hypoxia does not actually increase the generation of ROS. Instead, hypoxia alters the sub-cellular localization of the ROS generated by the Q_o site. Further investigation is required to determine exactly how hypoxia alters the partioning of these ROS

The data presented in this Chapter are in contrast with other groups that have proposed that the ability of mitochondria to consume oxygen is the major requirement for stabilization of the HIF-1α protein in hypoxic conditions. Their model proposes that respiring mitochondria generate an oxygen gradient, preventing hydroxylation, and thereby increasing stabilization of the HIF-1α protein (Doege et al., 2005; Hagen et al., 2003). According to this model, in the absence of a functioning respiratory chain, the oxygen gradient would be reduced resulting in hydroxylation and degradation of the HIF-1α protein. However, the cytochrome b null cells are respiratory incompetent, and therefore unable to generate an oxygen gradient. Contrary to this model, these cells still retain the ability to stabilize the HIF-1α protein during hypoxia. Furthermore, the mitochondrial targeted antioxidant MitoQ or the cytosolic antioxidant EUK-134 prevents stabilization of the HIF-1α protein in the cytochrome b deficient cells indicating ROS

involvement in HIF stabilization. MitoQ has been previously shown to prevent hypoxic stabilization of the HIF-1α protein in respiratory competent cells, demonstrating the importance of ROS in HIF-1α protein stabilization (Sanjuan-Pla et al., 2005). However, there are instances when an oxygen gradient created by the mitochondria during normoxia can create a hypoxic environment within cells causing HIF-1α protein accumulation (Doege et al., 2005). For example, if metabolically active cells are cultured at high confluency their demand for oxygen exceeds the supply of oxygen resulting in a local hypoxia. Under these conditions, respiratory inhibition would result in restoration of the oxygen levels to normoxia within the cells, resulting in the degradation of the HIF-1α protein. Cells that are cultured at a high confluency under hypoxia (1-2 % O_2) would experience anoxia (0 % O_2). Under these conditions respiratory inhibition would result in restoration of oxygen levels only to the hypoxic levels. If respiratory inhibition does not result in attenuating ROS generation, such as in the cytochrome b deficient cells, then cells would still be able to stabilize the HIF-1α protein in conditions of high confluency under hypoxia. Collectively, our data indicate that the ability of mitochondria to generate ROS and not an oxygen gradient is required for the stabilization of the HIF-1α protein during hypoxia.

In summary, Chapter 2 demonstrates that the Q_o site of complex III is necessary to increase cytosolic ROS in hypoxic conditions, which results in the inhibition of the ability of the PHDs to initiate degradation of HIF-1α protein (Figure 2.29). Furthermore, we present data indicating that the hypoxic increase in cytosolic ROS is actually due to altering the partioning of the ROS generated from the Q_o site from the matrix to the intermembrane space. We also conclusively demonstrate that the ability to consume

oxygen by mitochondria is not required for hypoxic stabilization of the HIF-1α protein.

The link between ROS and the PHDs is currently unknown. As oxygen levels fall, the

enzymatic activity of PHDs decrease (Schofield and Ratcliffe, 2004). It is possible that

the link could be an oxidant dependent signaling pathway in which a post-translational

modification of the PHDs, such as phosphorylation, turns off the catalytic activity. In fact

previous studies have implicated multiple signaling molecules that are required for

hypoxic activation of HIF-1 (Aragones et al., 2001; Emerling et al., 2005; Hirota and

Semenza, 2001; Hui et al., 2006; Turcotte et al., 2003). Alternatively, the link between

ROS and the PHDs could be due to changes in the cytosolic redox state. The ROS

may induce a shift in iron redox state from Fe^{+2} to Fe^{+3} due to the Fenton reaction,

thereby limiting an essential co-factor of the PHDs resulting in an inhibition of

hydroxylation of HIF protein (Gerald et al., 2004). It could also be that the low oxygen

levels decrease PHD activity and the ROS produced during hypoxia further decrease

PHD activity to prevent hydroxylation of HIFα protein. Furthermore, multiple factors

affecting cellular redox state and metabolism are likely to affect hydroxylation of the

HIF-1α protein (Pan et al. 2006). Our study also suggests that the targeting of

mitochondrial ROS could serve as a therapeutic target for many HIF dependent

pathological processes, including cancer. It will be of interest in future studies to

examine whether the Q_o site of complex III serves as part of a signal transduction

machinery for other hypoxia initiated cellular events, such as calcium signaling.
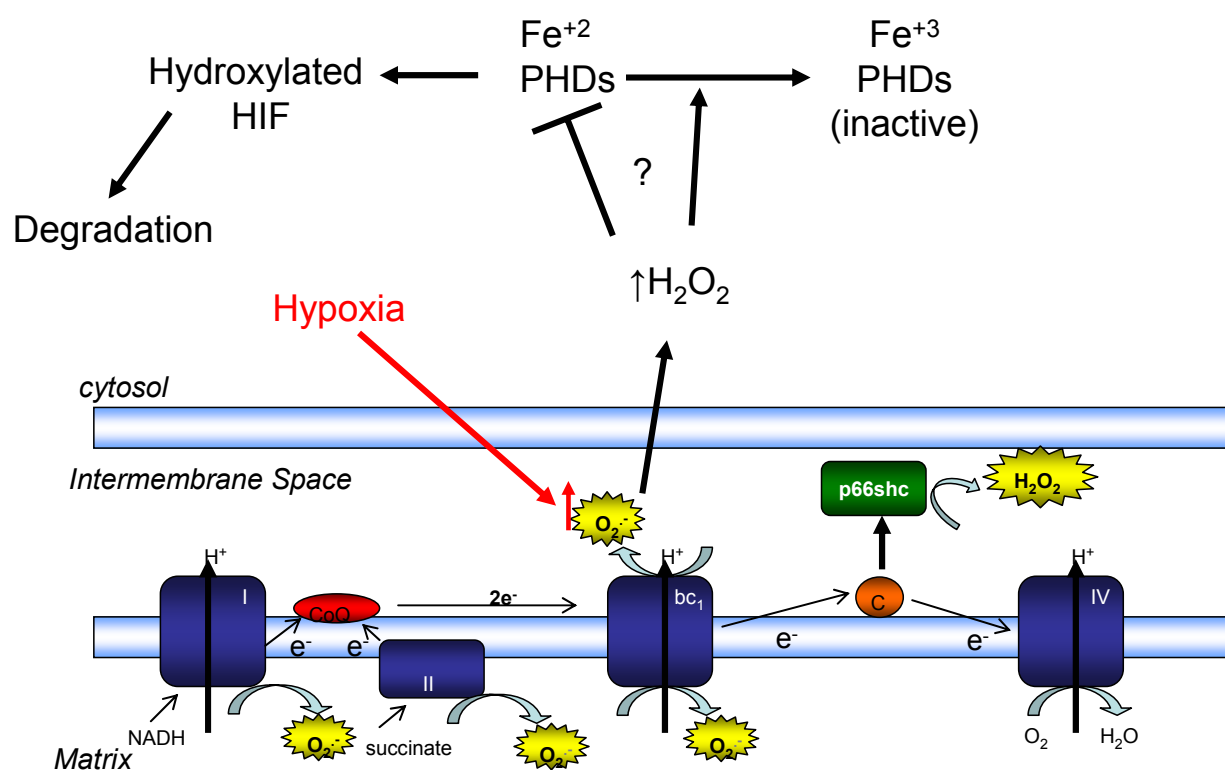
Figure 2.28 Schematic model of mitochondrial generated ROS stabilization of HIF-1α in hypoxic conditions. Hypoxia increases generation of ROS from the Q_o site of the bc_1 complex. These ROS are released into the inter-membrane space and enter the cytosol to decrease PHDs activity, thus preventing hydroxylation of the HIF-1α protein. We speculate ROS decrease the PHDs activity from a combination of a post-translational modification of the PHDs, such as phosphorylation or decreasing the availability of Fe (II) which is required for hydroxylation to occur.

Chapter 3: Mitochondrial ROS trigger HIF-dependent extension of replicative lifespan during hypoxia.

Introduction

Human cells have a finite capacity to replicate, and after a critical number of cell divisions, they reach a state in which further division cannot occur, termed replicative senescence (Hayflick, 1965).   Multiple mechanisms are postulated to explain the process of replicative senescence, including telomere attrition and accumulation of oxidative damage to DNA, lipids, and proteins from free radicals (Beckman and Ames, 1998; McEachern et al., 2000).   The latter hypothesis is known as the free radical theory. A seminal observation to support the free radical theory was made thirty years ago by Packer and Fuehr, who demonstrated that low oxygen concentration (hypoxia) extends replicative lifespan of cultured primary human fibroblasts (Packer and Fuehr, 1977).   The canonical interpretation of this finding is that hypoxia decreases the generation of reactive oxygen species (ROS) due to limiting oxygen levels. This results in diminished oxidative damage to DNA, lipids, and proteins, thus prolonging replicative senescence and extending the lifespan of cells. However, Chapter 2 of this dissertation clearly demonstrates that cytosolic ROS paradoxically increase under hypoxia. Therefore, the increase of replicative lifespan observed during hypoxia is not consistent with the free radical theory.

The source of the increased ROS generated under hypoxia is the mitochondria, and it has previously been demonstrated that these ROS are both necessary and sufficient to activate the transcription factor hypoxia inducible factor (HIF) (Chandel et al., 1998; Chandel et al., 2000). Hypoxia-inducible factor (HIF) is a transcription factor

that regulates physiological responses to hypoxia, including placental development, and pathophysiological processes such as cancer (Semenza, 2000).   HIF is a basic helix-loop-helix transcription factor comprised of the constitutively stable HIF-β/ARNT subunit and the oxygen regulated HIF-α subunit. In normal oxygen conditions, HIF-α is hydroxylated at two proline residues within the oxygen-dependent degradation domain (ODDD) by a family of prolyl hydroxylase enzymes (PHDs). The hydroxylated prolines are recognized by the Von Hippel Lindau tumor suppressor protein (pVHL), the recognition component of the VBC E3 ligase, which targets the alpha subunit for rapid ubiquitin-mediated proteasomal degradation (Safran and Kaelin, 2003).   Under hypoxic conditions, the generation of mitochondrial ROS prevents the hydroxylation of HIF-α, thereby stabilizing HIF-α and allowing it to translocate to the nucleus and dimerize with HIF-1β to initiate transcription of target genes (Brunelle et al., 2005a; Eric L. Bell, in press; Guzy et al., 2005; Mansfield et al., 2005a).

Recent studies in cancer cells demonstrate that telomerase activity and the mRNA of the rate limiting catalytic subunit of telomerase, human telomerase reverse transcriptase (hTERT), increases during hypoxia and that HIF is necessary for this increase (Minamino et al., 2001; Nishi et al., 2004; Yatabe et al., 2004). Telomerase is an enzyme that maintains telomere integrity and it is a major regulator of replicative lifespan in human cells. Multiple studies have demonstrated that the overexpression of hTERT is sufficient to increase telomerase activity and replicative lifespan of human fibroblasts (Bodnar et al., 1998; Counter et al., 1998a; Counter et al., 1998b; Vaziri and Benchimol, 1998). Conversely, the disruption of telomerase activity in normal human cells restricts replicative lifespan (Masutomi et al., 2003). In this Chapter, we

demonstrate that stabilization of HIF in normal oxygen conditions is sufficient to increase hTERT mRNA and telomerase activity in primary cells. More importantly, HIF is necessary to increase replicative lifespan in hypoxic conditions, and it is sufficient to increase replicative lifespan in normal oxygen conditions. These findings are in contrast to the free radical theory since hypoxic activation of HIF requires increased generation of mitochondrial ROS.   Data is presented which resolves this discrepancy by demonstrating that the levels of ROS generated under hypoxia are significantly less than the levels required to induce premature senescence, thereby providing a mechanism for the increase in replicative lifespan observed in hypoxic conditions.

Results

Hypoxia increases replicative lifespan and ROS.

Multiple reports have demonstrated that human fibroblasts extend their replicative lifespan during hypoxia (Forsyth et al., 2003; Packer and Fuehr, 1977; Saito et al., 1995). Consistent with previous reports, hypoxia (1.5% O_2) increased the replicative lifespan of primary human lung fibroblasts (PHLFs) by 10 population doublings (PD) compared to culture conditions mimicking normal physiological oxygen conditions of lung cells (ambient air) (Figure 3.1). Cells cultured under hypoxia also displayed an increase in the rate of proliferation. Senescence of cells under normoxia and hypoxia was confirmed by staining for β-galactosidase activity, a hallmark of the senescent phenotype (Dimri et al., 1995). As expected, proliferating cells under either normoxic or hypoxic conditions (PD29 and 27 respectively) did not stain blue when fixed and treated with X-Gal, indicating a lack of β-galactosidase activity. Normoxic cells that had stopped proliferating (PD56) clearly displayed β-galactosidase activity. In contrast, cells under hypoxic conditions at the same PD (PD55) failed to display β-galactosidase activity, but eventually demonstrate β-galactosidase activity at PD66 (Figure 3.2).

