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Signaling Pathways Regulating HIF-1

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ABSTRACT

Signaling Pathways Regulating HIF-1

Brooke Michele Emerling

Hypoxia is a reduction in the normal level of oxygen tension and occurs during acute and chronic vascular disease, pulmonary disease, and cancer. The key transcription factor that regulates the cellular responses to hypoxia is hypoxia-inducible-factor-1 (HIF-1). Understanding how HIF-1 is regulated is fundamental for understanding the biology of tumor growth. My thesis demonstrates two novel signaling pathways that regulate HIF-1. First, I demonstrate that *p38α*$^{-/-}$ cells fail to activate HIF-1 under hypoxic conditions. Cells deficient in Mkk3 and Mkk6, the upstream regulators of p38α, also fail to activate HIF-1 under hypoxic conditions. Furthermore, the hypoxic activation of p38α and HIF-1 was abolished by myxothiazol, a mitochondrial complex III inhibitor, and glutathione peroxidase 1 (GPX1), a scavenger of hydrogen peroxide. Thus, the activation of p38α and HIF-1 is dependent on the generation of mitochondrial reactive oxygen species (ROS). These results provide genetic evidence that the p38 MAPK signaling pathway is essential for HIF-1 activation. To further elucidate the direct targets of the ROS or the upstream regulators of p38 MAPK pathway a candidate approach was taken. Interestingly, the hypoxic activation of HIF-1 is independent of SRC, ASK1, and AMPK.

The tumor suppressor PTEN is mutated or deleted in many tumors, causing the activation of the PI3K pathway. Here, I show that the loss of PTEN increases the transcriptional activity of HIF-1 independent of mTOR signaling, but dependent on the inactivation of Forkhead transcription factors (FOXO) in PTEN null cells. Reintroduction of PTEN in the nucleus, overexpression of a non-phosphorylatable FOXO3a, which accumulates in the nucleus, or inhibition of nuclear

export of FOXO3a by leptomycin B repress HIF-1 transcriptional activity in PTEN null cells. PTEN and FOXO3a regulate the transactivation domain of HIF-1α. Co-immunoprecipitation shows that endogenous FOXO3a can associate with HIF-1α and p300 in the nucleus. Chromatin immunoprecipitation indicates that FOXO3a complexes with HIF-1α and p300 on the *Glut-1* promoter, a HIF-1 target gene. Overexpression of p300 reverses FOXO3a mediated repression of HIF-1 transcriptional activity. Thus, FOXO3a negatively regulates HIF-1 transcriptional activity by interfering with p300's ability to function as a transcriptional co-factor.

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CHAPTER 1: Introduction

Oxygen homeostasis is essential for normal development and physiology. As a result, cellular and systemic oxygen concentrations are precisely regulated. Disruption of oxygen homeostasis is a critical attribute in the pathophysiology of heart disease, vascular disease, pulmonary disease, and cancer. As mammalian cells encounter lower oxygen levels (hypoxia, 2-20 TORR or 0.3%-3% O_2) they have mechanisms to prevent depletion of oxygen to anoxic levels (0-2 TORR or 0-0.3% O_2). Cells that reside under hypoxia do not undergo cell death and cellular bioenergetics are not compromised. However, cells that encounter anoxia for a sustained period will commit to cell death (Graeber et al., 1996; Schroedl et al., 2002). Hypoxia induces a transcriptional program that promotes an aggressive tumor phenotype. Hypoxic cancer cells are resistant to both chemotherapy and radiation, thereby making them a major reason for the failure of cancer therapy. Like normal tissues, tumors require an adequate supply of oxygen. Hypoxia can limit tumor growth, and tumors with poor vascularization fail to grow and form metastases. However, hypoxia can also select for adaptive responses that allow tumor cells to survive. These responses include the induction of angiogenesis and a switch to anaerobic metabolism. The key transcription factor that regulates the cellular responses to hypoxia is hypoxia-inducible-factor-1 (HIF-1). How HIF-1 is regulated is a fundamental question in cancer biology and my research presented here illustrates two novel pathways that regulate HIF-1, thereby, providing significant information that may aid in designing effective targeted therapies for the treatment of cancer.

Mechanisms of Hypoxic Signaling

Hypoxia Inducible Factor -1 (HIF-1)

HIF-1 is a heterodimeric transcription factor that consists of HIF-1α and HIF-1β subunits (Wang et al., 1995). HIF-1β is also known as aryl hydrocarbon nuclear receptor translocator (ARNT) (Wang et al., 1995). Both HIF-1α and HIF-1β are members of the basic helix-loop-helix Per/Arnt/Sim (bHLH-PAS) transcription factor family (Wang et al., 1995; Wang and Semenza, 1995). The functions of HIF-1α are mediated by distinct protein domains (Figure 1.1). Dimerization of HIF-1α and HIF-1β occur via the bHLH and part of the PAS domain of HIF-1α. The basic domain is specifically required for DNA-binding activity (Jiang et al., 1996a). Two domains within the C-terminal half of HIF-1α mediate transcriptional activation and the interaction with coactivators. These domains are termed N-terminal (N-TAD) and C-terminal (C-TAD) transactivation domains (Pugh et al., 1997). Additionally, there is an oxygen-dependent degradation (ODD) domain, which partly overlaps with N-TAD (Figure 1.1). The ODD domain controls the protein stability of HIF-1α as a function of the oxygen tension (Huang et al., 1998; Jiang et al., 1997). HIF-1 activity is dependent upon the availability of the HIF-1α subunit, which is tightly controlled by cellular oxygen tension (Figure 1.2) (Huang et al., 1996; Kallio et al., 1997).

Under normal oxygen conditions (21% O_2), HIF-1α is polyubiquitinated and targeted for degradation by an E3 ubiquitin ligase complex that contains the von Hippel-Lindau tumor suppressor protein (pVHL), elongin B, elongin C, Cul2, and Rbx. This process is primarily regulated by prolyl hydroxylation at residues Pro402 and Pro564 of HIF-1α, which mediates the binding of pVHL (Jaakkola et al., 2001; Maxwell et al., 1999). Three closely related 2-

oxoglutarate-dependent oxygenases with the capacity to catalyze HIF prolyl hydroxylation at residues 402 and 564 have been identified (Bruick and McKnight, 2001; Epstein et al., 2001). They are known as prolyl hydroxylase domain (PHD) 1, PHD2, and PHD3. It has been shown that PHD2 is primarily responsible for determining HIF-1α stability as a function of oxygen availability (Berra et al., 2003). These HIF hydroxylases or PHDs require Fe^{++}, oxygen, and 2-oxoglutarate to catalyze the hydroxylation reaction. The requirement of iron and 2-oxoglutarate by the PHDs explains the same effects that can be seen by treatments known to mimic hypoxia-inducible expression, such as iron chelation (desferrioxamine-DFO), iron antagonists (cobalt chloride), and oxoglutarate analogues (dimethyloxaloylglycine-DMOG). Additionally, HIF-1α is hydroxylated at Asn803 by factor inhibiting HIF-1 (FIH-1) (Hewitson et al., 2002; Lando et al., 2002a; McNeill et al., 2002). FIH-1 prevents the binding of the coactivators p300 and CBP to HIF-1α, thus inhibiting HIF-1 mediated gene transcription. Under hypoxic conditions, the rate of hydroxylation is decreased. Consequently, pVHL cannot target HIF-1α for degradation, thereby allowing HIF-1α to accumulate in the cell. HIF-1α can then localize to the nucleus, where it dimerizes with HIF-1β and binds to hypoxia response elements (HREs) within the promoter regions of its target genes. The HRE motif (G/ACGTG) is associated with a broad range of transcriptional targets. To date, more than 60 HIF-1 target genes have been identified and many of these genes are involved in cancer biology, including angiogenesis, cell survival, oxygen transport, growth factor signaling, glucose metabolism, metastasis and invasion (Semenza, 2003).

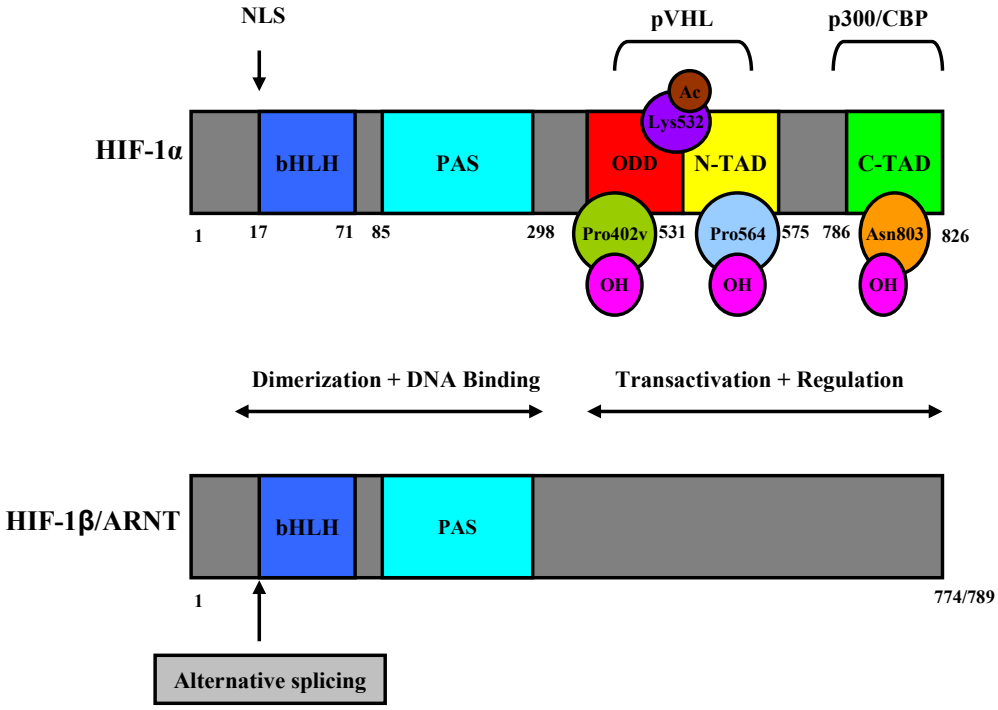

Figure 1.1. Structure of HIF-1α and HIF-1β/ARNT.

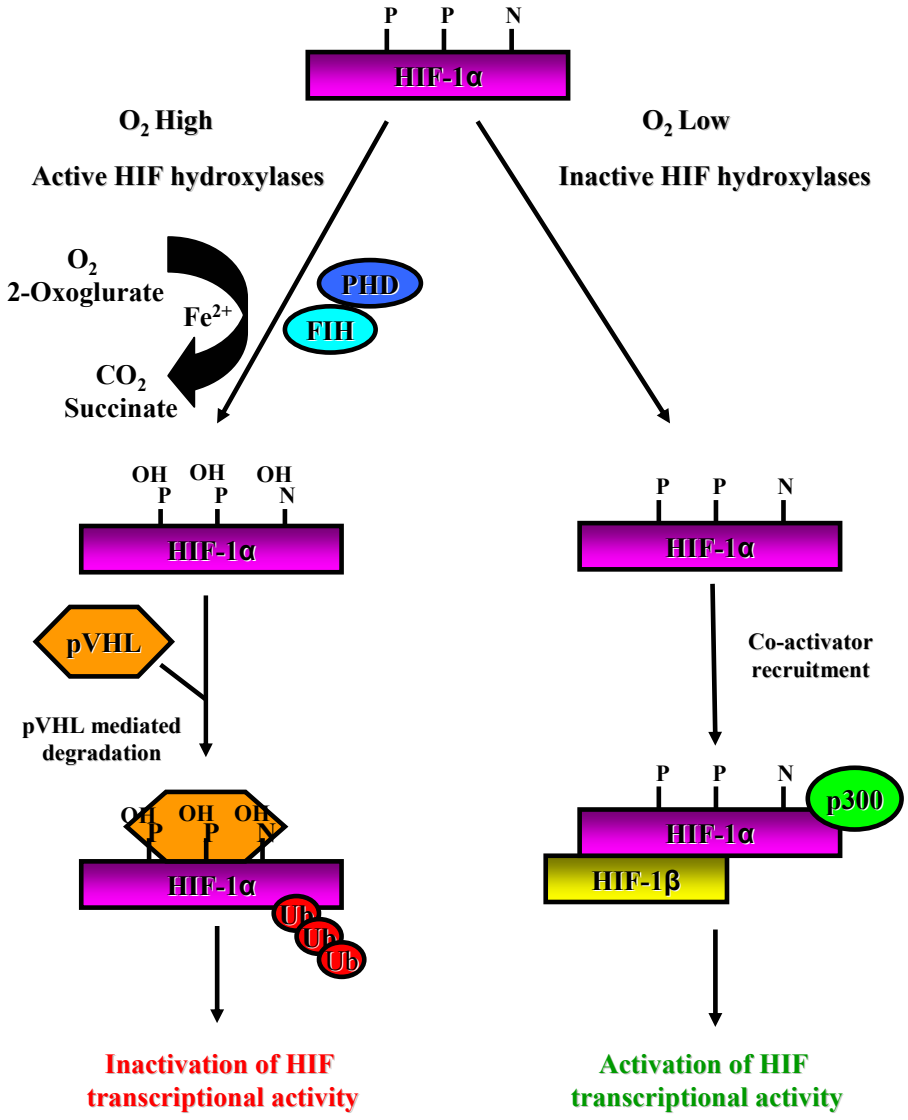

Figure 1.2. HIF-1α regulation by hydroxylation.

Oxygen sensing by the Mitochondria

A critical question concerning HIF-1 regulation is how cells sense the lack of oxygen and ultimately activate HIF-1. Our laboratory has proposed a model in which complex III of the mitochondrial electron transport chain serves as an oxygen sensor for HIF-1 activation under hypoxia. In support of this model, it has been demonstrated that hypoxia increases the generation of reactive oxygen species (ROS) within complex III to induce the stabilization of HIF-1α (Brunelle et al., 2005; Chandel et al., 2000; Guzy et al., 2005; Mansfield et al., 2005; Schroedl et al., 2002). Mitochondrial ROS that is generated within complex III can be blocked by using myxothiazol, an electron transport chain inhibitor (Figure 1.3). Myxothiazol blocks the stabilization of HIF-1α under hypoxic conditions (1.5% O_2), but not anoxia (0% O_2) or normoxia (21% O_2) in the presence of the iron chelator DFO (Chandel et al., 2000; Schroedl et al., 2002). Additionally, ρ^0 cells, cells depleted of their mitochondrial DNA, are unable to carry out functional electron transport and can not stabilize the HIF-1α protein under hypoxia (1.5% O_2) (Schroedl et al., 2002). In contrast, ρ^0 cells are able to stabilize HIF-1α under anoxia (0% O_2) or in the presence of DFO under normoxia (Schroedl et al., 2002). Moreover, it has been shown that antioxidants can abolish the HIF-1α response and that exogenous H_2O_2 during normoxia can activate HIF-1α (Chandel et al., 2000). These data suggest that mitochondrial ROS production may trigger a signal transduction pathway, leading to the stabilization of HIF-1α. The site of ROS generation during hypoxia is localized to complex III within the mitochondrial electron transport chain (Chandel et al., 1998; Chandel et al., 2000). The superoxide generated during the Ubiquinone (Q)-cycle within the mitochondrial electron complex III is considered the main site of ROS production within the electron transport chain (Figure 1.3). Superoxide anion is converted to hydrogen peroxide in the mitochondria by the manganese superoxide dismutase

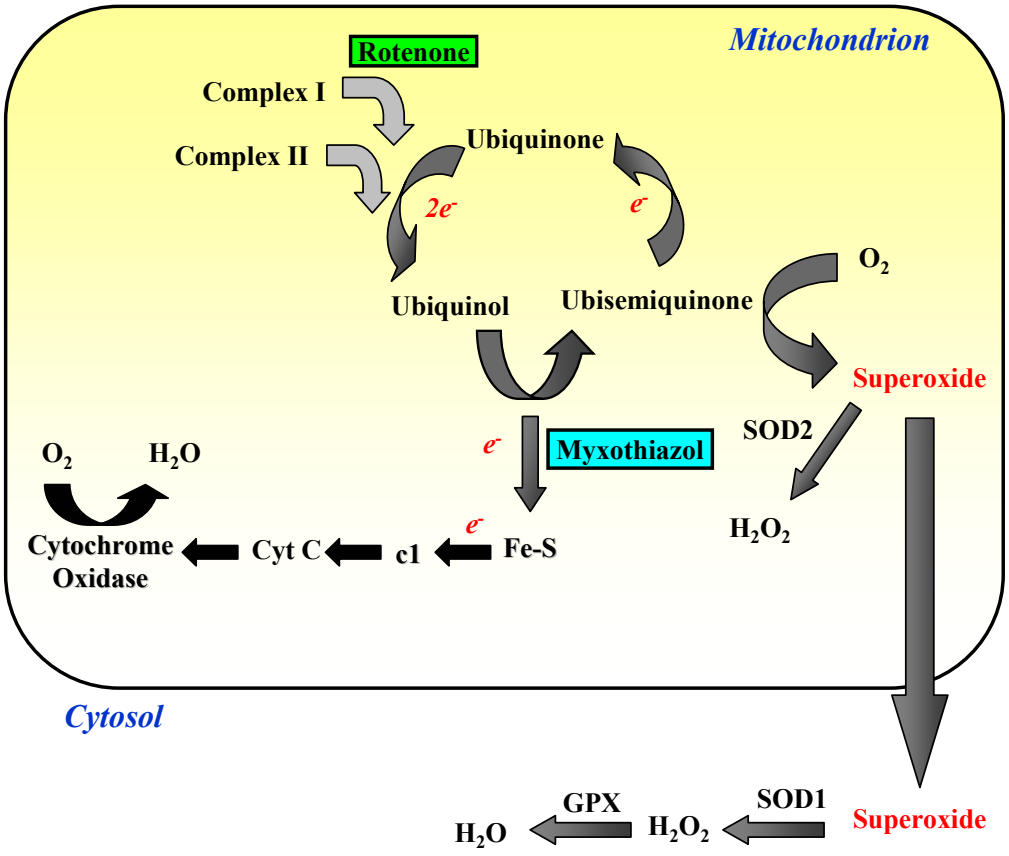

Figure 1.3. Mitochondrial model of oxygen sensing.

(SOD2) or in the cytosol by copper zinc superoxide dismutase (SOD1). In the cytosol, hydrogen peroxide is converted into water primarily by glutathione peroxidase 1 (GPX1) (Figure 1.3). Cells overexpressing SOD1 or SOD2 do not alter HIF-1α stabilization under hypoxia, while overexpression of GPX1 decreased HIF-1α stabilization under hypoxia (Brunelle et al., 2005). These results are consistent with findings demonstrating that catalase, an antioxidant enzyme, inhibits HIF-1α stabilization under hypoxic conditions, while exogenous H_2O_2 stabilizes HIF-1α under normoxia (Brunelle et al., 2005; Chandel et al., 2000). Thus, the hypoxic stabilization of HIF-1α requires H_2O_2 and not superoxide. Additionally, mitochondrial complex I inhibitors, such as rotenone, that prevent electron flux upstream of complex III, ablate ROS production during hypoxia and the hypoxic stabilization of HIF-1α (Figure 1.3).

Based on all the above results, we propose that under anoxic conditions the hydroxylation of residues within the HIF-1α protein by the hydroxylases can not occur and intracellular signaling molecules are not required for the stabilization of HIF-1α. However, during hypoxia intracellular signaling molecules, such as mitochondrial derived ROS regulate the stabilization of HIF-1α. An interesting corollary is that the mitochondria can not generate ROS during anoxia, due to the lack of oxygen as a substrate for electrons. The downstream signaling pathways that connect mitochondrial ROS to HIF-1α stabilization have not been identified. Cells recognize and respond to cellular stress by engaging specific signaling cascades, such as the p38 mitogen activated protein kinase (MAPK) signaling pathway (Figure 1.4). Several groups have shown that the p38 MAPK signaling pathway is activated by ROS and by hypoxia, although, p38 MAPK's relationship to mitochondrial ROS signals or to the activation of HIF-1 has not been described.

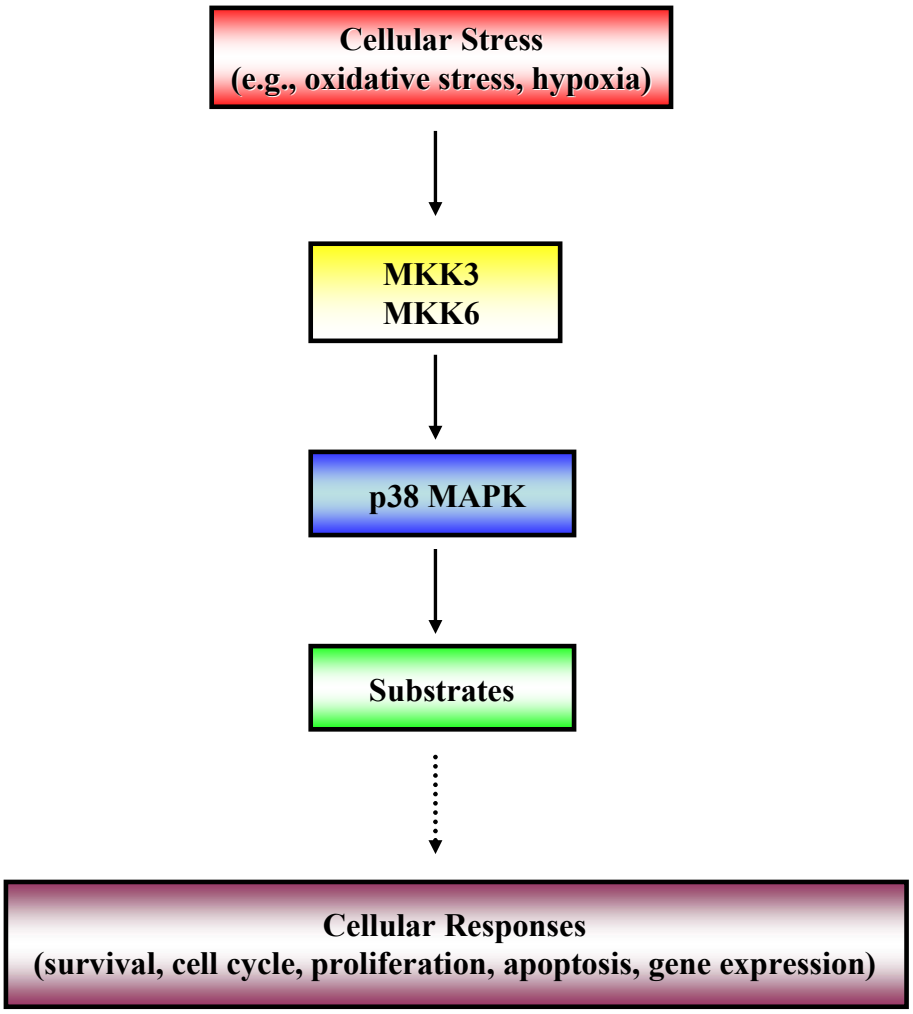

Figure 1.4. Overview of p38 MAPK signaling.

The first part of my thesis will focus on the role of the p38 MAPK signaling pathway in the hypoxic activation of HIF-1, thereby for the first time linking the mitochondrial generated ROS during hypoxia to the activation of HIF-1.

PI3K-PTEN signaling pathway

Hypoxia is a potent stimulus for triggering the 'angiogenic switch' and HIF-1 is the main transcription factor that regulates hypoxia induced angiogenesis (Semenza, 2000a). The activation of oncogenes and loss of tumor suppressor genes are also crucial for tumor angiogenesis. The tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) has been shown to regulate angiogenesis during normoxia and hypoxia and many PTEN null tumors are highly vascularized and display increased HIF-1 activity (Giri and Ittmann, 1999; Hsu et al., 1996; Jiang et al., 2001; Jiang et al., 2000; Wen et al., 2001; Wesseling et al., 1997; Zhong et al., 2000; Zundel et al., 2000). Therefore, activation of HIF-1, complemented by the loss of PTEN may result in a more aggressive cancer phenotype. The phosphatidylinositol 3-kinase (PI3K)-PTEN signaling pathway is one of the most frequently altered pathways in human tumors. Loss of the PTEN is the most common mechanism of activation of the PI3K pathway in human cancers. PTEN antagonizes PI3K signaling and regulates the subsequent activation of AKT (Vazquez and Sellers, 2000). In cells deficient in PTEN, substrates of AKT are aberrantly phosphorylated. Critical downstream targets of AKT include, the mammalian target of rapamycin (mTOR) and the Forkhead (FOXO) transcription factors (Brunet et al., 1999; Gao et al., 2002b; Inoki et al., 2002; Kops et al., 1999; Tee et al., 2003) (Figure 1.5). The connection between the loss of PTEN and HIF-1 activation in cancer has remained unclear. Therefore, understanding how the loss of PTEN and increase in HIF-1 activity

are correlated is fundamental for cancer biology. The second part of my thesis will explore the role of the tumor suppressor PTEN and its downstream target FOXO3a in regulating HIF-1. My findings highlight the importance of subcellular localization and suggest that by inhibiting FOXO3a nuclear export in PTEN null cancers may serve as a potential treatment in HIF-1 dependent tumors.

Consequently by defining the regulatory roles of the p38 MAPK and PI3K/PTEN/FOXO pathways in HIF-1 activation, my research has advanced the field of HIF-1 biology and has identified novel targets for cancer therapies.