In accordance with the free radical theory, the delay in replicative senescence under hypoxia is thought to be directly due to a decrease in ROS. Although hypoxia extends replicative lifespan of PHLFs, the levels of intracellular ROS did not diminish under hypoxia. The levels of intracellular ROS, as determined by Amplex Red, increased in cells exposed to hypoxia when compared to normoxic counterparts (Figure 3.3). As expected, PHLFs also increase intracellular ROS when incubated with the electron transport inhibitor antimycin a. Incubating these PHLFs with the mitochondria-

targeted antioxidant MitoQ abrogated the increase in ROS observed in PHLFs treated

with antimycin a, as well as in hypoxia, when compared to cells incubated with the

control compound Methyltriphenyl-phosphonium bromide (TPMP) (Figure 3.3). These

results demonstrate that an increase in mitochondrial ROS production during hypoxia is

associated with an extension of replicative lifespan.
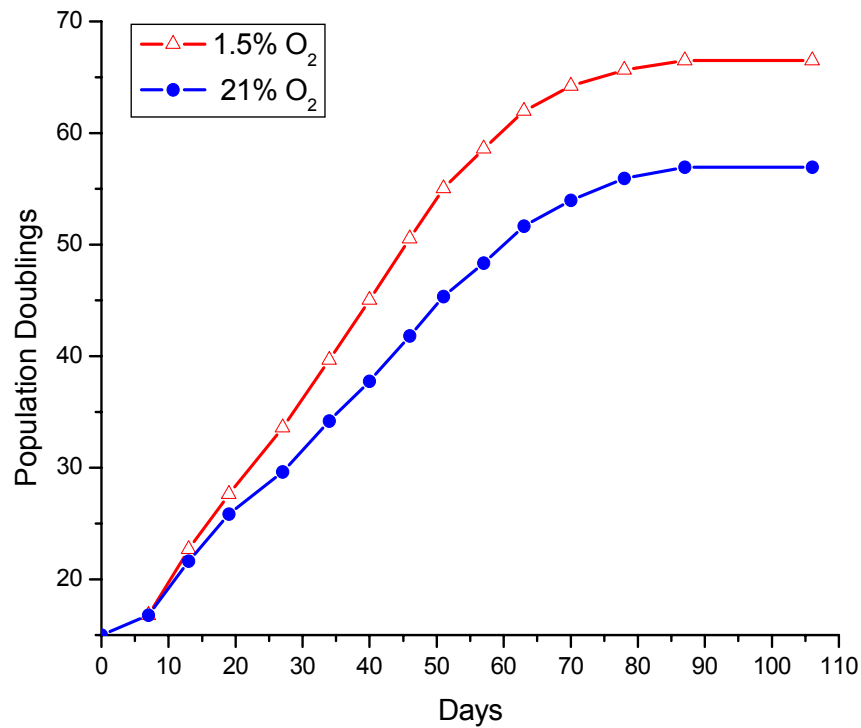
Figure 3.1 Hypoxia increases replicative lifespan of human lung fibroblasts. Primary human lung fibroblasts (PHLFs) were cultured in either 21% O_2 or 1.5% O_2 and their PD were monitored. Representative graph of three independent experiments.

PD 29   PD56

21% O$_2$

1.5% O$_2$
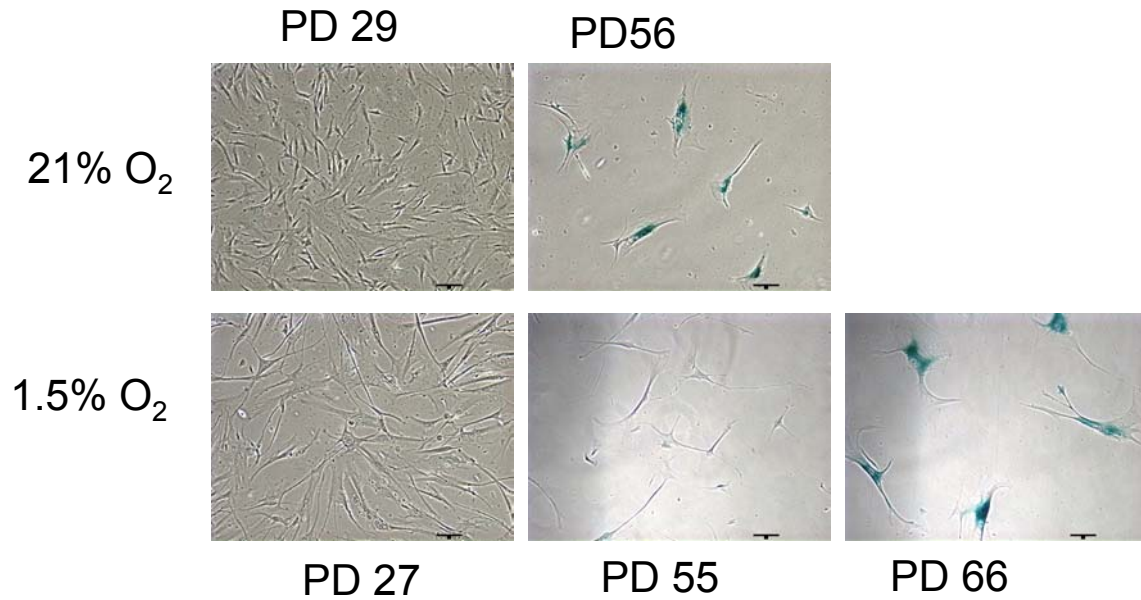
PD 27   PD 55   PD 66

Figure 3.2 Hypoxia delays the onset of senescence of human lung fibroblasts.   Senescence was detected by β-galactosidase activity in normoxia (PD29 and PD55) or hypoxia (PD27, PD55 and PD66). Scale bar is equivalent to 132 µm.
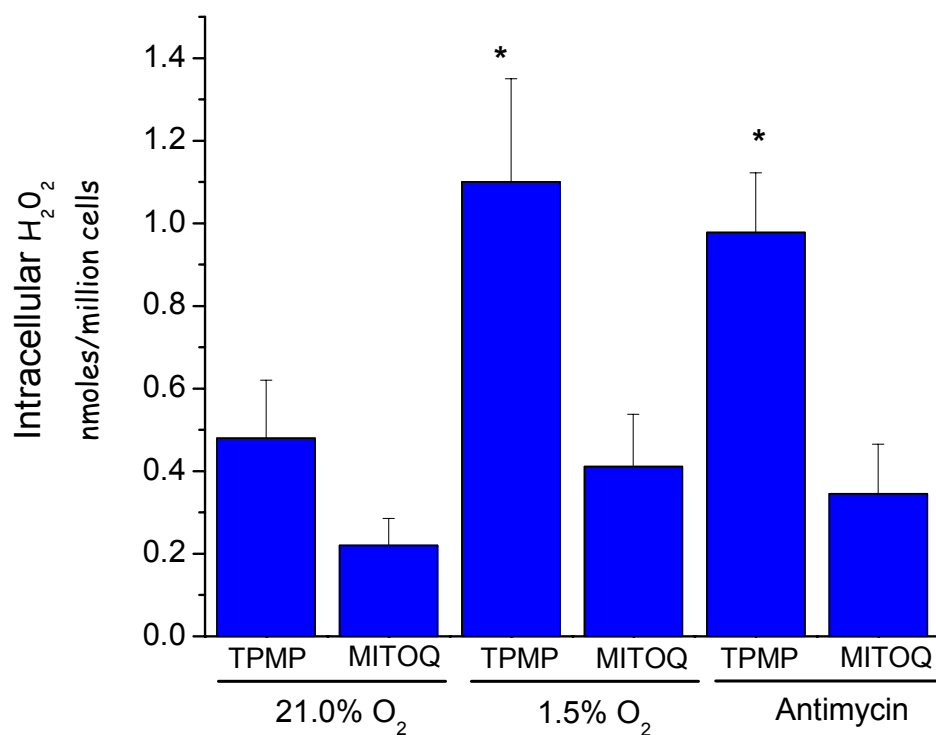
Figure 3.3 Hypoxia increases mitochondrial generated reactive oxygen species. Relative intracellular H_2O_2 levels of PHLFs, as determined by Amplex Red in 21% O_2, 1.5% O_2, or Antimycin A (1µg/mL) for 4 hrs in the presence of the mitochondrial targeted antioxidant MitoQ (1µM) or control compound TPMP (1µM) N=3 The *(TPMP at 21% O_2 compared to TPMP at 1.5 % O_2 or Antimycin A) indicates significance at $p < 0.05$.

Increase in cytosolic ROS during hypoxia does not damage DNA.

Previous studies have demonstrated that hypoxia increases ROS (Brunelle et al., 2005a; Chandel et al., 1998; Dirmeier et al., 2002; Duranteau et al., 1998; Guzy et al., 2005; Killilea et al., 2000; Mansfield et al., 2005a; Wood et al., 1999). However, other studies have indicated that ROS are decreased in hypoxic conditions (Fandrey et al., 1994; Michelakis et al., 2002; Vaux et al., 2001). Consistent with previously published data (Chandel et al., 1998), intracellular ROS is increased in an oxygen dose dependent manner (Figure 3.4A). ROS induce DNA double stranded breaks. To explore whether the ROS generated in hypoxic conditions damaged DNA we looked at phosphorylation of H2AX, a marker of DNA double stranded breaks (Paull et al., 2000). Culturing PHLFs in hypoxic conditions that increase intracellular ROS, 3% O_2 or 1.5% O_2, does not induce the phosphorylation of H2AX. However, treatment with the DNA damaging agent staurosporine does increase phosphorylation of H2AX (Figure 3.4B). These data indicate that the ROS generated in hypoxic conditions do not act to damage DNA, and probably serve as signaling molecules to initiate signal transduction cascades.
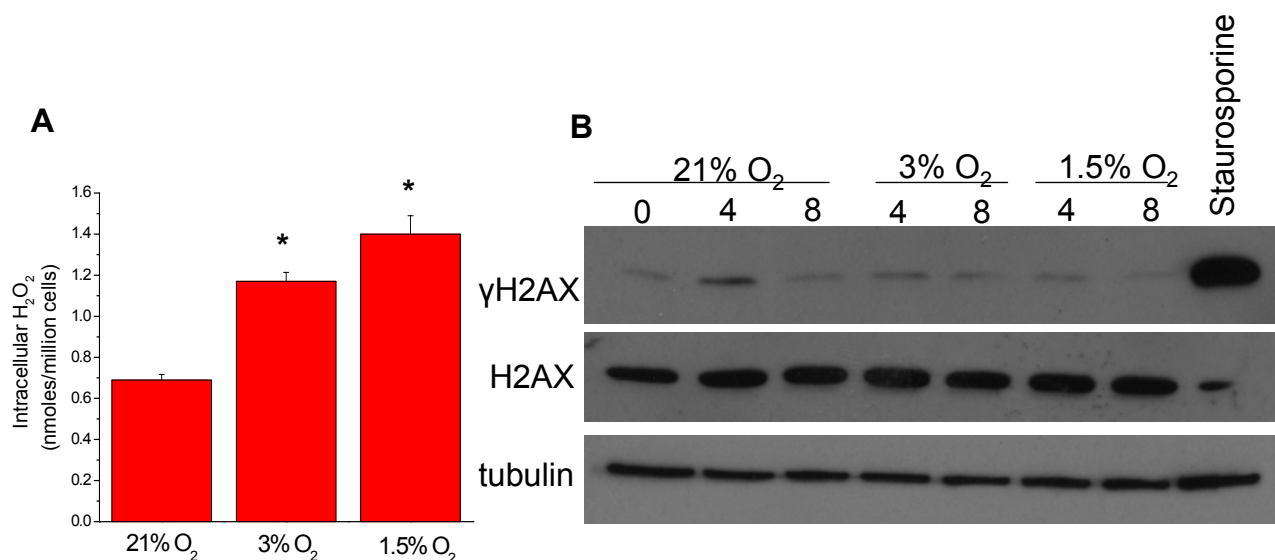
Figure 3.4 Hypoxia increase ROS at both 3% O_2 and 1.5% O_2 but does not induce the DNA double stranded break response. **(A)** Relative intracellular H_2O_2 levels of PHLFs, as determined by Amplex Red in 21% O_2, 3% O_2, or 1.5% O_2. N=3 *indicates significance at $p<0.05$ when 3% O_2, or 1.5% O_2 is compared to 21% O_2. **(B)** Phosphorylation status of H2AX in acid extracted proteins from PHLF exposed to 21% O_2, 3% O_2, 1.5% O_2, or staurosporine. Representative blot of three independent experiments

Mitochondrial generated ROS stabilize HIF-1α during hypoxia in PHLFs.