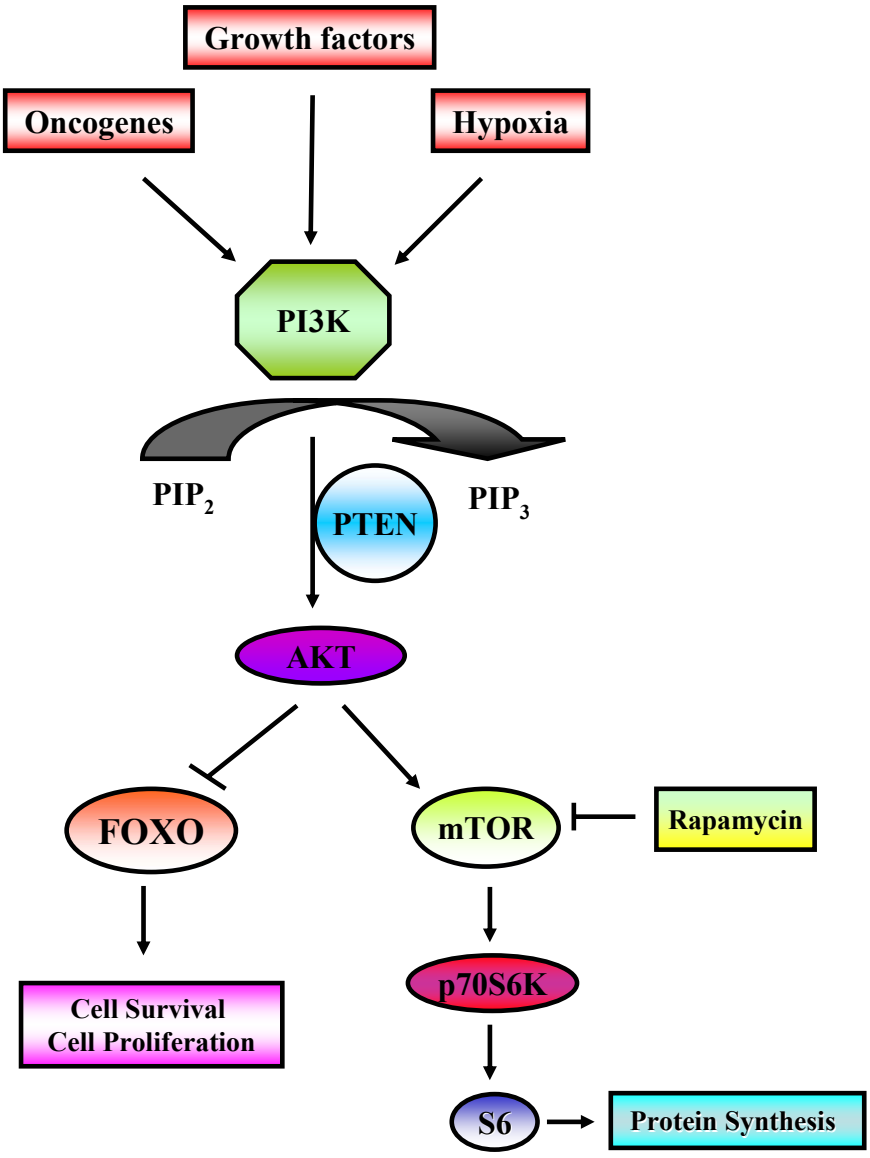

Figure 1.5. Overview of PI3K/PTEN signaling.

CHAPTER 2: Mitochondrial Reactive Oxygen Species Activation of p38 Mitogen-Activated Protein Kinase Is Required for Hypoxia Signaling

Introduction

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that regulates physiological responses to hypoxia, including placental development, and pathophysiological processes such as cancer (Semenza, 2000b). HIF-1 is composed of two subunits, an oxygen-sensitive HIF-1α subunit and a constitutively expressed HIF-1ß subunit. Under normal oxygen conditions, HIF-1α is polyubiquitinated and targeted for degradation by an E3 ubiquitin ligase complex that contains the von Hippel-Lindau tumor suppressor protein (pVHL), elongin B, elongin C, Cul2, and Rbx (Maxwell et al., 1999). This process is dependent on the hydroxylation of two proline residues by a family of prolyl hydroxylase (PHD) enzymes, which mediates the binding of pVHL (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001). PHDs utilize oxygen as a substrate and iron as a cofactor to hydroxylate proline residues of HIF-1α (Bruick and McKnight, 2001; Epstein et al., 2001). Oxygen tension also regulates the interaction of HIF-1α with the transcriptional coactivators p300 and CBP. Asparagine hydroxylation of residue 803 of HIF-1α by the enzyme FIH-1 (factor inhibiting HIF-1) blocks the binding of p300 and CBP to HIF-1α, thus inhibiting HIF-1-mediated gene transcription (Lando et al., 2002a; Lando et al., 2002b; Mahon et al., 2001). Under hypoxic conditions or in the presence of iron chelators, the rate of proline and asparagine hydroxylation is decreased. Consequently, pVHL cannot target HIF-1α for degradation, thereby allowing HIF-1α to accumulate and dimerize with HIF-1β in the cell. Moreover, p300 and CBP can then be recruited to the HIF-1 complex, allowing transcriptional activation of HIF-1 target genes.

The signaling mechanisms that regulate the hypoxic activation of HIF-1 are not fully understood. Current models suggest that signaling pathways are not involved in the hypoxic activation of HIF-1 (Semenza, 2001). This is based on the observation that during hypoxia the decline in oxygen levels directly decreases the activity of the PHDs, thereby preventing hydroxylation of the HIF-1α protein (Hirsila et al., 2003). The lack of hydroxylation inhibits pVHL from binding to HIF-1α, allowing HIF-1α to be stabilized. In contrast, there have been reports to indicate that intracellular signaling pathways are required for HIF-1 activation during hypoxia. These include but are not limited to the requirement of diacylglycerol kinase, small GTPases, mitochondrial reactive oxygen species (ROS), and phosphatidylinositol 3-kinase (PI3-K)/AKT (Aragones et al., 2001; Chandel et al., 2000; Gerald et al., 2004; Hirota and Semenza, 2001; Hudson et al., 2002; Turcotte et al., 2003; Zhong et al., 2000; Zundel et al., 2000). The requirement of signaling pathways in the activation of HIF-1 suggest that the hydroxylases are not the sole regulators of HIF-1 during hypoxia.

The p38α mitogen-activated protein kinase (MAPK) is a stress kinase that is activated by dual phosphorylation on Thr and Tyr in a Thr-X-Tyr motif located within the activation loop proximal to the ATP- and substrate-binding sites (Raingeaud et al., 1995). This phosphorylation is meditated by the upstream MAPK kinases (MAPKKs) MKK3 and MKK6 (Derijard et al., 1995; Raingeaud et al., 1996). Mice deficient in p38α MAPK demonstrate embryonic lethality at day 10.5 and display abnormal vascularization associated with the placenta, resembling the phenotype seen in the *Hif-1β*$^{-/-}$ embryos (Adams et al., 2000; Adelman et al., 2000; Allen et al., 2000; Maltepe et al., 1997; Mudgett et al., 2000; Tamura et al., 2000). The phenotype of the *Mkk3/6*$^{-/-}$ mice also resembles the *p38α*$^{-/-}$ embryos (Brancho et al., 2003). Pharmacologic

inhibition of p38α MAPK has been shown to decrease hypoxic activation of HIF-1 (Hirota and Semenza, 2001; Shemirani and Crowe, 2002). Nonhypoxic stimuli such as exposure to hepatocyte growth factor (HGF), chromium, or arsenite activates HIF-1 through oxidant induction of p38 MAPK (Derijard et al., 1995; Gao et al., 2002a; Tacchini et al., 2001). Furthermore, p38α MAPK is activated by mitochondrial ROS during hypoxia (Kulisz et al., 2002). Based on these previous reports, in the present study we genetically tested whether mitochondrial oxidant activation of the p38 MAPK signaling pathway is required for the hypoxic activation of HIF-1.

Results

p38α MAPK is required for hypoxic activation of HIF-1.

To elucidate the upstream signaling mechanisms that regulate the hypoxic activation of HIF-1, we used fibroblasts isolated from wild-type (WT) and p38α null embryos (Figure 2.1A) (Porras et al., 2004). HIF-1 activity is dependent on the stability of the oxygen sensitive subunit, HIF-1α. WT or *p38α*$^{-/-}$ cells were exposed to hypoxia (1.5% O_2) or normoxia (21% O_2) in the presence or absence of the iron chelator desferrioxamine (DFO; 100 μM) to examine whether p38α is required for the stabilization of the HIF-1α protein. Loss of *p38α* completely suppressed the stabilization of the HIF-1α protein under hypoxia, whereas HIF-1α protein stability was unaffected with treatment of DFO in either the WT or *p38α* -deficient cells (Figure 2.1B). Since PHDs require iron as a cofactor to execute hydroxylation, it is likely that DFO inhibits PHD activity directly by chelating intracellular iron. These results were further confirmed by examining HIF-1 activity using a luciferase reporter assay under the control of a promoter containing three hypoxic response element sites (HRE-Luciferase). Hypoxia and DFO increased HRE-dependent luciferase induction in WT cells (Figure 2.2). In contrast, *p38α*$^{-/-}$ cells displayed a marked attenuation of luciferase induction under hypoxia but not in the presence of DFO during normoxia (Figure 2.2). These results indicate that the hypoxic activation of HIF-1 is dependent on p38α MAPK and hypoxia and iron chelators have distinct mechanisms for activating HIF-1.

HIF-1α contains two oxygen regulated transactivation domains (TADs), which are termed N-TAD (amino acids 531 to 575) and C-TAD (amino acids 786 to 826) (Figure 1.1) (Jiang et al., 1997). The C-TAD is regulated by the hydroxylation of an asparagine residue by FIH to prevent

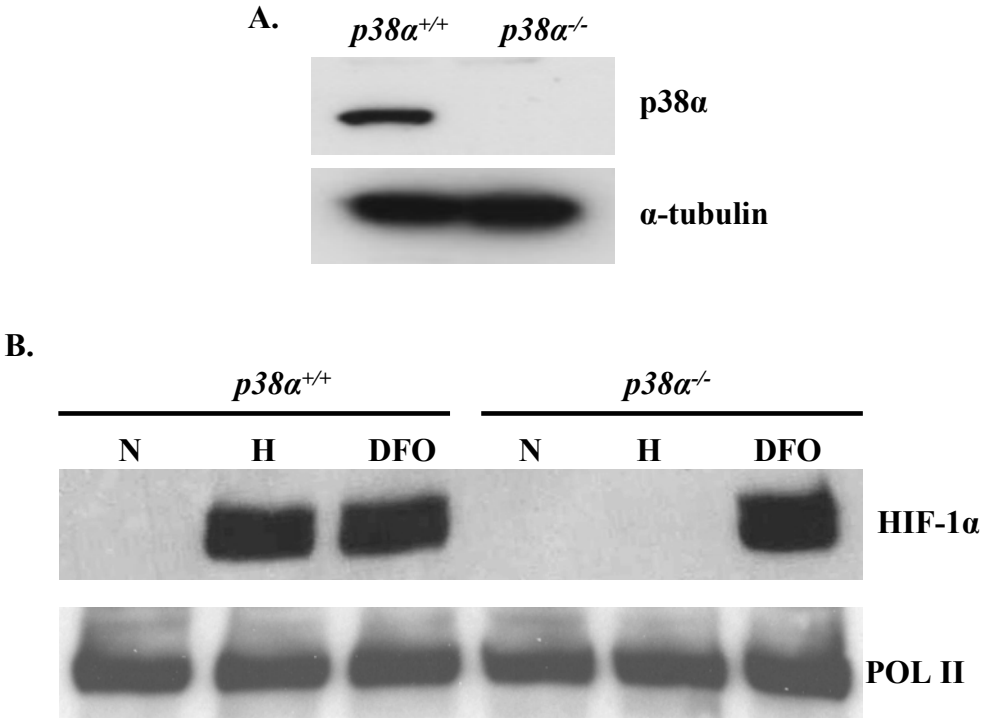

Figure 2.1. p38 α MAPK is required for HIF-1α protein stability.

(A). p38α MAPK protein levels in *p38α*$^{+/+}$ and *p38α*$^{-/-}$ cells**.** **(B).** HIF-1α protein levels in *p38α*$^{+/+}$ and *p38α*$^{-/-}$ cells exposed to 21% O_2 (N) ± 100 μM DFO or to 1.5% O_2 (H) for 2 h.