Hypoxic activation of HIF mediated transcription occurs in an oxygen dose dependent manner (Figure 3.5). The mitochondrial generated ROS produced under hypoxia initiate signaling cascades that results in cellular adaptation to environments with decreased oxygen levels (Emerling and Chandel, 2005). One such signaling cascade results in the activation of HIF to induce a transcriptional program. HIF is not activated in the absence of mitochondrial generated ROS indicating that the hypoxic activation of HIF during hypoxia requires mitochondrial ROS (Brunelle et al., 2005a; Chandel et al., 1998; Chandel et al., 2000; Guzy et al., 2005; Mansfield et al., 2005a). As expected, hypoxic stabilization of HIF-1α protein levels and HIF activity was abolished in the presence of the mitochondrial targeted antioxidant MitoQ (Figure 3.6 and 3.7). These data indicate that PHLFs require hypoxic induction of mitochondrial ROS to activate HIF
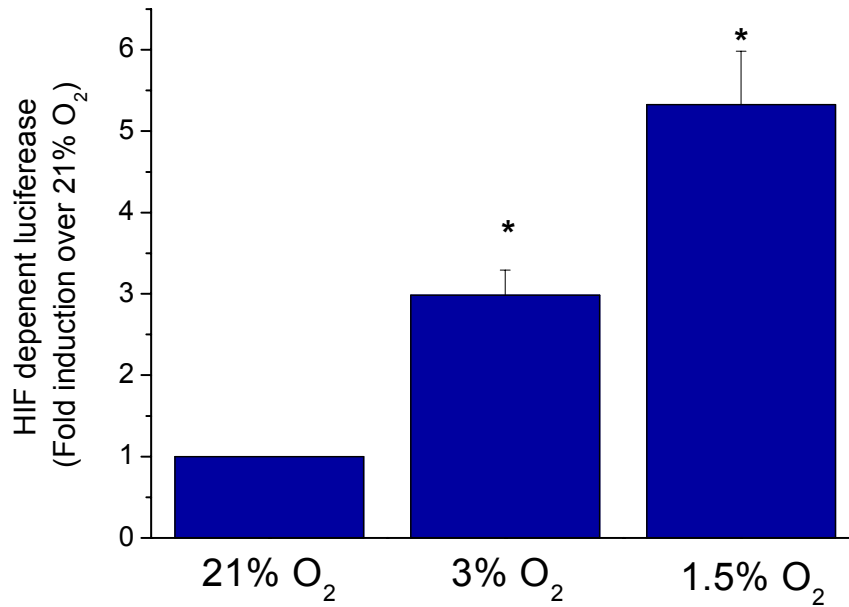
Figure 3.5 Hypoxia activates HIF transcription at 3% O_2 and 1.5% O_2. PHLFs transiently transfected with the firefly luciferase reporter construct driven by 3 hypoxic response elements and the *Renilla* luciferase construct driven by the thymidine kinase promoter exposed to 21% O_2, 3% O_2, or 1.5% O_2. N=3 *indicates significance at $p<0.05$ when 3% O_2, or 1.5% O_2 is compared to 21% O_2.
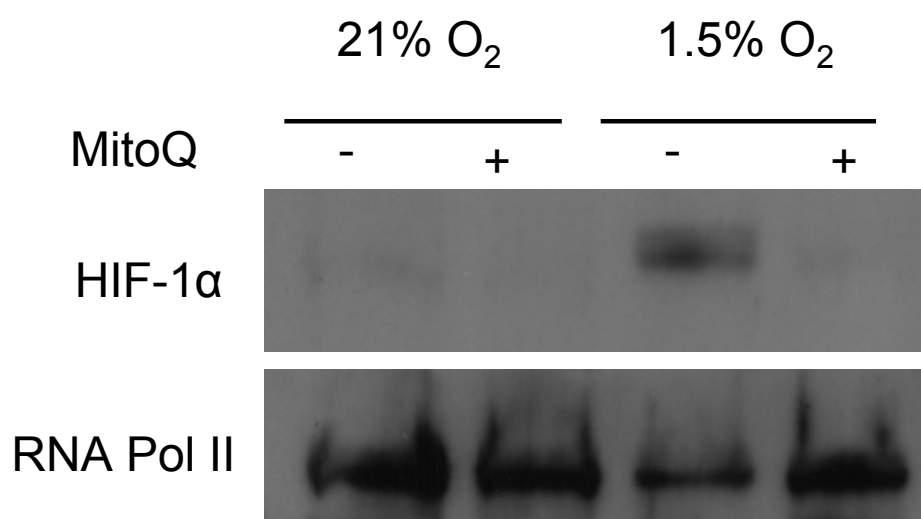
Figure 3.6 Hypoxia induced generation of mitochondrial ROS is necessary for HIF-1α protein stabilization in PHLF. HIF-1α and RNA polymerase II protein levels in nuclear extracts from PHLFs exposed to 21% O_2 and 1.5% O_2 for 4 hours in the presence of either 5μM MitoQ or TPMP. Representative blot of 3 independent experiments
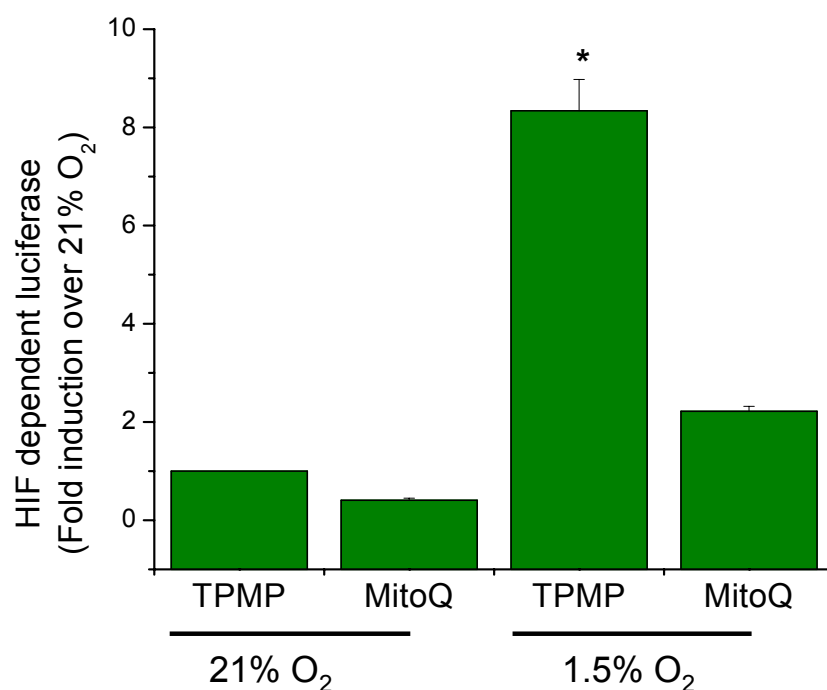
Figure 3.7 Hypoxic activation of HIF transcription requires mitochondrial generated ROS. PHLFs transiently transfected with the firefly luciferase reporter construct driven by 3 hypoxic response elements exposed to 2µM MitoQ or TPMP under 21% O_2 and 1.5% O_2 for 16 hours. Relative luciferase values were determined by normalizing firefly to *Renilla* luciferase. N=3 *(TPMP at 21% O_2 compared to TPMP at 1.5 % O_2) indicates significance at $p < 0.05$

HIF is sufficient to increase hTERT and telomerase activity in PHLFs.

Hypoxia increases telomerase activity and hTERT mRNA, and HIF is necessary for this increase (Yatabe et al., 2004). However, it is not established if HIF alone is sufficient to mediate these increases. To explore this possibility we utilized a technique that specifically stabilizes HIF protein in normal oxygen conditions. Overexpression of a peptide that corresponds to HIF ODDD effectively competes with endogenous HIF for its E3 ligase pVHL, allowing HIF to accumulate, translocate to the nucleus, and induce transcription. Stabilization of endogenous HIF under normoxia, achieved by overexpression of the HIF ODDD (ODDD wt), but not a mutant lacking proline 564 (ODDD P564A) was sufficient to increase HIF-1 α protein levels (Figure 3.8). Stabilization of HIF-1α protein by overexpressing the ODDD wt increased hTERT mRNA and telomerase activity compared to cells that overexpressing ODDD P564A (Figure 3.9 and 3.10). These data demonstrate that HIF activation is sufficient to induce hTERT expression and telomerase activity in primary cells.
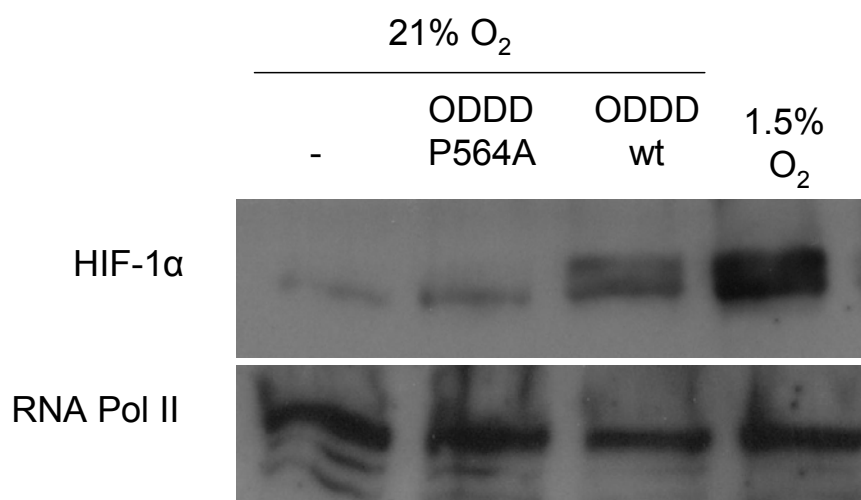
Figure 3.8 Overexpression of the HIF ODDD stabilizes HIF-1α protein in normoxic conditions. HIF-1α protein levels in PHLF exposed to 21% O_2, PHLF infected with adenovirus encoding wt or mutant (P564A) oxygen dependent degradation domain of HIF-1α (ODDD, a.a. 531-575) in 21% O_2, or PHLF in 1.5% O_2. Representative blot of four independent experiments.
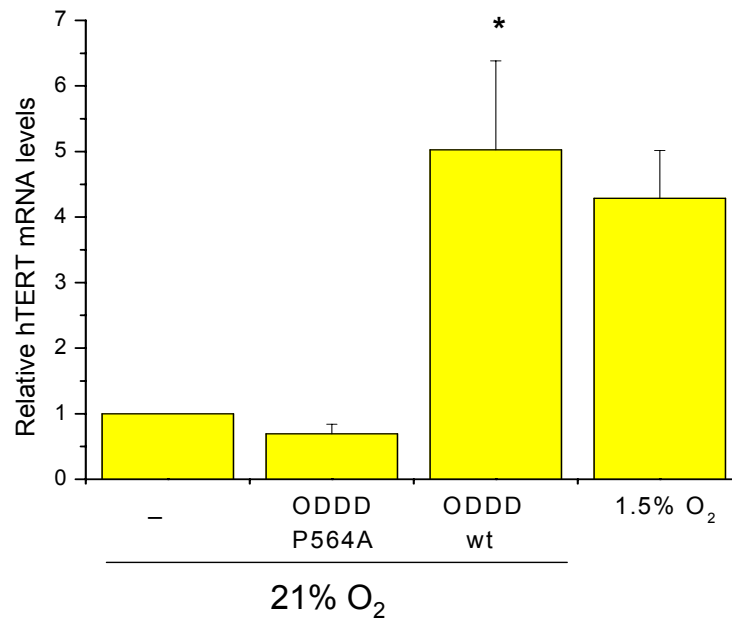
Figure 3.9 Stabilization of HIF in normal oxygen conditions is sufficient to increase hTERT transcription. Relative hTERT mRNA levels normalized to the ribosomal protein L19 in PHLF exposed to 21% O_2, PHLF infected with adenovirus encoding wt or mutant (P564A) oxygen dependent degradation domain of HIF-1α (ODDD, a.a. 531-575) in 21% O_2, or PHLF in 1.5% O_2. N=3. * (ODDDwt compared to ODDDP564A) indicates significance at $p<0.05$.
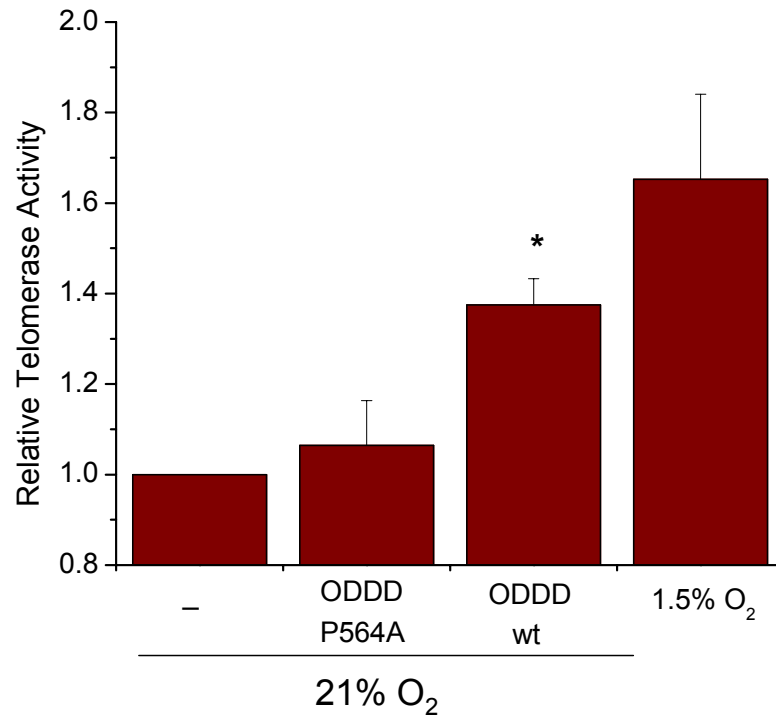
Figure 3.10 Stabilization of HIF in normal oxygen conditions is sufficient to increase telomerase activity.   Telomerase activity in PHLF exposed to 21% O_2, PHLF infected with adenovirus encoding wt or mutant (P564A) oxygen dependent degradation domain of HIF-1α (ODDD, a.a. 531-575) in 21% O_2, or PHLF in 1.5% O_2. N=3. * (ODDDwt compared to ODDDP564A) indicates significance at p<0.05.