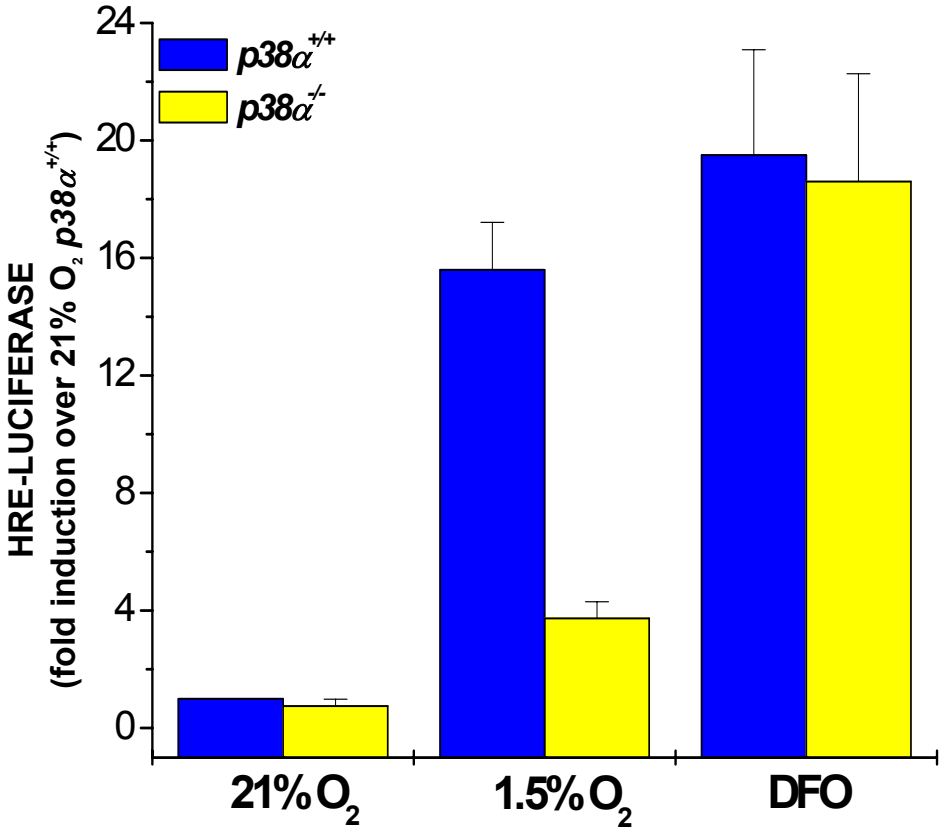

Figure 2.2. p38α MAPK is required for hypoxic activation of HIF-1.

p38α$^{+/+}$ and *p38α*$^{-/-}$ cells transfected with the HRE-Luciferase reporter gene construct and exposed to 21% O_2 ± 100 μM DFO or to 1.5% O_2 for 16 h. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to *p38α*$^{+/+}$ cells. The data presented are the mean (±SEM) of four independent transfections.

the interaction of the HIF-1α protein with transcriptional coactivators such as p300. To examine the activity of the transactivation domains a GAL4 DNA-binding domain (amino acids 1 to 147) fused to HIF-1α (531 to 826) protein can be utilized. Previous work shows that steady-state levels of this fusion protein do not change between normoxic and hypoxic conditions (Jiang et al., 1997). Therefore, we used the GAL4-HIF-1α (531 to 826) fusion construct to investigate the transcriptional activity of HIF-1α independent of its protein expression level. Transactivation by GAL4-HIF-1α (531 to 826) was induced by hypoxia and DFO in WT cells (Figure 2.3). In contrast, cells deficient in *p38α* failed to induce transactivation under hypoxia mediated by GAL4-HIF-1α (531 to 826). Furthermore, transactivation by GAL4-HIF-1α (531 to 826) was induced by DFO in *p38α* null cells, similar to the induction seen in the WT cells. HIF-1 transcriptional activity was further assessed by examining the induction of HIF-1 target genes, phosphoglycerate kinase 1 (*Pgk1*) and glucose transporter 1 (*Glut-1*), using real-time quantitative RT-PCR. WT cells exposed to hypoxia displayed an eightfold increase in both *Pgk1* and *Glut-1* levels, whereas $p38\alpha^{-/-}$ cells had diminished the transcription of *Pgk1* and *Glut-1* under hypoxic conditions (Figure 2.4). Moreover, *Pgk1* and *Glut-1* gene induction in WT and $p38\alpha^{-/-}$ cells exposed to DFO were significantly increased to similar levels. These results suggest that p38α MAPK is specifically required for HIF-1α transcriptional activity that is induced by hypoxia, but not by DFO.

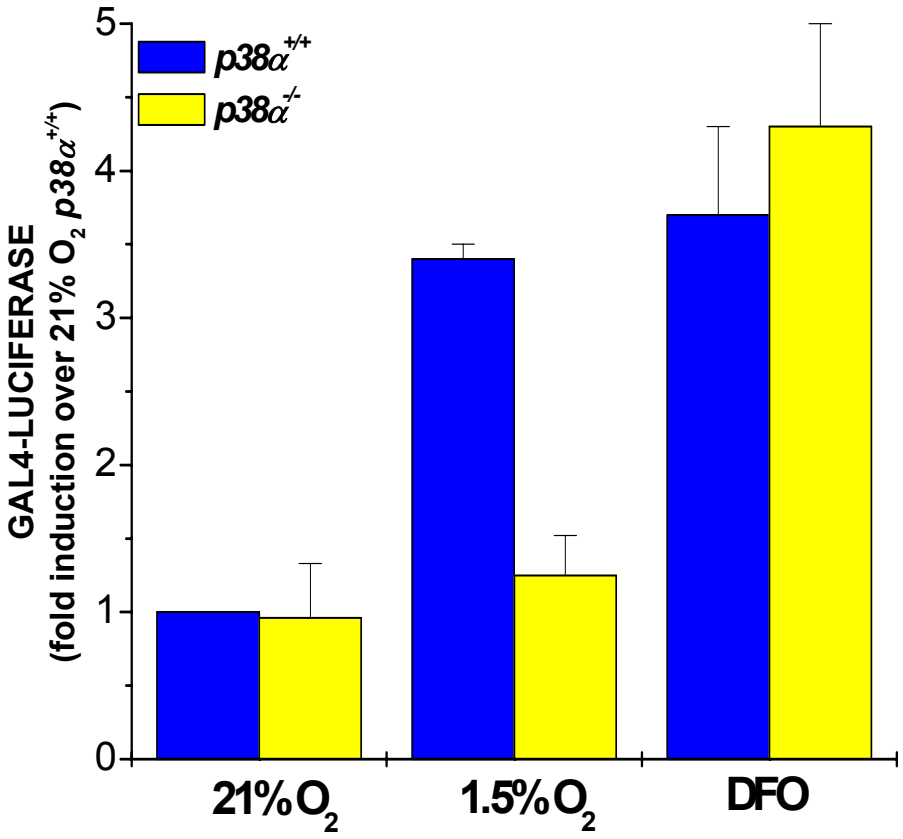

Figure 2.3. p38α MAPK is required for HIF-1α transactivation.

p38α$^{+/+}$ and *p38α*$^{-/-}$ cells were transfected with a GAL4 (1-147) DNA-binding domain fused to HIF-1α (531-826) construct and a reporter gene construct encoding five GAL4-binding sites. Cells were incubated at 21% O_2 for 20 hours, followed by 36 hours at 21% O_2 ± 100 μM DFO or to 1.5% O_2. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to *p38α*$^{+/+}$ cells. The data presented are the mean (±SEM) of four independent transfections.

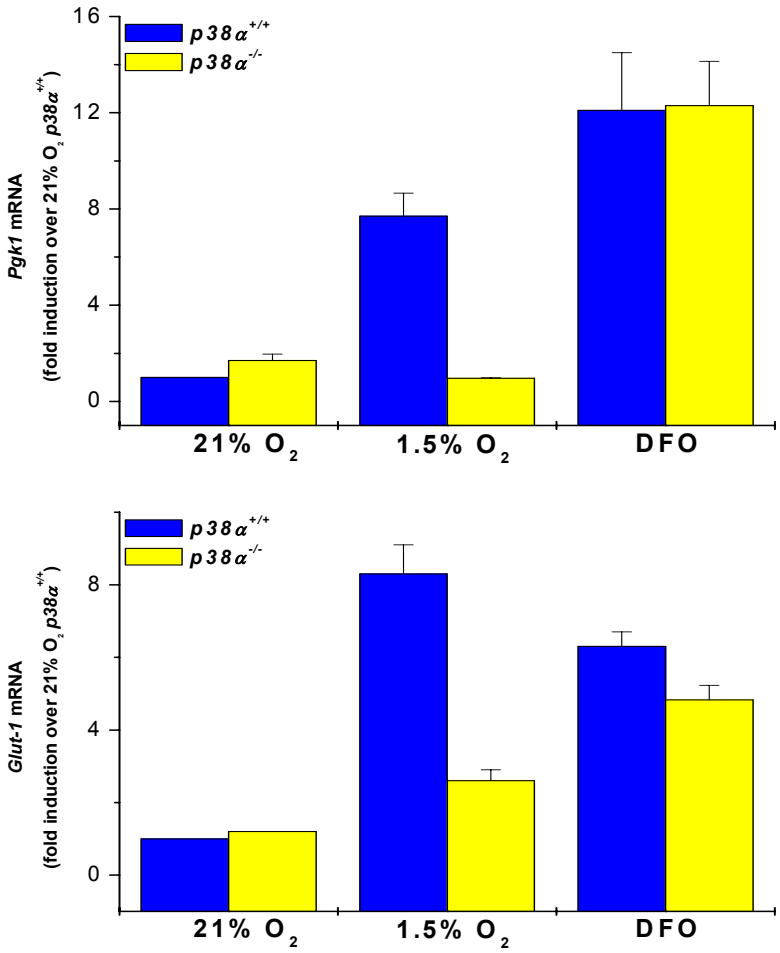

Figure 2.4. p38α MAPK is required for hypoxic induction of HIF-1 target genes. Cells were cultured for 16 hours under 21% O_2 ± 100 μM DFO or to 1.5% O_2, harvested, and transcription levels of the target genes *Pgk1* and *Glut-1* were determined by quantitative real-time RT-PCR analysis. Cycle Threshold (Ct) values were normalized for amplification of the mitochondrial ribosomal protein *L19*. The data presented are the result of triplicate analyses and the error bars indicate SEM.

Anoxia activation of HIF-1 does not require p38α MAPK.

We have previously reported that cells exposed to anoxia (~0% O_2) differ in their mechanism of HIF-1 activation compared to cells exposed to hypoxia (Schroedl et al., 2002). Cells exposed to hypoxia fail to activate HIF-1 in the presence of mitochondrial inhibitors or in cells that lack mitochondrial DNA [*rho*^{0}] cells). In contrast, cells exposed to anoxia retain their ability to activate HIF-1 even in the presence of mitochondrial inhibitors or in [*rho*^{0}] cells. These results suggest that hypoxia but not anoxia utilizes a functional electron transport chain to activate HIF-1. Anoxia directly limits oxygen availability to the hydroxylases to stabilize HIF-1α protein and thus would not require activation of any signaling mechanisms. To further test whether anoxia requires p38 MAPK signaling, WT or *p38α*$^{-/-}$ cells were exposed to anoxia or normoxia. Loss of p38α did not affect the stabilization of the HIF-1α protein under anoxia (Figure 2.5A). Anoxia also increased HRE-dependent luciferase to similar levels in both WT and *p38α*$^{-/-}$ cells (Figure 2.5B). These results indicate that the anoxic activation of HIF-1 is independent of p38α MAPK and that hypoxia and anoxia have distinct mechanisms for activating HIF-1.

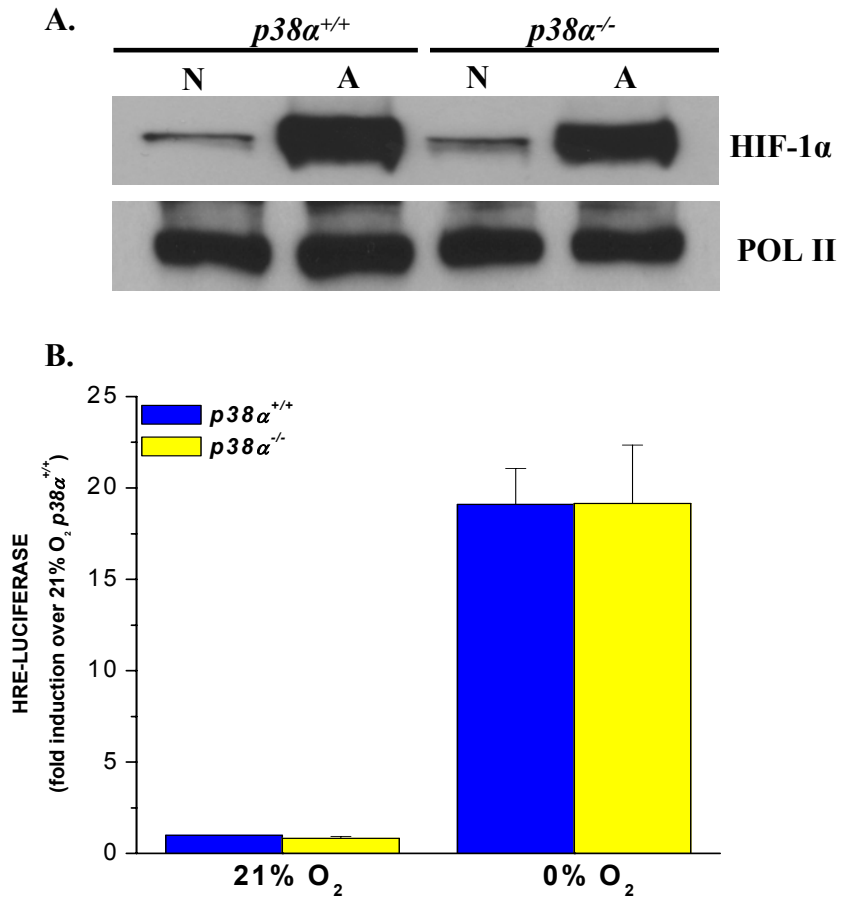

Figure 2.5. p38α MAPK is not required for anoxic activation of HIF-1.