HIF transcriptional activity is necessary for hypoxic increase in replicative lifespan.

Since HIF is sufficient to increase telomerase activity in primary cells and telomerase activity increases replicative lifespan, we examined whether HIF transcriptional activity is necessary for the hypoxic increase in replicative lifespan. PHLFs stably expressing a HIF transcriptional dominant negative (HIF-DN) (Forsythe et al., 1996) demonstrated impaired HIF transcriptional activity under hypoxia (Figure 3.11). Furthermore, these cells were not able to increase telomerase activity in hypoxic conditions (Figure 3.12). No difference in PDs was observed between cells containing the HIF-DN and the vector control cells (pLXIN) cultured in normoxic conditions (Figure 3.13). By contrast, cells expressing the HIF-DN cultured under hypoxia had a significantly lower PD compared to control cells (Figure 3.14), indicating HIF is necessary for the hypoxic increase in replicative lifespan.
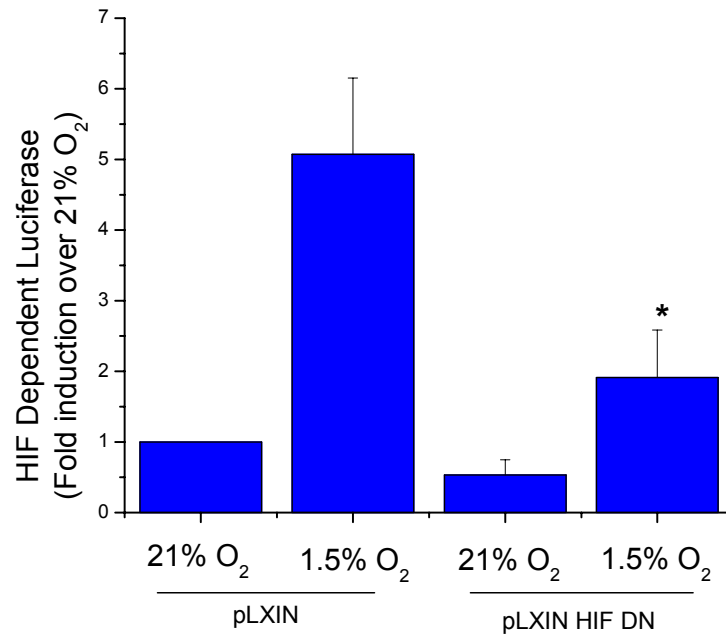
Figure 3.11 Stable expression of HIF Dominant Negative attenuates hypoxic induction of HIF mediated transcription. HIF transcriptional activity of PHLFs stably expressing a HIF dominant negative (HIFDN) construct or vector control (pLXIN) exposed to 21% O_2 or 1.5% O_2. N=3. * (pLXIN HIF DN 1.5%O_2 compared to pLXIN 1.5% O_2) indicates significance at p<0.05.
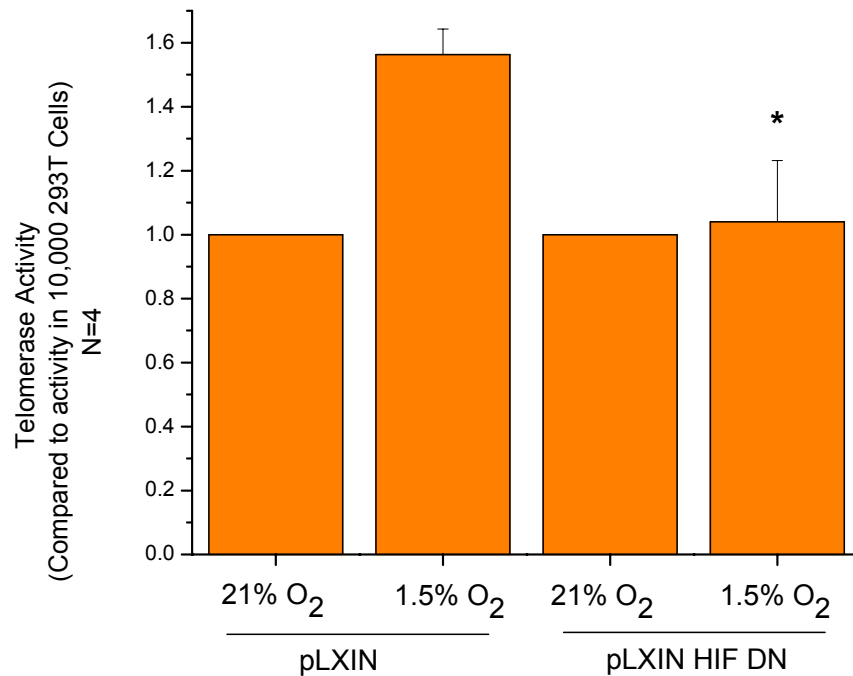
Figure 3.12 HIF transcriptional activity is necessary for hypoxic increase in telomerase activity. Telomerase activity of PHLFs stably expressing a HIF dominant negative (HIFDN) construct or vector control (pLXIN) exposed to 21% O_2 or 1.5% O_2. N=3. * (pLXIN HIF DN 1.5%O2 compared to pLXIN 1.5% O2) indicates significance at $p<0.05$.
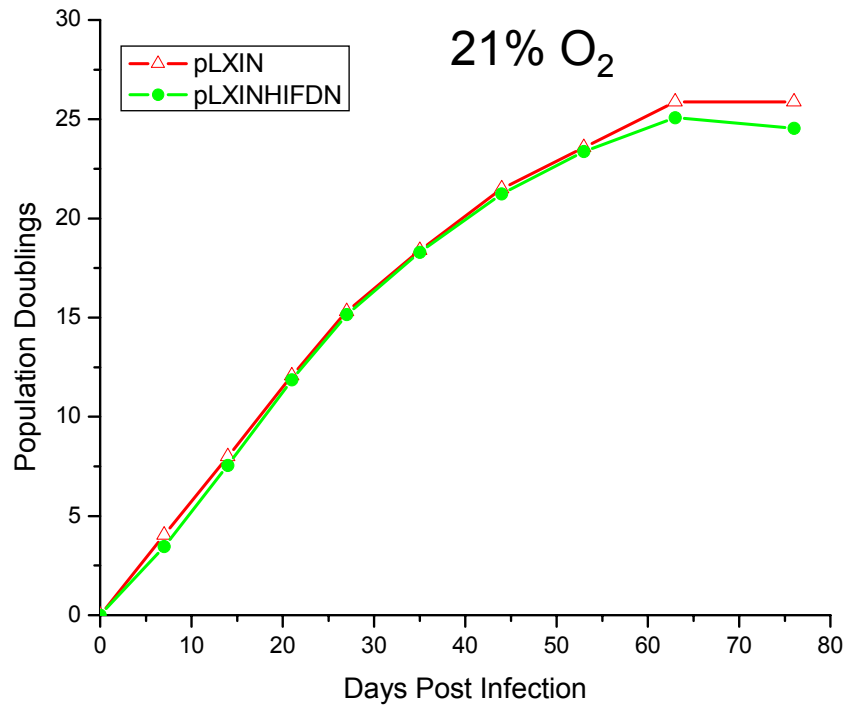
Figure 3.13 Attenuating HIF mediated transcription does not alter replicative lifespan of PHLFs. Population doublings of PHLFs stably expressing a HIF dominant negative (HIFDN) construct or vector control in 21% O_2 and their population doublings were monitored post infection. Representative graph of three independent experiments.
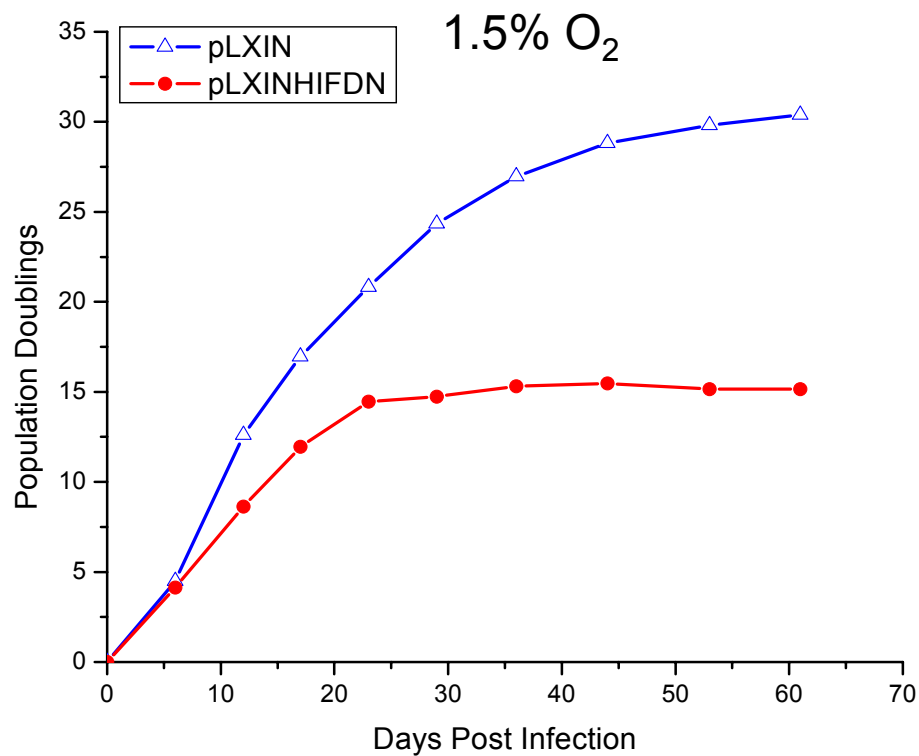
Figure 3.14 HIF mediated transcription is necessary for hypoxic increase in replicative lifespan of PHLFs. Population doublings of PHLFs stably expressing a HIF dominant negative (HIFDN) construct or vector control in 1.5% O_2 and their population doublings were monitored post infection. Representative graph of three independent experiments

Activation of HIF or loss of pVHL is sufficient to extend replicative lifespan.

To determine whether stabilization of endogenous HIF under normoxia is sufficient to extend replicative lifespan, PHLFs stably expressing wild-type ODDD or P564A-ODDD were cultured until they senesced. Cells expressing the wild-type ODDD display an increase in HIF transcriptional activity (Figure 3.15). PHLFs stably expressing the wild-type ODDD undergo 5 more PDs compared to cells that express the P564A-ODDD (Figure 3.16). These data clearly demonstrate that HIF is sufficient to increase replicative lifespan in normal oxygen conditions, providing a novel role for HIF in the regulation of replicative lifespan.