(A). HIF-1α protein levels in *p38α*$^{+/+}$ and *p38α*$^{-/-}$ cells exposed to 21% O_2 (N) or to 0%O_2 (A) for 2 h. **(B).** *p38α*$^{+/+}$ and *p38α*$^{-/-}$ cells transfected with the HRE-Luciferase reporter gene construct and exposed to 21% O_2 or to 0%O_2 for 16 h. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to *p38α*$^{+/+}$ cells. The data presented are the mean (±SEM) of four independent transfections.

Reintroduction of p38α MAPK rescues hypoxic activation of HIF-1.

To confirm that the hypoxic activation of HIF-1 was directly due to the absence of *p38α*, *p38α* $^{-/-}$ cells were stably reconstituted with a p38α cDNA or with vector alone. Immunoblot analysis demonstrates that the reconstituted cells stably express p38α (Figure 2.6A). The reintroduction of p38α rescued the hypoxic stabilization of the HIF-1α protein in *p38α*$^{-/-}$ cells (Figure 2.6B). The transcriptional activity of HIF-1, as assessed by HRE-Luciferase and the GAL4-HIF-1α (531 to 826) fusion construct, during hypoxia in p38*α*$^{-/-}$ cells was also rescued by the expression of p38α (Figures 2.7 and 2.8). Furthermore, hypoxia induced gene expression of *Pgk1* and *Glut-1* in the p38α reconstituted cells (Figure 2.9). Cells treated with DFO during normoxia activated HIF-1 irrespective of the presence or absence of p38α. These results indicate that the suppression of HIF-1 activation under hypoxia in the *p38α*$^{-/-}$ cells was due to loss of p38α MAPK.

Upstream MAPKKs MKK3 and MKK6 are required for HIF-1 activation during hypoxia.

MKK3 and MKK6 are the upstream MAPKK isoforms that specifically activate p38α MAPK (Brancho et al., 2003; Derijard et al., 1995; Raingeaud et al., 1996). To investigate if MKK3 and MKK6 are required for the hypoxic activation of p38 MAPK, we used the *Mkk3/6*$^{+/+}$ and *Mkk3/6*$^{-/-}$ cells (Figure 2.10A), exposed them to hypoxia, and performed immunoblot analysis using a phosphospecific antibody for p38 MAPK. During hypoxia, the WT cells demonstrated activation of p38 MAPK (Figure 2.10B). In contrast, cells deficient in both *Mkk3* and *Mkk6* failed to activate p38 MAPK during hypoxia. Next, we examined whether MKK3 and MKK6 were required for the stabilization of HIF-1α. Similar to the *p38α* null cells, the *Mkk3/6*$^{-/-}$ cells did not stabilize HIF-1α during hypoxia, whereas cells treated with DFO stabilized HIF-1α

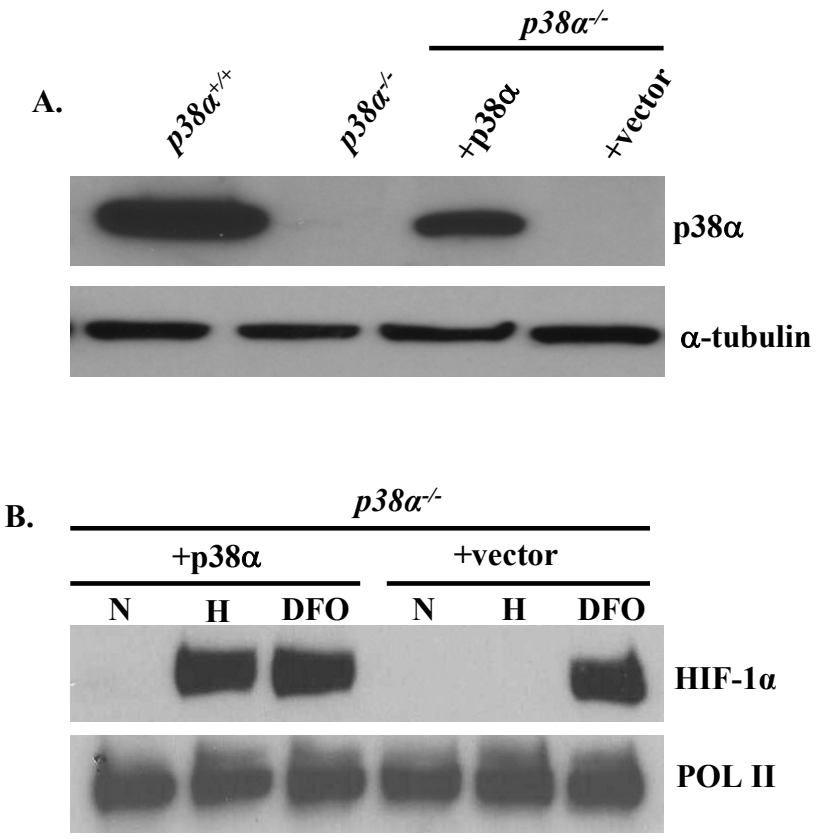

Figure 2.6. Reintroduction of p38α rescues hypoxic stabilization of the HIF-1α protein in p38α null cells.

(A). *p38α*$^{-/-}$ cells stably reconstituted with a p38α cDNA or with vector alone. **(B).** HIF-1α protein levels in reconstituted cells exposed to 21% O_2 (N) ± 100 μM DFO or to 1.5% O_2 (H) for 2 h.

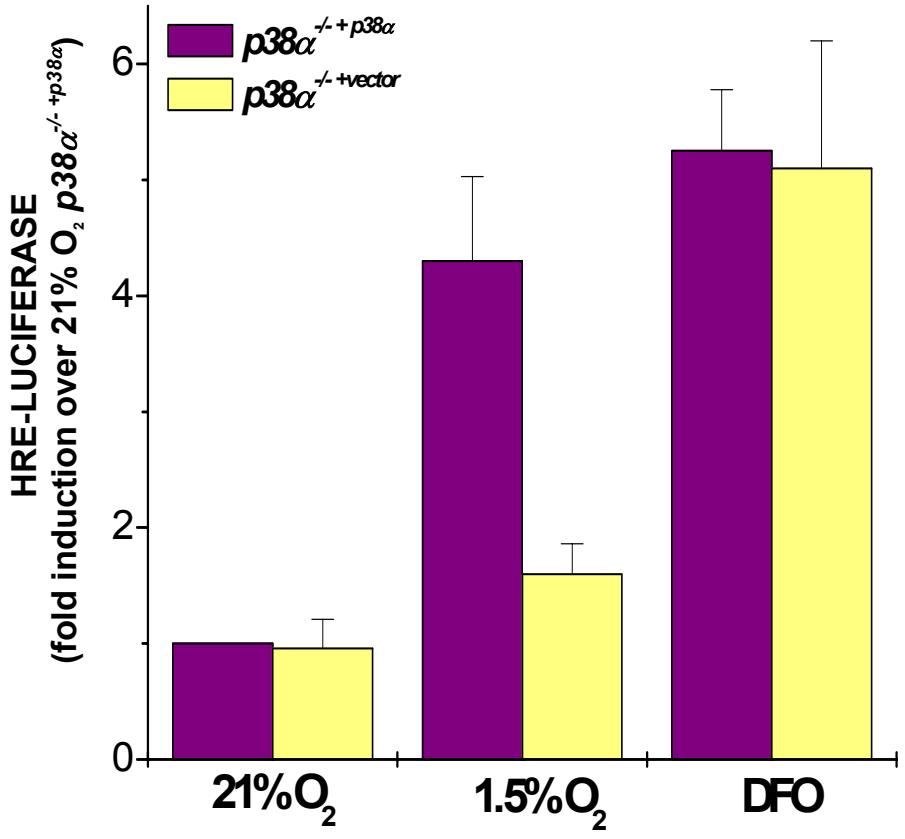

Figure 2.7. Reintroduction of p38α rescues hypoxic activation of HIF-1 in p38α null cells.

Cells transfected with HRE-Luciferase reporter gene construct and exposed to 21% O_2 ± 100 μM DFO or to 1.5% O_2 for 16 h. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to $p38\alpha^{-/-+p38\alpha}$ cells. The data presented are the mean (±SEM) of four independent transfections.

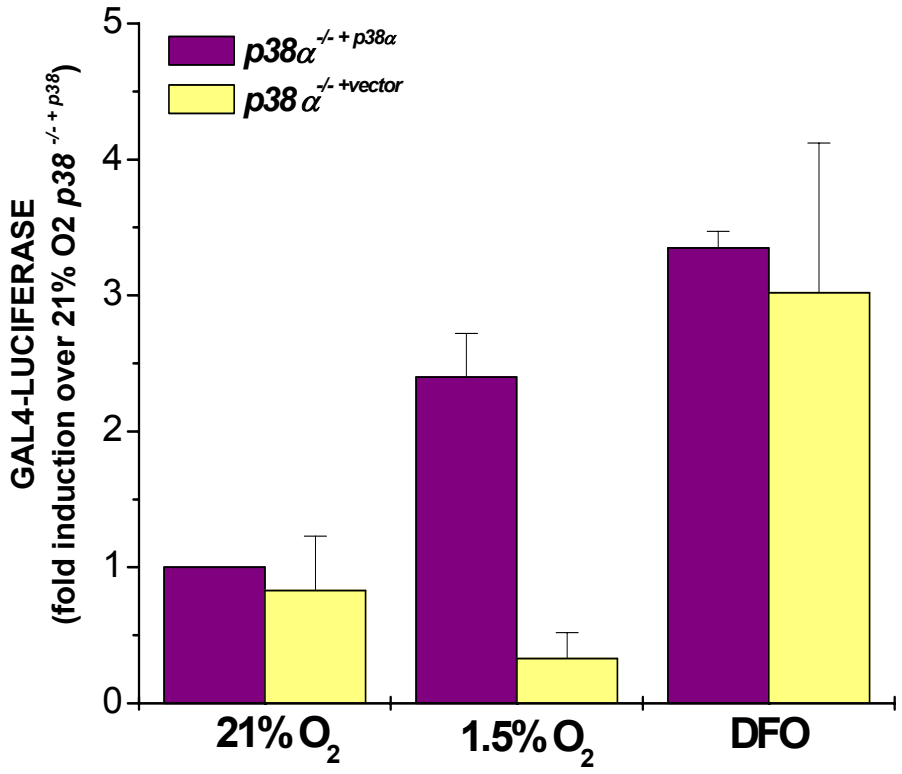

Figure 2.8. Reintroduction of p38α rescues hypoxic transactivation of HIF-1α in p38α null cells.

Cells were transfected with a GAL4 (1-147) DNA-binding domain fused to HIF-1α (531-826) construct and a reporter gene construct encoding five GAL4-binding sites. Cells were incubated at 21% O_2 for 20 hours, followed by 36 hours at 21% O_2 ± 100 μM DFO or to 1.5% O_2. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to *p38α*$^{-/-+p38α}$ cells. The data presented are the mean (±SEM) of four independent transfections.

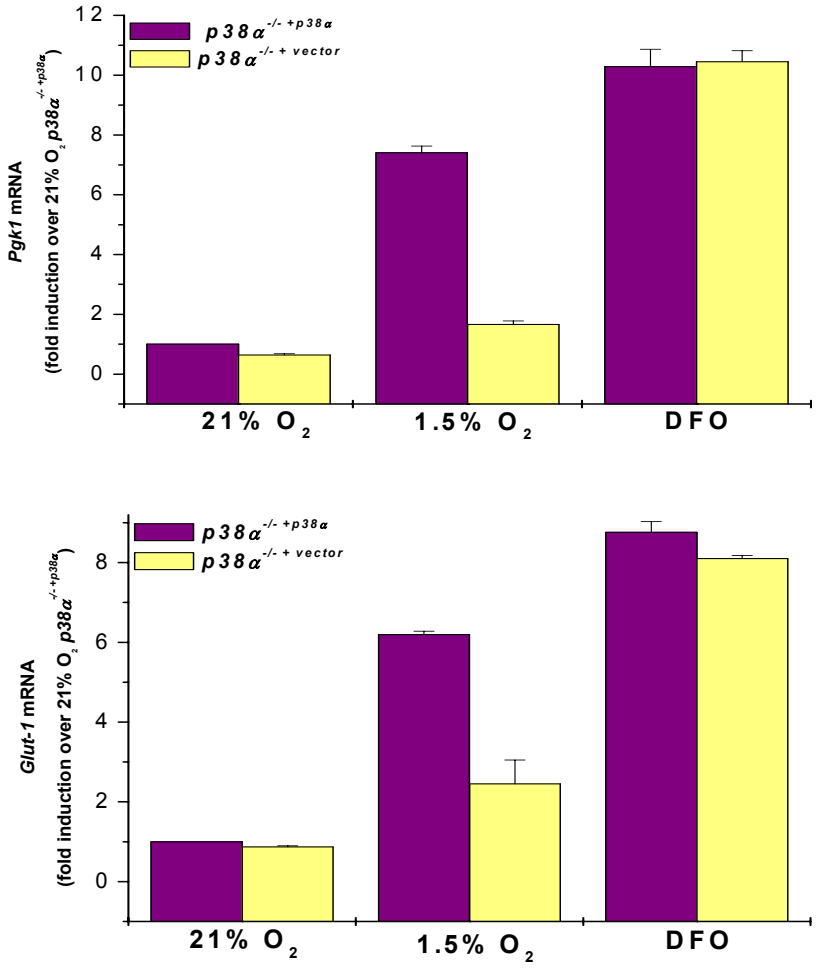

Figure 2.9. Reintroduction of p38α rescues hypoxic induction of HIF-1 target genes. Reconstituted cells were cultured for 16 hours under 21% O_2 ± 100 μM DFO or to 1.5% O_2, harvested, and transcription levels of the target genes *Pgk1* and *Glut-1* were determined by quantitative real-time RT-PCR analysis. Cycle Threshold (Ct) values were normalized for amplification of the mitochondrial ribosomal protein *L19*. The data presented are the result of triplicate analyses and the error bars indicate SEM.

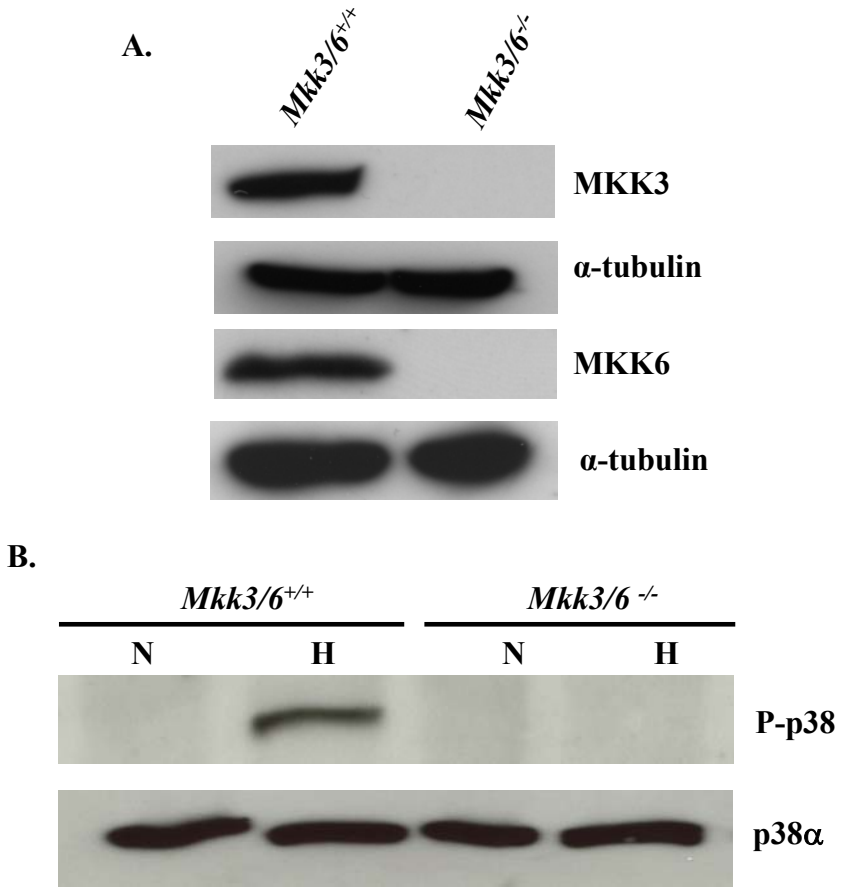

Figure 2.10. Hypoxic activation of p38 MAPK is MKK3 and MKK6 dependent. **(A).** MKK3 and MKK6 protein levels in WT and *Mkk3/6*$^{-/-}$ cells. **(B).** p38 MAPK activation in WT and *Mkk3/6*$^{-/-}$ cells exposed to 21% O_2 (N) or to 1.5% O_2 (H) for 30 min.

irrespective of the status of MKK3/6 (Figure 2.11). To test the requirement of MKK3 and MKK6 for the hypoxic activation of HIF-1, WT and *Mkk3/6*$^{-/-}$ cells were transfected with HRE-Luciferase under normoxia, hypoxia, and DFO (Figure 2.12). Hypoxia and DFO induced HRE-Luciferase expression in WT cells. In contrast, *Mkk3/6*$^{-/-}$ cells displayed a severe decline of luciferase induction under hypoxia but not in the presence of DFO during normoxia. Furthermore, cells deficient in *Mkk3/6* failed to induce transactivation of HIF-1 under hypoxia (Figure 2.13). Consistent with these data are the observation that MKK3 and MKK6 are essential for the hypoxic induction of the HIF-1 target genes but not for DFO (Figure 2.14). Together these results indicate that the hypoxic activation of p38 MAPK and HIF-1 is dependent on MKK3 and MKK6.

Mitochondrial ROS are required for both the hypoxic activation of HIF-1 and p38 MAPK. The upstream regulators of the MKK3/6-p38α MAPK signaling pathway remain unknown during hypoxia. Mitochondrial ROS generated within complex III have been implicated as potential regulators of p38 MAPK activation during hypoxia (Kulisz et al., 2002). Hypoxia increases the generation of oxidants during the ubiquinione cycle within complex III. Indeed, hypoxia generated ROS, as detected by the oxidation of the 2',7'-dichlorofluorescin (DCFH) dye, in WT and *p38α*$^{-/-}$ cells (Figure 2.15). To demonstrate the source of the ROS generation during hypoxia, cells were treated with myxothiazol, a complex III inhibitor. Myxothiazol significantly decreased the oxidation of the DCFH dye during hypoxia (Figure 2.15). To demonstrate the requirement of the mitochondrial complex III for the activation of p38 MAPK, WT cells were treated with myxothiazol under hypoxic conditions. The phosphorylation of p38 MAPK under hypoxic conditions was also suppressed by myxothiazol (Figure 2.16). To demonstrate whether ROS are

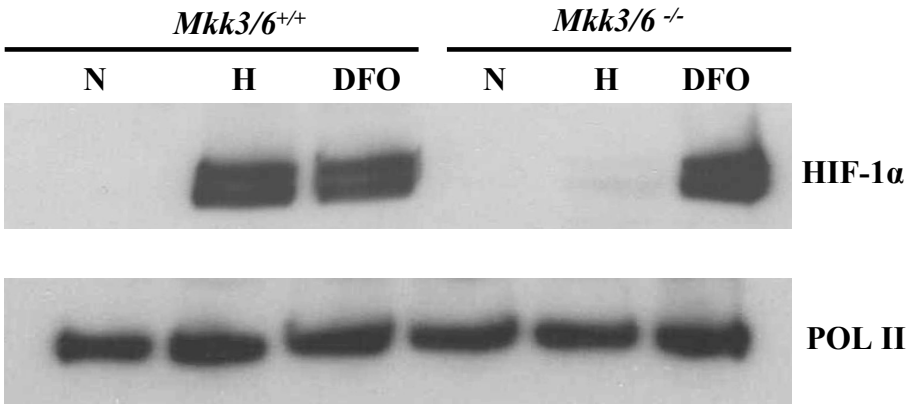

Figure 2.11. MKK3 and MKK6 are required for HIF-1α protein stability.

HIF-1α protein levels in WT and *Mkk3/6*$^{-/-}$cells exposed to 21% O_2 (N) ± 100 μM DFO or to 1.5% O_2 (H) for 2 h.

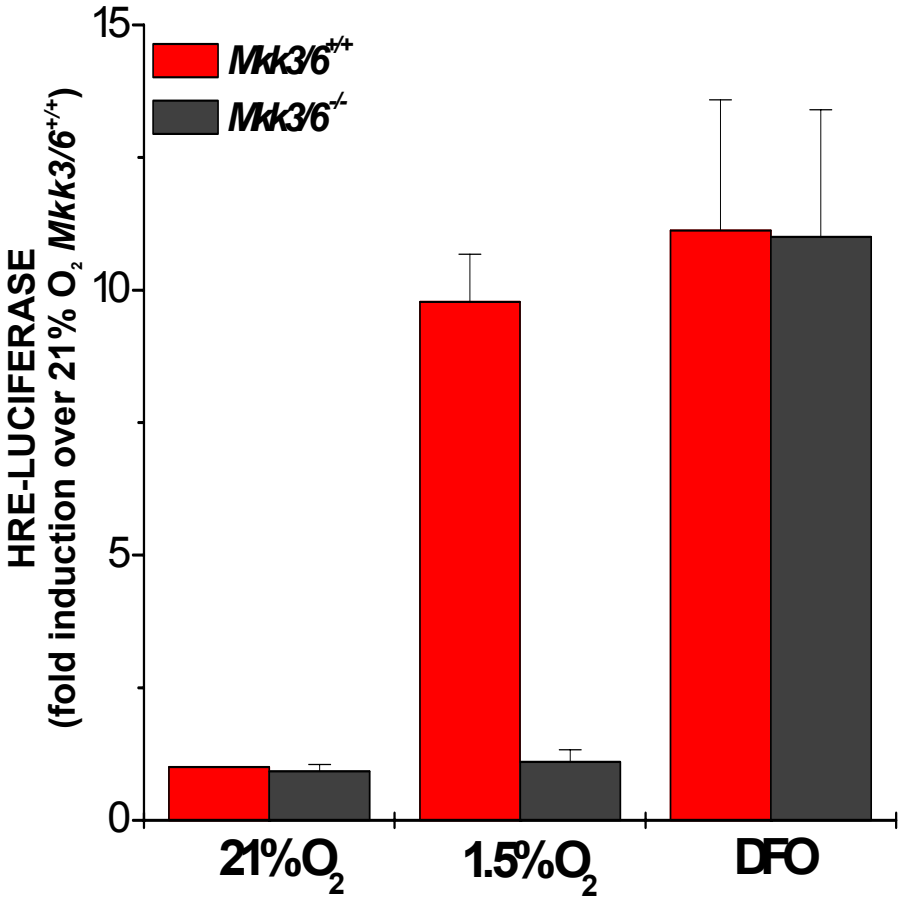

Figure 2.12. MKK3 and MKK6 are required for hypoxic activation of HIF-1.

WT and *Mkk3/6*$^{-/-}$ cells transfected with the HRE-Luciferase reporter gene construct and exposed to 21% O_2 ± 100 μM DFO or to 1.5% O_2 for 16 h. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to WT cells. The data presented are the mean (±SEM) of four independent transfections.

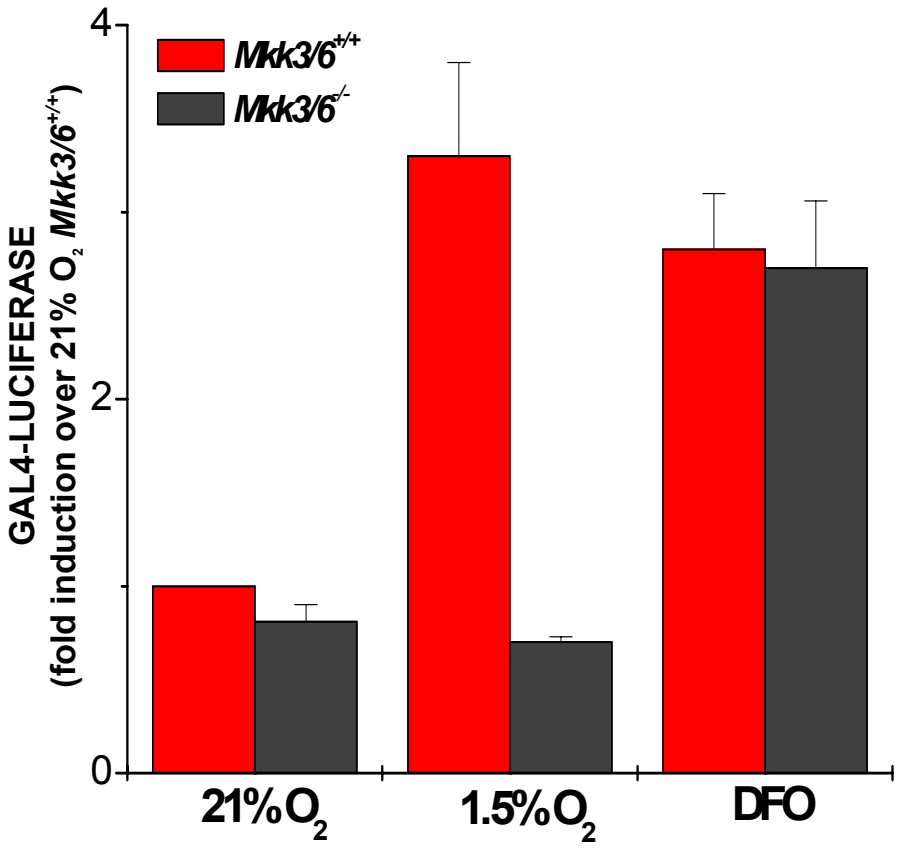

Figure 2.13. MKK3 and MKK6 are required for HIF-1α transactivation.