The loss of tumor suppressor VHL increases HIF activity under normoxia. To test whether the loss of pVHL would regulate senescence, PHLFs were created to stably express a shRNA against pVHL (Figure 3.17). Cells expressing the shRNA against pVHL displayed an increase in HIF-1α protein in normal oxygen conditions (Figure 3.18). The pVHL knockdown cells also displayed an increase in PDs compared to cells that expressed the control shRNA against *Drosophila melanogastor* HIF (dHIF) in normal oxygen conditions (Figure 3.19). Thus, loss of pVHL is sufficient to increase replicative lifespan in normoxic conditions.
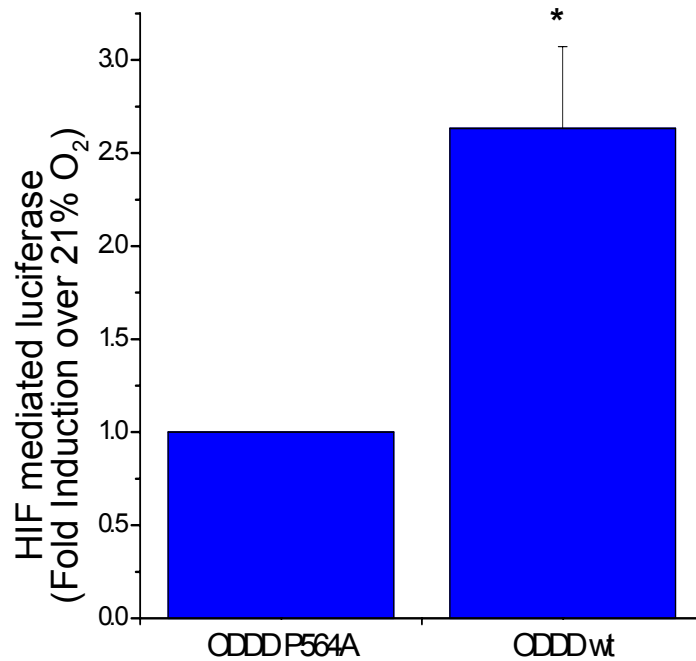
Figure 3.15 Stable expression of HIF ODDD increases HIF activity in normal oxygen conditions. Transcriptional activity of HIF was assessed at 21% O_2 in cells stably expressing HIF ODDD wt or the mutant HIF ODDDP564A. * (ODDDwt compared to ODDDP564A) indicates significance at $p < 0.05$ N=3.
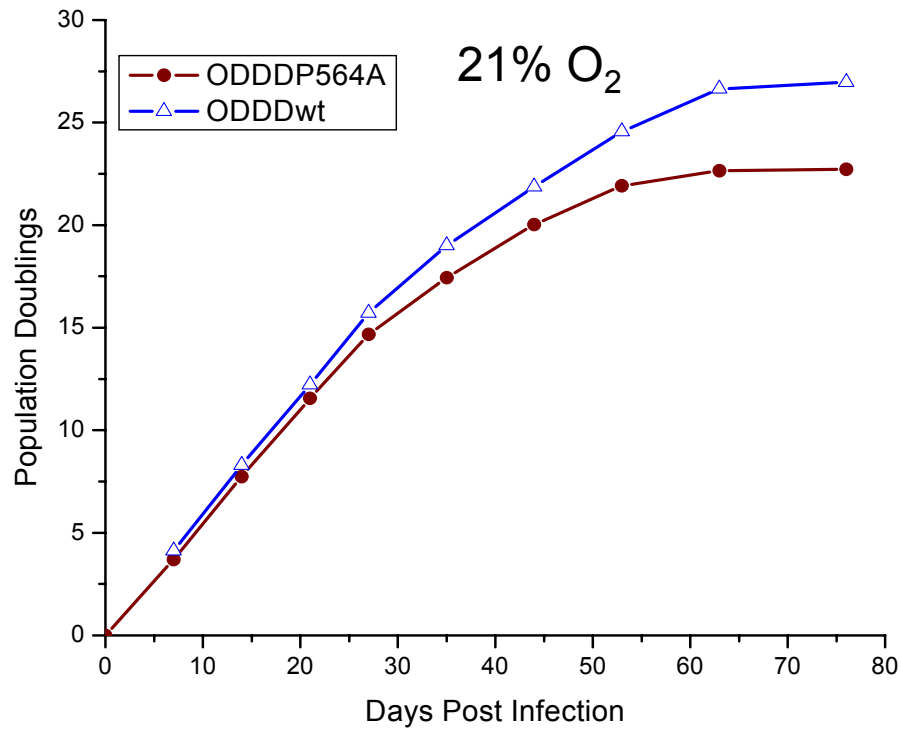
Figure 3.16 HIF is sufficient for the increase in replicative lifespan. PHLF cells with activated endogenous HIF were generated by stably expressing the ODDD construct, with ODDDP564A as control. Population doublings were monitored under 21% O_2 at days post infection. Representative graph of three independent experiments.

shRNA

dHIF   VHL

pVHL

α tubulin
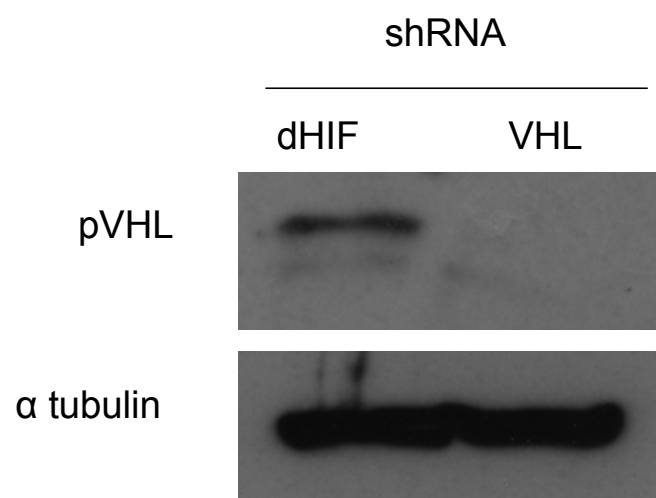
Figure 3.17 shRNA targeting pVHL knocks down endogenous pVHL. pVHL protein levels from whole cell lysates of PHLF stably expressing a shRNA against pVHL.
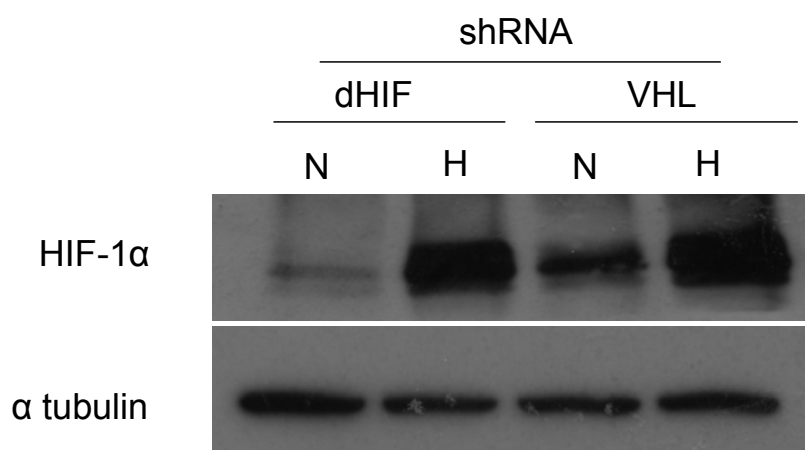
Figure 3.18 Stable expression of a pVHL shRNA stabilzes HIF-1α protein in normal oxygen conditions. HIF-1α protein levels in cells stably expressing shRNA for dHIF and pVHL that were exposed to 21%O_2 or 1.5%O_2.
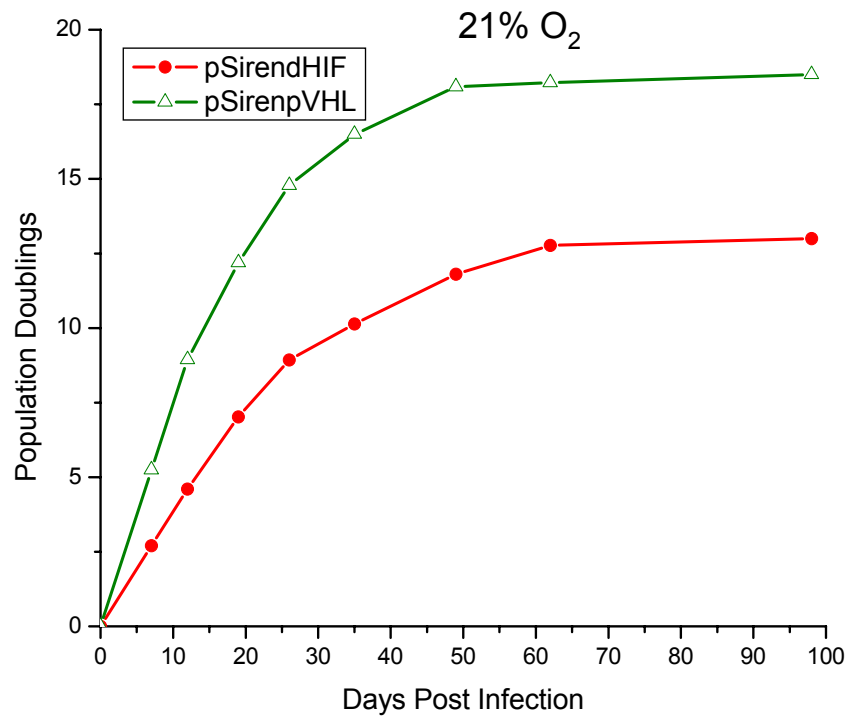
Figure 3.19 Loss of the pVHL tumor suppressor protein that regulates HIF increases replicative lifespan in PHLF under normal oxygen conditions. PHLFs expressing a shRNA against pVHL or *D. melanogaster* HIF (control) were grown in 21% O_2, and their population doublings were monitored days post infection. Representative graph of three independent experiments

Hypoxic cytosolic ROS levels are less than the levels required to induce premature senescence.

Our data provide a rare example where increases in ROS extend replicative lifespan, which contradicts the existing body of literature indicating that oxidants induce senescence (Chen et al., 1995). This discrepancy may be due to different effects elicited by varying levels of ROS. To test how the levels of intracellular ROS generated under hypoxia compare with those associated with the induction of senescence, we infected PHLFs with an adenovirus expressing a redox sensitive GFP (roGFP). This probe serves as a surrogate biological target of ROS in living cells (Dooley et al., 2004). PHLFs displayed increased levels of ROS in hypoxic conditions at both 24 and 48 hours (Figure 3.20). Interestingly, H_2O_2 (100 μM) treatment of PHLFs produced significantly greater oxidation of the roGFP sensor than did hypoxia (Figure 3.20). These data support the premise that there is a threshold level of oxidant production required for the induction of senescence, and that the levels produced in hypoxia, though higher than normoxic conditions, are below this threshold.

A

0 μM H$_2$O$_2$ 100 μM H$_2$O$_2$

\-

Cat

T. Klimova

B
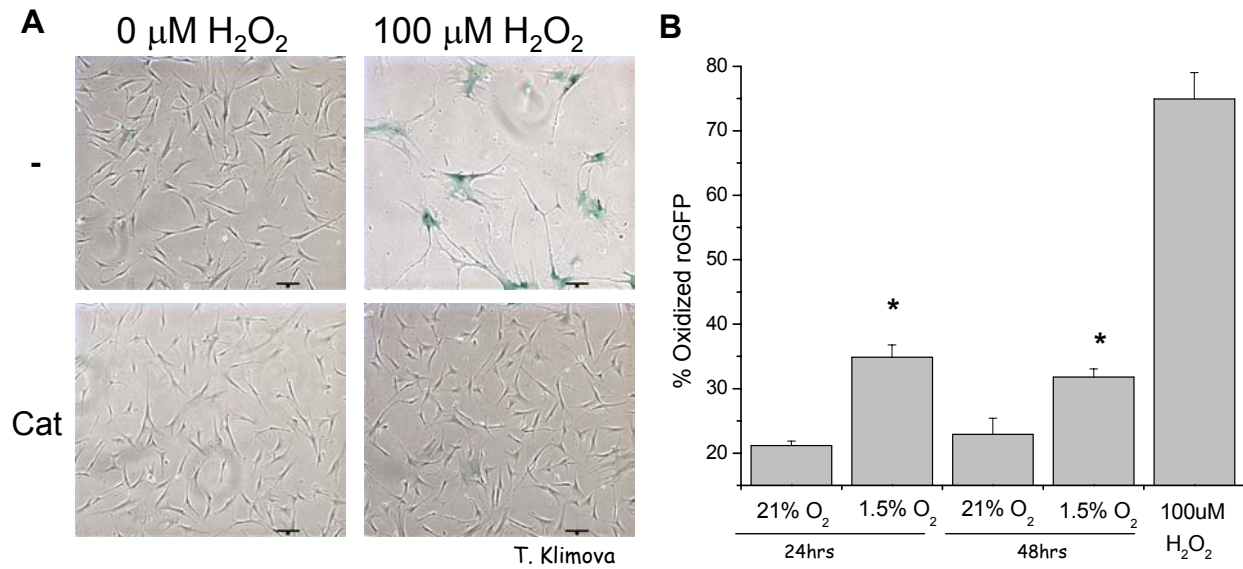
Figure 3.20 The levels of ROS generated in hypoxic conditions are lower than those required to induce senescence. (a) β-galactosidase activity in low-passage primary human lung fibroblasts incubated with a two hour treatment of H$_2$O$_2$ with or without catalase. Scale bar is equivalent to 132 μm. (b) Cytosolic ROS levels were detected by roGFP in PHLFs exposed to 21% O$_2$ and 1.5% O$_2$ for 24 and 48hrs. Intracellular oxidant levels are displayed as percent of roGFP maximally oxidized by treating cells with 1mM H$_2$O$_2$ for 5 minutes. N=3 The * indicates significance at p<0.05.

Discussion

These results reveal that mitochondrial ROS behave as signaling molecules to activate HIF dependent increase in telomerase activity, thus contributing to the positive regulation of replicative lifespan in human cells. ROS have been previously demonstrated paradoxically to increase during hypoxia (Brunelle et al., 2005a; Chandel et al., 1998; Chandel et al., 2000; Guzy et al., 2005; Mansfield et al., 2005a). The increase in ROS during hypoxia occurs at the Q_o site of mitochondrial complex III (Eric L. Bell, 2007). Consistent with previous observations, our current study indicates that hypoxia increases both ROS and HIF activity as a function of decreasing oxygen levels (Chandel et al., 1998). Typically, ROS are thought to be exclusively damaging and are associated with the induction of senescence (Beckman and Ames, 1998). However, we demonstrate in the current study that mitochondrial ROS generated under hypoxic conditions do not activate the DNA double stranded break response. Instead they activate HIF, resulting in the induction of hTERT mRNA and subsequent telomerase activity. Previous studies have demonstrated that hTERT is HIF target gene. Therefore, our study highlights that ROS can serve as signaling molecules to regulate HIF dependent replicative senescence.

Multiple studies have demonstrated that low levels of ROS can serve as signaling molecules and are required for cell proliferation and gene expression by inactivating phosphatases and activating kinases (Abe et al., 1997; Finkel, 2003; Irani et al., 1997; Kamata et al., 2005; Meng et al., 2002; Tobiume et al., 2001). Our results suggest that there is a graded response to senescence with respect to ROS. Accordingly, increased levels of oxidative stress during hypoxia would function as

adaptive signaling molecules to extend replicative lifespan via activation of HIF, whereas higher levels of oxidative stress would trigger premature senescence (Figure 3.21). We speculate that the generation of mitochondrial ROS has not been completely eliminated in evolution because they are involved in activating genes that help in the adaptation to stress such as changes in oxygen levels. In fact ROS have been shown to activate transcription factors OxyR and Yap1 in *E.Coli* and *S. Cervesia*, respectively (for review see(Georgiou, 2002). Furthermore, there are multiple studies indicating redox based activation of transcription factors in mammalian cells as well (for review see (Liu et al., 2005).

The finding that HIF is involved in the regulation of senescence is significant with respect to cancer. Reports indicate that overcoming senescence is critical for tumor suppression (Campisi, 2005). Activation of oncogenes such as Ras, or loss of the tumor suppressor PTEN, can activate p53-dependent senescence to prevent the progression of cells to a transformed state (Chen et al., 2005; Serrano et al., 1997). Dysregulation of p53 occurs frequently in cancer, as does HIF overexpression (Semenza, 2003). A causal role of HIF has been established in renal cell carcinoma (RCC) where a genetic defect results in loss of function of the tumor suppressor pVHL (Kondo et al., 2002; Maranchie et al., 2002b). The loss of pVHL function results in an increase of HIF levels under normoxia, thereby contributing to the tumorigenicity of RCC via aberrant activation of HIF (Kondo et al., 2002). The finding that HIF is sufficient in normal oxygen conditions to increase replicative lifespan demonstrates that HIF is capable of regulating replicative capacity to further promote the ability of pre-cancerous cells to overcome the tumor suppressing mechanism of senescence. The ability of HIF

to increase replicative lifespan would provide a growth advantage in cancers that have aberrant upregulation of HIF due to loss of tumor suppressors or activation of oncogenes. Recent report also indicated that HIF regulates senescence in mouse cells (Welford et al., 2006). Collectively, these data identify a novel role of HIF in the regulation of cancer in addition to its established role in regulating tumor angiogenesis and metabolism.

In summary, the data demonstrating that HIF is necessary for hypoxic increase in replicative lifespan provides an alternative mechanism to the free radical theory for explaining the observed increase in replicative lifespan. Moreover, the finding that HIF is sufficient to increase replicative lifespan in normal oxygen conditions demonstrates that HIF is capable of regulating replicative capacity, and would further implicate HIF as a positive regulator in diseases associated with increased proliferation such as cancer and pulmonary hypertension. Finally, hypoxia and HIF have been implicated in maintaining stem cells in an undifferentiated state (Cowden Dahl et al., 2005; Gustafsson et al., 2005). The ability of hypoxia and HIF to extend replicative lifespan would further provide an environmental advantage for stem cells to reside in hypoxic niches like the bone marrow.
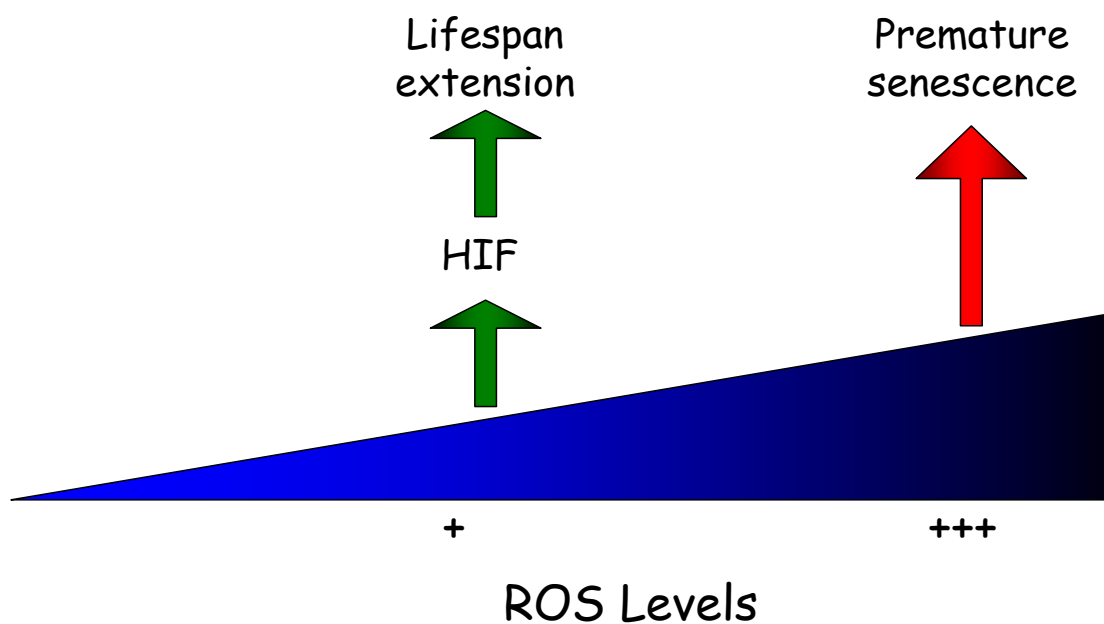
Figure 3.21 PHLFs demonstrate a graded response to levels of ROS. We propose a model that there is a graded response to ROS. A small increase in ROS levels serve as signaling molecules to extend replicative lifespan, whereas higher levels of ROS trigger premature senescence.

Chapter 4: Conclusions

The body of work presented in this dissertation provide data that make progress towards understanding how higher eukaryotes maintain oxygen homeostasis. First, it defines how mitochondria regulate the activity of HIF through the generation of ROS. Second, it demonstrates a novel role of HIF in the control of replicative lifespan. Third, it provides the first association of an increase in free radicals with an increase lifespan.

Oxygen Sensing

Higher eukaryotes depend on oxygen for survival, therefore making oxygen homeostasis a critical component for maintaining life in higher organisms. The stress of reduced oxygen levels induces the transcription factor HIF. The transcriptional program induced by HIF facilitates adaptation to decreased oxygen availability, making HIF the master regulator of oxygen homeostasis. The importance of HIF in oxygen homeostasis is highlighted by the fact that mice which have either component of HIF genetically deleted are embryonic lethal. HIF was discovered in the 1990s as the factor necessary for the induction of erythropoietin, a protein that induces systemic adaptation of hypoxia through the process of erythropoiesis. Initial studies demonstrated the HIF activity is regulated through the oxygen dependent stabilization of the HIFα subunit. It was later demonstrated that in normal oxygen conditions, the HIFα subunit is degraded by the proteasome and hypoxic conditions inhibited this degradation. In the beginning of the 21st century, two studies independently discovered a family of enzymes (the PHDs) that facilitate the proteasomal degradation of HIFα through the hydroxylation of this subunit on proline residues. In the absence of hydroxylation, the HIFα subunit is

stabilized and consequently activated. Hypoxic conditions inhibit HIFα hydroxylation, however the mechanism(s) by which cells sense decreased oxygen levels to inhibit hydroxylation and stabilize HIFα protein were not known.

In order for cells to sense hypoxic conditions and consequently activate HIF, cells must possess a functional mitochondrial electron transport chain. When this body of work began, two competing theories existed on the function of mitochondrial electron transport in oxygen sensing. One hypothesizes that mitochondrial oxygen consumption is necessary for oxygen sensing (Hagen et al., 2003). The second hypothesizes that ROS generated by the mitochondria under hypoxic conditions is necessary for oxygen sensing (Chandel et al., 1998). During the course of this work, our group published a paper demonstrating that cells which are deficient in oxygen consumption are able to activate HIF, thereby supporting the ROS model (Brunelle et al., 2005b). However, this study did not demonstrate that cells deficient in mitochondrial oxygen consumption are able to generate ROS in hypoxic conditions. Using cells that are deficient in the mitochondrial encoded protein cytochrome b, and are unable to consume oxygen, data presented in Chapter 2 demonstrate that oxygen consumption is not necessary for ROS generation under hypoxia. The levels of cytosolic ROS increase in the cytochrome b null cells when placed in hypoxic conditions. This novel data differentiates between the two functions of mitochondria independently proposed to be required for oxygen sensing, thereby supporting the model in which mitochondria serve as the cellular oxygen sensor by generating ROS and not oxygen consumption. Furthermore, the data described in Chapter 2 provide the first genetic evidence identifying the source of the ROS. Using genetic interventions, the Q_o site of Complex III is identified as the source

of the ROS required to activate HIF in hypoxic conditions, thereby demonstrating

that the Q_o site is an essential component of the cellular oxygen sensor.

The increase in cytosolic ROS generated by the Q_o during hypoxic conditions

can be attributed to an increase in the generation of ubisemiquinone and/or a shift in the

partitioning of the ROS made by the Q_o site. By examining the localization of ROS

generated in normoxic and hypoxic conditions in the presence or absence of antimycin

a, the data presented in Chapter 2 demonstrate for the first time that hypoxia actually

induces a shift in the localization of ROS generated by the Q_o site. ROS localized in the

matrix decreases when cells are exposed to hypoxic conditions. Conversely, ROS

localized to the cytosol increases in hypoxic conditions. This data does not exclude the

possibility that total ROS levels are increased, and these ROS are preferentially

localized to the cytosol. Whether hypoxia induces a conformational change in the Q_o

site, or whether there is a change in the dynamics of the lipids within the intermembrane

to allow for the partitioning of the ROS to the cytosol is a subject for future studies. The

interpretation of this data is that hypoxia induces ROS generated from the Q_o site to be

released into the intermembrane space where it then migrates to the cytosol to activate

HIF through the inhibition of hydroxylation. Chapter 2 presents data demonstrating that

the absence of mitochondrial derived ROS in hypoxia results in the continued

hydroxylation of HIFα protein, and therefore proteasomal degradation. This indicates

that the ROS affect the ability of the PHDs to hydroxylate HIFα, but the mechanism is

not understood. It is possible that the ROS alter the cellular redox state. Specifically,

they may induce a change in the state one of the necessary co-factors of the PHDs,

Fe^{+2} to Fe^{+3} through the Fenton reaction. Alternatively, they may initiate signaling

cascades that ultimately lead to the inhibition, or decrease, in PHD enzymatic activity. Previous data indicates the importance of signaling cascades. It is known that the hypoxic increase in cytosolic ROS activates p38, and this is necessary for the hypoxic activation of HIF (Emerling et al., 2005). The requirement of p38 for hypoxic activation of HIF supports the model in which the ROS activate a signal transduction cascade to limit hydroxylation of HIFα.

The data presented in Chapter 2 provides the Q_o site as a target for drug discovery in the treatment of pathologies regulated by hypoxic activation of HIF. Furthermore, the data demonstrating that ROS generation and oxygen consumption are not interrelated in hypoxic activation of HIF indicate that treatments targeting ROS generation by the Q_o site of the mitochondria can be developed without altering cellular bioenergetics. Therefore, such treatments can specifically targeting hypoxic cells without altering the bioenergetics in the surrounding cells due to inhibition of oxidative phosphorylation (OXPHOS). Electron transport inhibitors inhibit the ability of cells to generate ATP through OXPHOS as well as mitochondria to generate ROS. However, using inhibitors of ROS generation at the Q_o site, or mitochondrial targeted antioxidants would impair the ability of mitochondria to generate the ROS necessary to activate HIF in the target cells while allowing OXPHOS to continue in the surrounding cells. Thus, dissociating the two functions of mitochondria in terms of hypoxic activation of HIF provides a rationale to develop treatments that preferentially target cells in regions of hypoxia without affecting the surrounding cells.

Hypoxia and Replicative Lifespan

The activation of HIF is not only important to maintain oxygen homeostasis and proper development; it is also involved in the development of pathophysiological processes such as pulmonary hypertension and cancer. Numerous HIF target genes promote the proliferation and survival of cells. These processes are required for the development of pulmonary hypertension and cancer. In order for cells to become cancerous, they need to overcome their programmed limit on replicative lifespan. It is less clear whether this is a hurdle for the development of pulmonary hypertension. However, this disease is manifested by hyperplasia, therefore it is very possible that cells must acquire additional replicative capacity. The work presented in Chapter 3 of this dissertation demonstrate a novel role for HIF in mediating replicative lifespan.