WT and *Mkk3/6*$^{-/-}$ *c*ells were transfected with a GAL4 (1-147) DNA-binding domain fused to HIF-1α (531-826) construct and a reporter gene construct encoding five GAL4-binding sites. Cells were incubated at 21% O_2 for 20 hours, followed by 36 hours at 21% O_2 ± 100 μM DFO or to 1.5% O_2. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to WT cells. The data presented are the mean (±SEM) of four independent transfections.

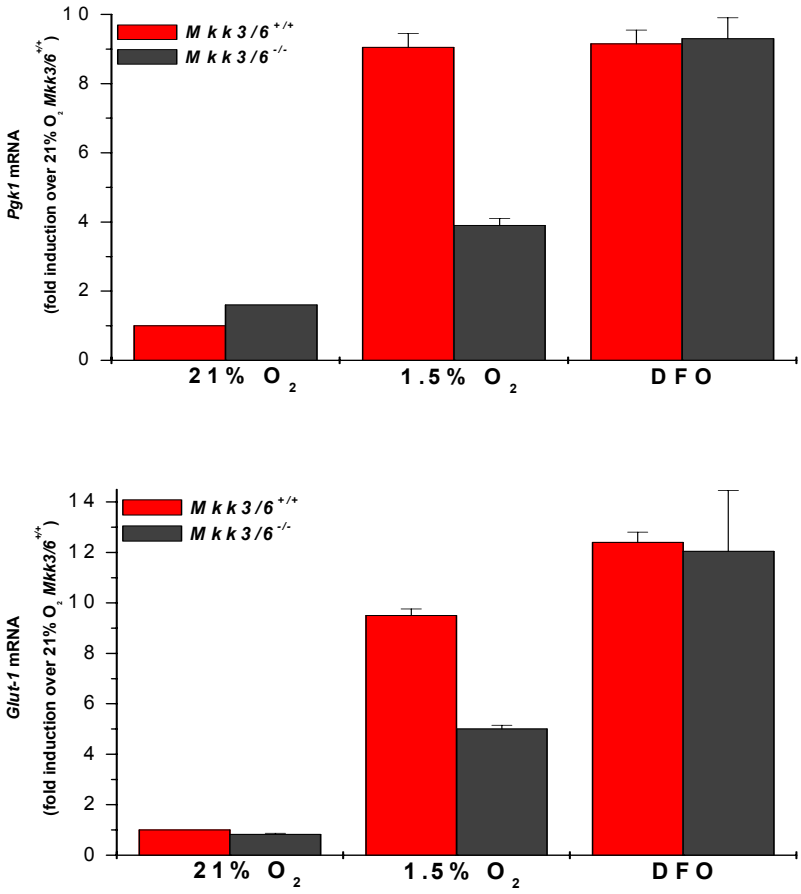

Figure 2.14. MKK3 and MKK6 are required for hypoxic induction of HIF-1 target genes.

Cells were cultured for 16 hours under 21% O_2 ± 100 µM DFO or to 1.5% O_2, harvested, and transcription levels of the target genes *Pgk1* and *Glut-1* were determined by quantitative real-time RT-PCR analysis. Cycle Threshold (Ct) values were normalized for amplification of the mitochondrial ribosomal protein *L19*. The data presented are the result of triplicate analyses and the error bars indicate SEM.

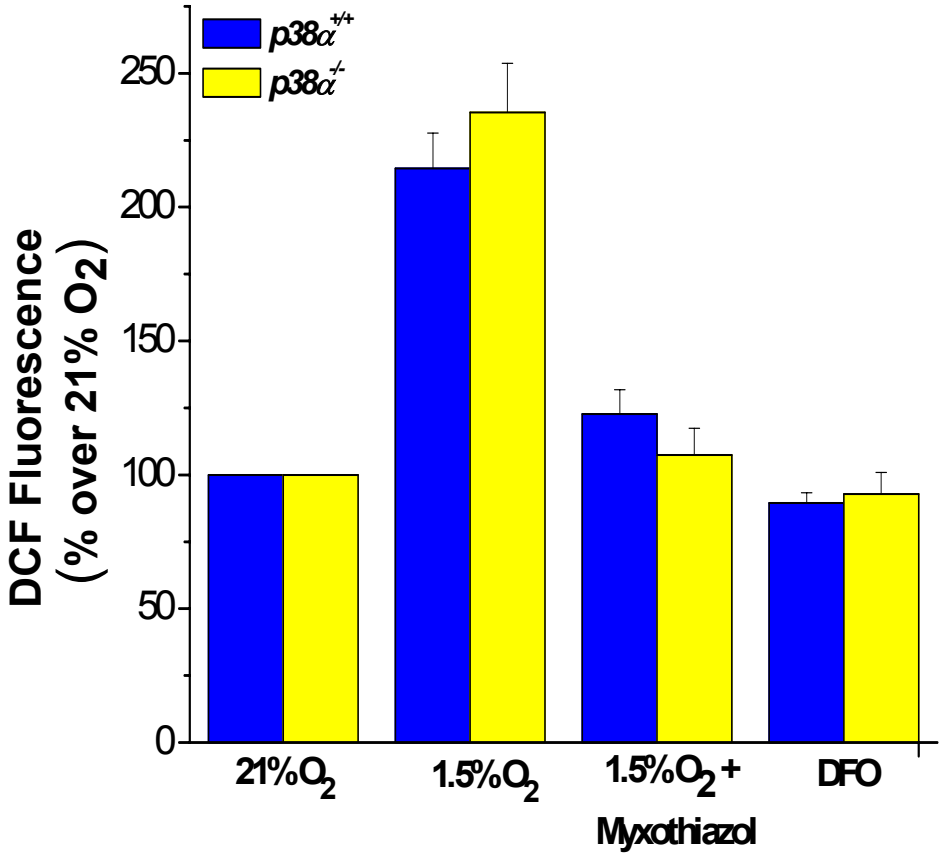

Figure 2.15. Hypoxia stimulates mitochondrial generated oxidants.

ROS were determined by incubating *p38α*$^{+/+}$ and *p38α*$^{-/-}$ cells with DCFH-DA (10 μM) exposed to 21% O_2 ± 100 μM DFO or to 1.5% O_2 ± myxothiazol (1μM), a complex III inhibitor, for 4 h.

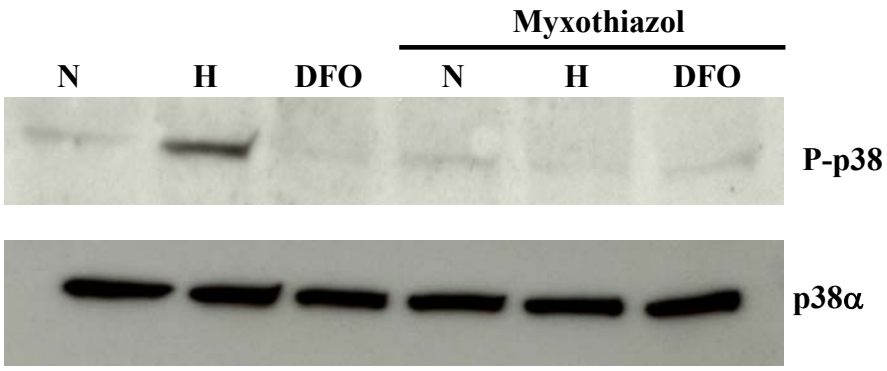

Figure 2.16. Hypoxic activation of p38 MAPK requires mitochondrial generated oxidants.

p38 MAPK activation in WT cells exposed to 21% O_2 (N) ± 100 μM DFO or 1.5% O_2 (H) ± myxothiazol (1 μM) for 30 min.

required for the hypoxic stabilization of HIF-1α and the activation of p38 MAPK, cells were infected with an adenovirus encoding glutathione peroxidase 1 (GPX1), an antioxidant enzyme that converts hydrogen peroxide into water (Figure 2.17A). Cells overexpressing GPX1 failed to activate p38 MAPK during hypoxia (Figure 2.17B). The activation of HIF-1 during hypoxia has also been shown to be regulated by mitochondrial ROS (Chandel et al., 1998; Chandel et al., 2000). The increase in oxidant production is required for the hypoxic stabilization of HIF-1α protein. To demonstrate the requirement of the mitochondrial complex III for the stabilization of HIF-1α, WT cells were treated with myxothiazol under hypoxic conditions. Myxothiazol prevented the hypoxic stabilization of the HIF-1α protein but not in response to DFO (Figure 2.18). Cells overexpressing GPX1 failed to stabilize HIF-1α protein during hypoxia (Figure 2.19). These results demonstrate that activation of p38 MAPK signaling links mitochondrial generated ROS to the activation of HIF-1 during hypoxia.

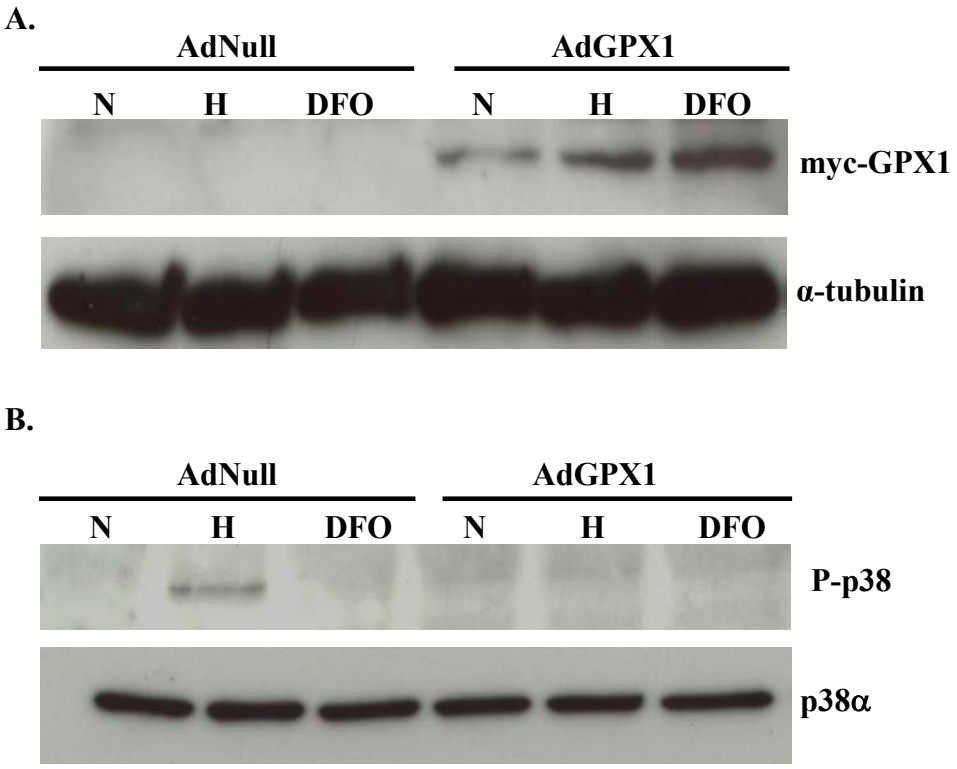

Figure 2.17. Antioxidant enzyme expression prevents activation of p38 MAPK during hypoxia.

A. and B. GPX1 levels and p38 MAPK activation in WT cells infected with null adenovirus (control) or adenovirus expressing myc-tagged GPX1 and subsequently exposed to 21% O_2 (N) ± 100 μM DFO or 1.5% O_2 (H) for 2 h.

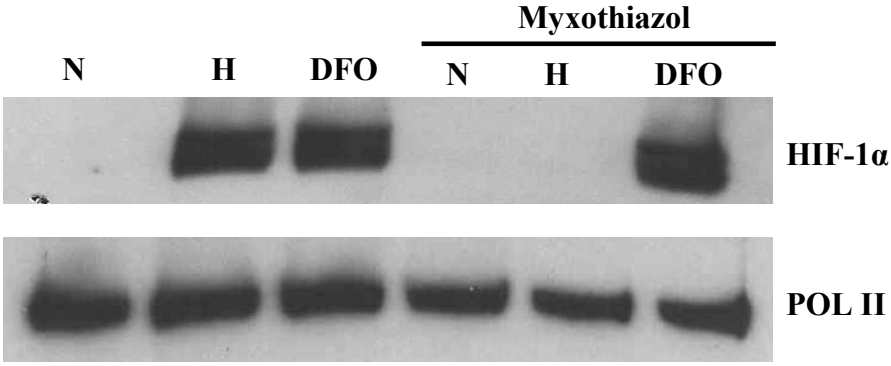

Figure 2.18. Hypoxic activation of HIF-1α protein levels requires mitochondrial generated oxidants.