It was demonstrated more than thirty years ago that hypoxia increase replicative lifespan of cells in culture (Packer and Fuehr, 1977). It has been assumed that this was due to decreased levels of damaging oxygen radicals, since there was a decrease in the levels of oxygen. Although, as previously published and confirmed in Chapter 2, ROS are not decreased in hypoxic conditions. Cytosolic levels of ROS are paradoxically increased under hypoxia and these ROS are necessary to activate HIF. Hypoxia increases the activity of an enzyme that, when the rate-limiting catalytic subunit hTERT is expressed in normal primary cells, increases replicative lifespan. It has been previously demonstrated that HIF is necessary for the hypoxic increase in telomerase activity in cancerous cells. Chapter 3 presents the first data demonstrating that HIF is sufficient to increase the transcription of hTERT and telomerase activity in normal human lung fibroblasts under normal oxygen conditions. Moreover, this data

demonstrates that HIF transcriptional activity is necessary for hypoxic increase in replicative lifespan. This data raises the question of whether activation of HIF in normal oxygen conditions can regulate replicative lifespan. In fact, data presented in Chapter 3 demonstrates that activation of HIF in normal oxygen conditions increases replicative lifespan. Stabilization of endogenous HIFα protein by overexpressing a peptide which competes away the E3 ligase pVHL, or stable knock down of pVHL itself increases replicative lifespan. This is the first demonstration of a positive role of HIF transcription on replicative lifespan, further implicating the HIF in promoting pathophysiological processes associated with increased proliferation/replicative lifespan. As far as cancer, activation of HIF is primarily thought to help the cell avoid death and induce proliferation. Now, not only does HIF regulate these processes, but it is also able to help cells overcome their inherent limit on the number of times that they can divide. A gain of function of HIF in a cell now compromises three different checkpoints that are meant to inhibit unregulated cellular growth, thereby allowing for the development of cancer. This highlights the importance of developing treatments that modulate the activity of HIF in order to treat diseases associated with hypoxia and/or the aberrant activation of HIF mediated transcription such as cancer and possibly pulmonary hypertension. Moreover, this data may explain why stem cells like to reside in regions of hypoxia. The induction of HIF mediated extension of replicative lifespan by hypoxic conditions would allow the stem cells to remain in a state of unlimited replicative lifespan thereby maintaining pluripotency. Therefore, culturing stem cells in hypoxic conditions may provide investigators a tool for the continued study of stem cells in treatment of multiple pathologies.

ROS and Aging

The free radical theory of aging postulates that there is a direct relationship between the levels of ROS and the onset of aging phenotypes such as replicative senescence. On a cellular level, ROS cause damage to proteins, lipids, and DNA that results in a loss of normal cellular function to induce senescence. Over the lifespan of a cell, ROS are continually made as byproducts of mitochondrial electron transport. Therefore, the cell will accumulate damage to cellular proteins, lipids, and DNA over its lifespan ultimately causing senescence. It was previously thought that senescence was an artifact of cell culture, but recent studies demonstrate that senescence indeed occurs *in vivo* and actually plays an important role in tumor suppression (Campisi, 2005; Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005). However, the role of cellular senescence in organism aging is less clear, but it is thought that the free radical theory of aging, ROS induced senescence, plays an important role in organism aging.

Recently published data both support and dispute the free radical theory of aging. Exposing Drosophilia melanogaster to antioxidants does not increase their lifespan (Magwere et al., 2006). Furthermore, increased dysfunction of mitochondrial electron transport by increasing the mutation rate in mitochondrial DNA results in decreased lifespan of mice, but is not associated with increased oxidative stress (Kujoth et al., 2005). However, genetically targeting the protein antioxidant catalase to the mitochondria in mice does delay the aging process (Schriner et al., 2005). Furthermore, Chapter 3 provides data indicating that ROS do not always have negative affects on lifespan by demonstrating that increased levels of cytosolic ROS are associated with an increase in replicative lifespan. Importantly, the increase in cytosolic ROS in hypoxic

conditions is not sufficient to damage DNA. This leads to the speculation that the affects of increased ROS on lifespan vary according to the levels of ROS. In fact, data is presented that demonstrate the increase in cytosolic ROS under hypoxia is significantly less than those levels required to induce premature senescence, indicating that there is a threshold of ROS required to induce the damage required to decrease replicative lifespan. The levels of cytosolic ROS attained in hypoxic conditions are below the threshold required to induce cellular damage and subsequently decrease replicative lifespan. However, they are sufficient to increase the activity of HIF to increase replicative lifespan in hypoxic conditions through the upregulation of hTERT and telomerase activity.

These data indicate that the free radical theory of aging is not absolute and may need to be updated. However, further experimentation needs to be performed in order to determine the relationship between ROS and aging according to the free radical theory. Furthermore, antioxidant supplements are currently being marketed as products that decrease the aging process. The data presented in Chapter 3 indicate that antioxidants may have deleterious affects on cells which preferentially reside in hypoxic conditions, such as stem cells. The stem cells may reside in hypoxic conditions because the ROS mediated activation of HIF maintains an increased replicative lifespan. Attenuating ROS with antioxidants could have deleterious effects on normal physiology and actually promote aging instead of delaying the aging phenotype.

Concluding Remarks

Collectively, the data presented in this dissertation have broad implications in the fields of hypoxia, oxygen sensing, aging, and the pathologies associated with the activation of HIF. Chapter 2 presents data that distinguishes between the two models of mitochondrial oxygen sensing by genetically demonstrating that the generation of ROS can be dissociated from oxygen consumption by the mitochondria in terms of hypoxic activation of HIF. The ability of cells to sense decreased levels of oxygen to activate HIF is independent of mitochondrial oxygen consumption. Therefore, these data provide further support for the ROS model of mitochondrial oxygen sensing. The Q_o site of the Complex III is defined to be necessary for cellular oxygen sensing, thereby providing a drug discovery target for therapies targeting hypoxic activation of HIF. The data imply that the mechanism by which hypoxia induces an increase in cytosolic ROS is by inducing a shift in the partitioning of the ROS from both the mitochondrial matrix and intermembrane space to exclusively the intermembrane space. Further experiments need to be performed in order to determine how hypoxia induces this change in the localization of ROS, and whether this change is sufficient for oxygen sensing. Chapter 3 demonstrates that HIF is necessary for the increase in replicative lifespan observed in hypoxic conditions, thereby providing the first example of a situation where increased cytosolic ROS is directly responsible for an increase in replicative lifespan. Furthermore, activation of HIF in normal oxygen conditions is sufficient to increase replicative lifespan. The novel link between HIF and replicative lifespan provides further rationale for targeting HIF in the treatment of pathologies requiring an increase in replicative lifespan. Moreover, it provides a rationale for using

HIF activating therapies in situations where the limiting of replicative lifespan promotes the onset of pathological processes such as during development.

Overall, these studies identified how the mitochondria serves as the cellular oxygen sensor and describes a novel function of the cellular oxygen sensing network in response to decreased oxygen availability by controlling replicative lifespan. Finally, the fact that ROS have withstood the selective pressures of evolution indicates that they are not exclusively damaging. The data presented here put forth a rationale for the survival of ROS through evolution; they promote the transcriptional activity of HIF, which can increase the replicative lifespan of cells.

Chapter 5: Materials and Methods

Cell Culture:

Wild type A549 and wild type 143B cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml penicillin, 100µg/ml streptomycin, 0.25 µg/ml amphotericin B and 20 mM HEPES. Wild type cybrids, Δ cytochrome b cybrids, A549 ρ^0, and 143B ρ^0 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml penicillin, 100µg/ml streptomycin, 0.25 µg/ml amphotericin B, 20 mM HEPES, and 100 µg/ml Uridine. Normal human diploid lung fibroblasts were obtained from Cambrex and grown in Fibroblast Growth Media 2 (Cambrex). Cells were maintained at 37°C in 5% CO_2 humidified incubators.

Measurement of Replicative Lifespan

Replicative lifespan of the cells was determined by calculating the number of population doublings (PD) the cells endured. Cells were split when they reached approximately 70% confluence, counted and seeded at a density of 4×10^4 cells. PD was calculated using the formula: PD= log(number of cells counted/number of cells seeded)/log2 + previous PD.

Oxygen Conditions

Hypoxic conditions (1.5% O_2, 5% CO_2, balanced with N_2 or 3% O_2, 5% CO_2, balanced with N_2) were achieved in a humidified variable aerobic workstation (InVivo$_2$, Biotrace). This workstation maintains an internal temperature of 37 ^{o}C and contains an oxygen sensor that continuously monitors the oxygen tension.

Senescence Associated β-galactosidase Staining

PHLF at various PDs were plated in 60mm dishes. Three days later senescence associated beta-galactosidase activity was determined with the Senescence Associated beta-Galactosidase Staining Kit (Cell Signaling) according to manufacturer's protocol. For premature senescence, early passage PHLFs that were incubated overnight with or without 200 units of Polyethylene glycol (PEG) conjugated catalase (Sigma) were treated with a bolus of 100μM H_2O_2 for 2 hrs and assessed for senescence associated beta-galactosidase activity 72 hours later. Images were obtained with a Nikon Eclipse TE200 inverted microscope at 10X magnification.

Stable Cell Lines

Stable lines were generated using retroviral methods with the PT67 packaging cell line (Clontech). PT67 cells which were ~50% confluent were transfected with 10-15 μg of plasmid using TransIT LT-1 (Mirus). 100μl of serum free DMEM was incubated with TransIT LT-1 (2.5μl/μg of plasmid) for 15 minuntes. Plasmid was added to the reaction mixture and incubated at room temperature for 30 minutes. Transfection mixture was added drop wise to the PT67 cells (10cm dish) in 2.5 mL of DMEM supplemented with 25μM chloroquine. 24 hours later cells were washed with PBS and 4mL of appropriate

media was added to collect retrovirus. 24 hours later viral containing media was collected, centrifuged for 5 minutes at 1000 rpm, filtered through a 0.45 μm membrane, supplemented with 8μg/ml of polybrene, and then added to target cells (~50% confluent) for 24 hrs. 48 hours after viral infection, cells were selected with appropriate antibiotic. Generation of stable cell lines in the PHLF were performed in early passage diploid fibroblasts (~PD15-PD25). The pLXIN vector (Clontech) was used to overexpress HIF-DN (ATCC) and the oxygen dependent degradation domains (HIF-1α a.a 531-575 fused to GFP). The pSiren vector (Clontech) was used to express shRNA sequences for pVHL (5'-GGAGCGCATTGCACATCAACG-3'), Drosophila melanogaster HIF (5'-GCCTACATCCCGATCGATGATG-3'), the Rieske Fe-S protein (5'-AAGGTGCCTGACTTCTCTGAA -3'), and TFAM (5'-GTTGTCCAAAGAAACCTGT-3').

Adenoviral Infections

Cells at 70% confluence in 100 cm cell culture dishes were placed in 2ml of appropriate basal media (no FBS, antibiotics, supplements) for viral infection. Appropriate volume of adenovirus was added to in order to get proper particle forming units (pfu) per cell, and incubated for 6 hours with gentle agitation every hour and then 7 ml of complete media was added to the plates. Ad-ODDD wt and Ad-ODDD P564A (used at 50 or 100 pfu) were obtained from ViraQuest. Ad-roGFP (used at 20 pfu) was obtained from Paul Schumacher.

ROS Measurement

Intracellular ROS was measured using multiple assays. To measure acute H_2O_2 production we used Amplex Red (Molecular Probes) according to manufacturer's protocol. Briefly, 5×10^6 cells were lysed in Amplex Red solution (100µM) supplemented with 200 mUnits/ml of SOD (OXIS International) and incubated in the dark for 30 minutes. Fluorescence was measured in the Spectra Max Gemini (Molecular Probes) plate reader with excitation of 540 nm and emission of 590 nm. In the MitoQ experiment, cells were pre-incubated with MitoQ or TPMP for 30 minutes and then exposed to 21% O_2 and 1.5% O_2 for 4 hours in the presence of either 2µM MitoQ or TPMP. To assess the localization of ROS in live cells we used dihydroethidium (DHE) (Invitrogen) and MitoSOX Red (Invitrogen). DHE and MitoSOX Red react with superoxide localized to the cytosol and mitochondrial matrix respectively. Cells were incubated with DHE (5 mM) or MitoSOX Red (1mM) for 1 hour and subsequently exposed to normoxia or hypoxia in the presence of either dye for 4 hours. Fluorescence was measured at excitation of 488 and emission of 575 with the CyanADP Flow Cytometry Analyzer (Dako) immediately after. A minimum of 10000 cells were counted for each sample. For longer time points we used the redox sensitive GFP (roGFP) previously described (Dooley et al., 2004). PHLFs were infected with 20pfu adenovirus encoding roGFP targeted to the cytosol. Cells were harvested by for analysis with the CyanADP Flow Cytometry Analyzer (Dako) 24 or 48 hours after being placed in conditions. The mean fluorescent channel for the ratio of violet excitable to blue excitable was determined with Summit v4.2 software (Dako). The percent oxidized

probe is determined as the ratio of the sample mean to the mean from probe oxidized by 1mM H_2O_2.

Gene Reporter Assay

HIF mediated transcriptional activity was measured using the pGL2 vector containing 3XHRE from the pgk-1 gene upstream of firefly luciferase. The pRLTK plasmid was used as a control for transfection efficiency. Cells were transfected using TransIT-LT1 (Mirus). 100µl of serum free DMEM was incubated with TransIT LT-1 (2.5µl/µg of plasmid) for 15 minuntes. Plasmid was added to the reaction mixture and incubated at room temperature for 30 minutes. Transfection mixture was added drop wise to the cells of interest (60mm dish) in 2 mL of DMEM. 36 hours later, cells were subjected to conditions for 16 hours before lysates were collected. Luciferase values were determined using the Dual-luciferase reporter assay kit (Promega) according to manufacturer's protocol. Values for firefly luciferase were normalized to Renilla luciferase under the control of the thymidine kinase promoter in the pRLTK vector. In the MitoQ experiment, cells were pre-incubated with MitoQ or TPMP for 30 minutes and then exposed to 21% O_2 and 1.5% O_2 for 16 hours in the presence of either 2µM MitoQ or TPMP.

Real-Time PCR

Total RNA was isolated using the Aurum Mini Kit (Bio-Rad) and cDNA was generated from 1µg of RNA using the RNAqueous-4PCR system (Ambion) according to manufacturer's protocol. Prepared cDNA was analyzed for TFAM (1µl cDNA) using

SYBR Green Master Mix (BioRad) and the Platinum SYBR Green qPCR SuperMix

UDG (Invitrogen) for hTERT (10µl cDNA). Cycle threshold (C_t) values for TFAM were

normalized to C_t values for ribosomal protein L19 and data was analyzed using the

Pfaffl method (Pfaffl, 2001).

Primers:

TFAM   5'-   AAGATTCCAAGAAGCTAAGGGTGA   -3'   and   5'-
CAGAGTCAGACAGATTTTTCCAGTTT -3'

hTERT   5'-CGTTTGGTGGATGATTTCTTGTT-3'   and   5'-
TCGTCTTCTACAGGGAAGTTCA-3'

RPL19   5'-GTATGCTCAGGCTTCAGAAGA-3'   and   5'-
CATTGGTCTCATTGGGGTCTAAC-3'

Nuclear Extract Preparation

A minimum of 8 hours before experimentation 100 mm cell culture dishes containing 10

mL of appropriate culture media were placed in either a cell culture incubator (5% CO_2)

or in the hypoxic workstation (1.5% O_2, 5% CO_2, balanced with N_2) to pre-equilibrate.

Cells at 70% confluence cultured in 100 mm cell culture dishes were incubated in 10 mL

of pre-equilibrated culture media in either hypoxic or normoxic conditions for 4 hrs. To

prepare a positive control sample, cells were incubated with 1mM DMOG for 4 hr. After

incubation culture dishes were placed on ice and media is aspirated. Cells were

washed once with 3 mL cold PBS and then scraped in 1 mL of cold Buffer A (10 mM

HEPES-KOH, 1.5 mM $MgCl_2$, 10 mM KCL, 50 µg/mL phenylmethylsulfonyl fluoride

(PMSF), 1 mM dithiothreitol (DTT), and cocktail of protease inhibitors (Roche)). The cell

suspension was then transferred to 1.5 mL microfuge tubes, briefly vortexed, and incubated on ice for 30 min. Cells were pelleted at 12,000g for 1 minute, and the supernatant is aspirated. Depending on size of the resulting pellet, the nuclei were re-suspended in 30-100 μL of cold Buffer C (20 mM HEPES-KOH, 1.5 mM $MgCl_2$, 420 mM KCL, 25% (v/v) glycerol, 0.2 mM EDTA, 50 μg/mL phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and cocktail of protease inhibitors (Roche) (25 μL of Buffer C per $1x10^6$ cells), vortexted and then incubated on ice for 30 min. After incubation the soluble fraction was collected by centrifuging samples for 1 min at 12,000g and the supernatant (nuclear extract) was transferred to new tubes and stored at -20^oC until they were analyzed by gel electrophoresis. In the MitoQ experiment, cells were pre-incubated with MitoQ or TPMP for 30 minutes and then exposed to 21% O_2 and 1.5% O_2 for 4 hours in the presence of either 5μM MitoQ or TPMP.

Whole Cell Lysate Preparation

A minimum of 8 hours before experimentation 100 mm cell culture dishes containing 10 mL of appropriate culture media were placed in either a cell culture incubator (5% CO_2) or in the hypoxic workstation (1.5% O_2, 5% CO_2, balanced with N_2) to pre-equilibrate. Cells at 70% confluence cultured in 100 mm cell culture dishes were incubated in 10 mL of pre-equilibrated culture media in either hypoxic or normoxic conditions for 4 hrs. To prepare a positive control sample, cells were incubated with 1mM DMOG for 4 hr. After incubation, culture dishes were immediately placed on ice and media was aspirated. Cells were washed with 3 mL cold PBS and scraped in 100-400 μL of 1x cold cell lysis

buffer containing 100 µM PMSF (100 µL of 1x cell lysis buffer per 1x10^6 cells). Cell lysates were transferred to microfuge tubes and incubated on ice for 15 min. Before analysis by gel electrophoresis lysates were subjected to one freeze thaw cycle or the lysates were stored at -20°C until they were analyzed by gel electrophoresis.

Isolation of Mitochondria and Measurement of Cyctochrome c Redox State

2 x 10^7 cells were pelleted by centrifugation at 120 x g and resuspended in ice cold HIM buffer (200mM mannitol, 70 mM sucrose, 1mM EGTA and 10mM HEPES pH7.5) supplemented with 1mM PMSF and cocktail of protease inhibitors (Roche). Cells were lysed with 20 strokes of a dounce homogenizer. Nuclei and unlysed cells were pelleted at 120 x g for 10 minutes. The supernatant was transferred to a new tube and centrifuged at 7500 x g for 15 minutes. The mitochondrial pellet was washed 2X in ice cold HIM buffer and then resuspended in 200µl of HIM buffer. The redox state of cytochrome c was determined by measuring the difference in 550nm and 540nm optical density of the mitochondrial fraction on the tunable VersaMax microplate reader (Molecuarl Devices).

Mitochondria Copy Number Assay

The number of mitochondria was determined by analyzing the abundance of the mitochondrial gene COX1 relative to the nuclear gene 18S. Total DNA was isolated using the DNEasy Tissue kit (Qiagen) according to manufacturer's protocol. 10ng of total DNA was subjected to quantitative Real-Time PCR using Sybr Green Chemistry.

Pimers: 18S forward 5'ACAGGATTGACAGATTGATAGCTC-3' 18S reverse 5'-CAAATCGCTCCACCAACTAAGAA-3' and COX1 forward 5'-CCCACCGGCGTCAAAGTATT-3' reverse 5'-TTTGCTAATACAATGCCAGTCAGG3'.

Oxygen Consumption

Cellular O_2 consumption rates were measured in aliquots of 1-3 X 10^6 sub-confluent cells removed from flasks and studied in a magnetically stirred, water-jacketed (37^oC) anaerobic respirometer fitted with a polarographic O_2 electrode (Oxytherm system, Hansatech Instruments). Oxygraph Plus software was used to determine oxygen consumption rate.

Immunoblotting

Nuclear extracts (30μg) or whole cell lysates (50-80μg) were resolved on a SDS polyacrylamide gel (7.5% gels for HIF-1α, RNA polymerase II, and α-tubulin; 12% gels SHC, cytochrome c, H2AX, γH2AX, pVHL, and Rieske Fe-S protein). Proteins were transferred to nitrocellulose membrane using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Biorad) (15volts for 1 hour and 5 minutes for HIF-1α, RNA polymerase II, catalase, and α-tubulin; 15 volts for 45 minutes SHC, cytochrome c, H2AX, γH2AX, pVHL, CuZnSOD, and Rieske Fe-S protein). Membranes were then blocked for 1 hour in 5% milk in TBS-T. Primary antibody was applied to the membrane in a solution of 5% milk TBS-T overnight at 4^oC with gentile agitation. The following day, membranes were washed 3X with 10mL 5% milk TBS-T for 10 minutes, and then secondary antibodies were applied in 5% milk TBS-T and incubated at room

temperature for 1 hour. Following incubation, membranes were washed 2X for 30 minutes each time with TBS-T. Proteins were visualized by applying SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Antibodies

HIF-1α (BD Biosciences) 1:250, Cytochrome c (BD Biosciences) 2µg/ml, Shc (BD Biosciences) 1:1000, H2AX (Upstate) 1:500, γH2AX (Upstate clone JBW301) 2µg/ml, Rieske Fe-S protein (Molecular Probes) 2µg/ml, pVHL (BD Biosciences) 1:250, α-tubulin (Sigma) 1:2000, RNA polymerase II (Santa Cruz) 1:200, catalase (abcam) 1:500, CuZnSOD (BD Biosciences) 2µg/ml.

Telomerase Activity

Telomerase activity was determined using a quantitative real-time Telomerase Repeat Amplification Protocol method previously described (Wege et al., 2003). Briefly, PHLFs were cultured for 24 hours at 21% O_2 alone or with 100pfu of ODDDwt or ODDDP564A adenovirus, or at 1.5% O_2. Cells were then harvested and washed in 5 ml cold PBS and then 1ml PBS. An aliquot of the 1ml PBS cell suspension was used to count the total number of cells in order to suspended the cells in CHAPS buffer (10mM Tris-HCL pH 7.5, 1mM $MgCl_2$, 1mM EDTA, 5µM β-mercaptoethanol, 10% glycerol, 0.1mM PMSF, 0.5% CHAPS) at 10^5 cells/µl. Suspension was incubated on ice for 30 minutes and then centrifuged at 16,000g for 30 minutes. Supernatant was harvested and 1µl was mixed on ice with 0.1 ug of telomerase primer TS (5'-AATCCGTCGAGCAGAGTT-3'), 0.05 ug of anchored return primer ACX (5'-GCGCGG[CTTACC]₃CTAACC-3'), 25 ul of

SYBR Green PCR Mastermix (Bio-Rad), and water to 50 μl. Using the iQ Icycler (Bio-Rad) samples were incubated at 25°C for 20 minutes and then amplified by PCR using a 2 step PCR for 35 cycles (30sec at 95°C then 90sec at 60°C). Sample C_t values were compared to a standard curve generated by serial dilution of extract obtained from telomerase positive 293T cells.

Statistical analysis

The data presented are means ± standard errors of the means. Data were analyzed by two-way analysis of variance using Graph Pad Prism 4. When the analysis of variance indicated a significant difference, individual differences were explored with paired t test. Statistical significance was determined at the 0.05 level.

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Publications (in chronological order):

Research Papers

1. Doyle DD, Upshaw-Earley J, **Bell EL**, Palfrey HC. Natriuretic peptide receptor-B in adult rat ventricle is predominantly confined to the nonmyocyte population. Am J Physiol Heart Circ Physiol. 2002 Jun;282(6):H2117-23.

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7. Robert D. Guzy, Bhumika Sharma, **Eric L. Bell**, Navdeep S. Chandel,Paul T. Schumacker. Alterations in ROS signaling mediate the effects of mitochondrial complex II disruption on HIF-1α stabilization and tumor growth (Submitted to EMBO)

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3. **Eric L. Bell,** Tatyana A. Klimova, and Navdeep S. Chandel. Mitochondrial Oxygen Sensing: Regulation of Hypoxia Inducible Factor by Mitochondrial Generated Reactive Oxygen Species. (Submitted Essays in Biochemistry)

4. **Eric L. Bell,** and Navdeep S. Chandel. Mitochondrial electron transport through the complex III and oxygen sensing. (Submitted Methods In Enzymology)

Abstracts

1. **Eric L. Bell** and Navdeep S. Chandel. Hypoxia Increases Reactive Oxygen Species and Cellular Lifespan. Cellular Senescence and Cell Death Keystone Meeting 2005

2. **Eric L. Bell**, Tatyana A Klimova, and Navdeep S. Chandel. The Q_o site of the bc_1 complex is necessary for hypoxic stabilization of the HIF-1α protein. Hypoxia and Development, Physiology and Disease Keystone Meeting 2006

3. **Eric L. Bell**, Tatyana A. Klimova, James Eisenbart, Paul T. Schumacker, and Navdeep S. Chandel. Mitochondrial ROS Trigger HIF Dependent Extension of Replicative Lifespan During Hypoxia. Mechanisms and Models of Cancer Cold Spring Harbor 2006