HIF-1α protein levels in WT cells exposed to 21% O_2 (N) ± 100 μM DFO or 1.5% O_2 (H) ± myxothiazol (1 μM) for 2 h.

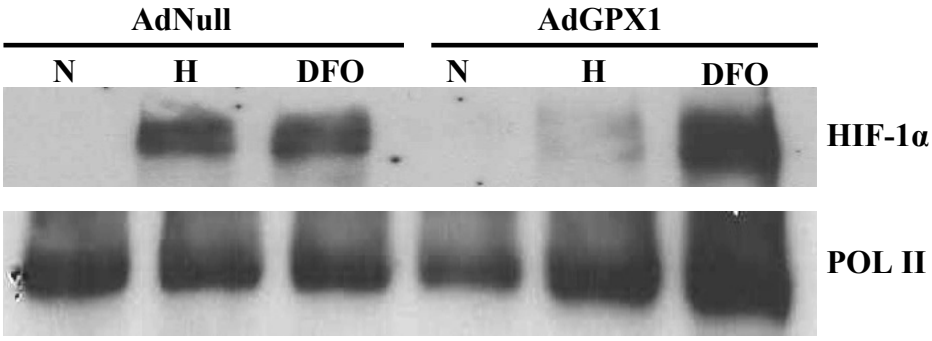

Figure 2.19. Antioxidant enzyme expression prevents stabilization of the HIF-1α protein during hypoxia.

HIF-1α protein levels in WT cells infected with null adenovirus (control) or adenovirus expressing myc-tagged GPX1 and subsequently exposed to 21% O_2 (N) ± 100 μM DFO or 1.5% O_2 (H) for 2 h.

Discussion

The signal transduction pathways that regulate the stabilization of HIF-1α, as well as, the subsequent expression of HIF-1 regulated genes are not fully understood. How cells sense the decrease in oxygen to activate signaling pathways resulting in the activation of HIF-1 remains unknown. Many signaling molecules have been implicated in the regulation of HIF-1, although most studies have relied on the use of pharmacological agents in cancer cell lines (Hirota and Semenza, 2001; Shemirani and Crowe, 2002). In the present study we provide genetic evidence, using cells from *p38α*$^{-/-}$ or *Mkk3/6*$^{-/-}$ knockout mice, that the p38 MAPK signaling cascade is necessary for the hypoxic activation of HIF-1. The reconstitution of p38α null cells with a p38α cDNA restores the ability to activate HIF-1 during hypoxia. This indicates that the inability of hypoxia to activate HIF-1 was due to a loss of p38α as opposed to an adaptation of p38α null cells in the absence of the kinase. The p38 MAPK activation during hypoxia was dependent on oxidant generation within mitochondrial complex III. Thus, p38 MAPK signaling provides a mechanistic link between the generation of oxidant production and the activation of HIF-1 during hypoxia (Figure 2.20).

Presently there are two models that account for the activation of HIF-1. One model proposes that the hydroxylases, PHDs and FIH, serve as oxygen sensors that control HIF-1 activation during hypoxia (Epstein et al., 2001; Lando et al., 2002b). According to this model, the hydroxylases reduce their activity directly as a function of declining oxygen levels resulting in a decrease in hydroxylation of proline and asparagine residues within the HIF-1α protein. The decrease in hydroxylation of the HIF-1α protein results in both the stabilization of the protein as well as recruitment of coactivators to induce gene expression. The fact that the hydroxylases could serve

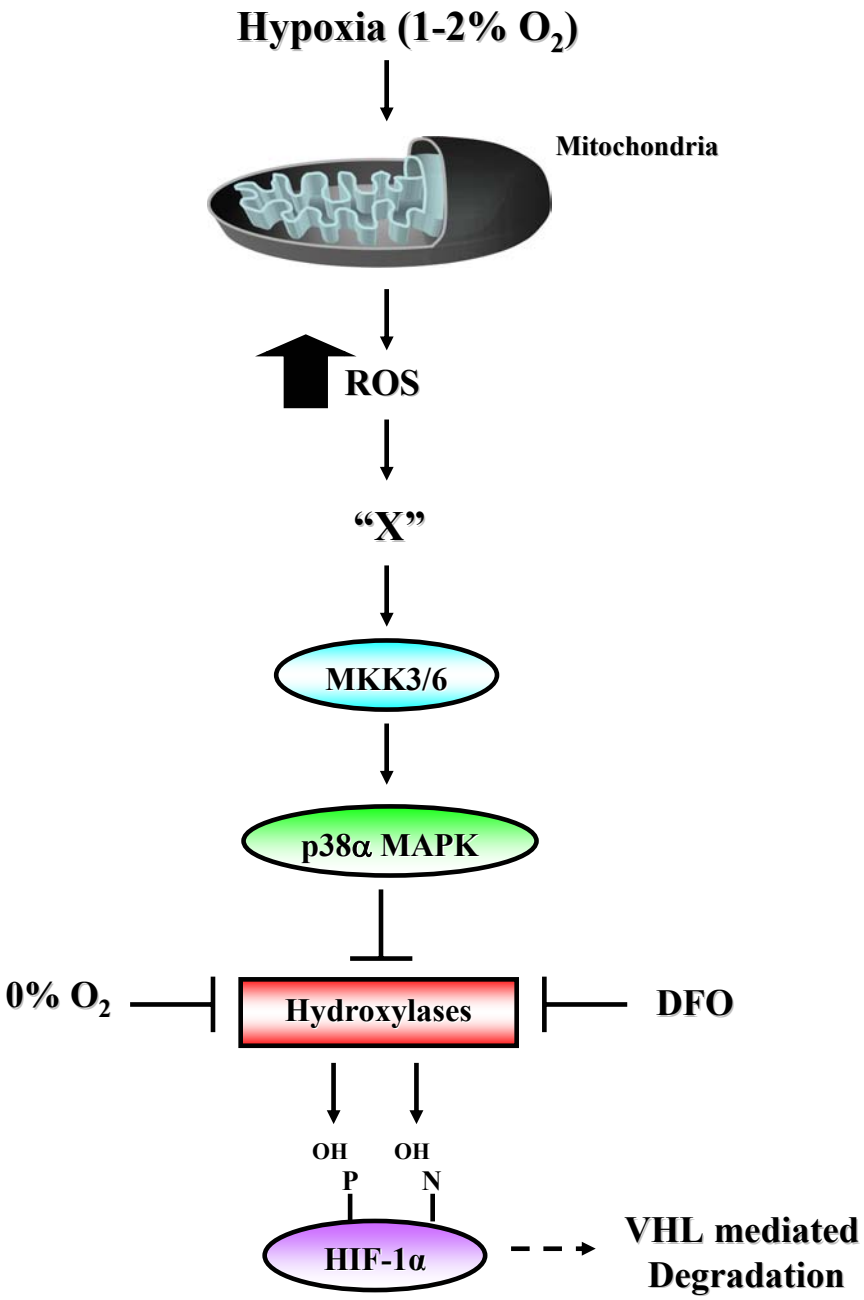
Hypoxia (1-2% O_2)
Mitochondria
ROS
"X"
MKK3/6
p38α MAPK
0% O_2
Hydroxylases
DFO
OH
P
OH
N
HIF-1α
VHL mediated
Degradation

Figure 2.20. A signaling model for hypoxic activation of HIF-1.

Based on our findings we propose that hypoxia stimulates oxidant production within mitochondria. These oxidants interact with an unknown protein "X" in the cytosol to activate MKK3/6 and p38α MAPK. The activation of the p38α MAPK signaling pathway results in the decrease in hydroxylation of the HIF-1α protein at proline and asparagine residues resulting in the activation of HIF-1. By contrast, anoxia or iron chelators (DFO) during normoxia inhibit hydroxylation directly by limiting the availability of oxygen as a substrate and iron as a cofactor, respectively. Thus, anoxia or DFO does not require signaling pathways for the activation of HIF-1. Hypoxia and anoxia have distinct signaling mechanisms to activation HIF-1.

as oxygen sensors also suggests that there would be no requirement for signaling pathways to be initiated for the activation of HIF-1 during hypoxia. In order to fulfill the role of oxygen sensors the hydroxylases would have to have a K_m in the hypoxic region. Recombinant prolyl hydroxylases have a K_m of ambient air (20.9% O_2) in vitro while asparaginyl hydroxylase (FIH) has a K_m of 40% of ambient air in vitro, indicating that the hydroxylases decrease their enzymatic activity throughout the physiological range of PO_2 (Hirsila et al., 2003). Therefore, if the hydroxylases were in fact the sensors, one would predict a continuous increase in the accumulation of HIF-1α protein as oxygen levels fall from 21% O_2 to 0% O_2. However, HIF-1α protein begins to accumulate around 5% O_2, and its concentration increases as the oxygen levels approach anoxia (Jiang et al., 1996b). Thus, the K_m of the hydroxylases is not compatible with the oxygen dependence of HIF-1α protein stabilization. Our current finding that p38 MAPK signaling is required for the activation of HIF-1 during hypoxia further suggests that the hydroxylases are not likely to be the sole regulators of HIF-1.

A second model proposes that the hydroxylases are only proximal regulators of the HIF-1α protein. According to this model there would be upstream regulators of the hydroxylases. Our present results are in agreement with this model. Loss of p38 MAPK signaling prevented both the hypoxic stabilization of HIF-1α protein as well as the transcriptional activity of the protein. The stabilization of HIF-1α protein is primarily regulated by hydroxylation of proline residues by PHDs while the transcriptional activity is regulated by asparagine hydroxylation by FIH. The activation of p38 MAPK signaling during hypoxia is likely to prevent PHDs as well as FIH from hydroxylating proline and asparagine residues. Our results are also consistent with previous studies indicating that signaling molecules are necessary for HIF-1α protein stabilization during

hypoxia. These signaling pathways include but are not limited to the requirement of diacylglycerol kinase, small GTPases, and PI3-K/AKT (Aragones et al., 2001; Hirota and Semenza, 2001; Turcotte et al., 2003; Zhong et al., 2000; Zundel et al., 2000). Many of these signal transduction molecules can activate p38 MAPK signaling pathways. Moreover, the transactivation potential of HIF-1α depends on phosphorylation of the conserved residue Threonine-796 (Gradin et al., 2002). The modification of this residue increases the affinity of HIF-1α to the transcriptional coactivator CBP. Whether this modification does not allow FIH mediated hydroxylation at Asparagine 803 remains unknown. Also, p42/p44 MAPK can directly phosphorylate HIF-1α and increase the transcriptional activity of the protein (Richard et al., 1999). We interpret these findings to suggest that the hydroxylases are likely to have enough oxygen to carry out hydroxylation of HIF-1α protein throughout the physiological range but fail to hydroxylate HIF-1α protein as they approach oxygen levels in the hypoxic region due to modification by p38 MAPK signaling (Figure 2.20).

Our results also indicate that cells utilize different mechanisms to activate HIF-1 during hypoxia compared with anoxia or exposure to iron chelators. The p38 MAPK signaling is not required for the activation of HIF-1 in cells exposed to anoxia or iron chelators under normoxia. Oxygen is required as a substrate and iron as a cofactor for the hydroxylation of the HIF-1α protein, and in the absence of oxygen this reaction will not occur. Therefore, HIF-1α protein will not be hydroxylated and targeted for ubiquitin mediated degradation under anoxia due to substrate limitation. The hydroxylases ultimately serve as oxygen sensors under anoxic conditions. Similarly, the hydroxylases are likely to be the only regulators of HIF-1 by iron chelators, such as DFO. Iron chelators can inhibit the hydroxylation reaction directly by limiting the availability

of iron. Previous observations have indicated that hypoxia and anoxia or iron chelators have distinct mechanisms for the activation of HIF-1. For example, cells exposed to hypoxia fail to activate HIF-1 in the presence of mitochondrial inhibitors or in cells that lack mitochondrial DNA [*rho*^{0}] cells). In contrast, cells exposed to anoxia retain their ability to activate HIF-1 even in the presence of mitochondrial inhibitors or in [*rho*^{0}] cells (Schroedl et al., 2002). Furthermore, diphenylene iodonium (DPI), an inhibitor of a wide range of flavoproteins including mitochondrial complex I, prevents stabilization of HIF-1α protein and HIF-1 target genes at oxygen levels of 1% but not in the presence of iron chelators under normoxia (Gleadle et al., 1995).

A major finding of the present study is that ROS generated within mitochondrial complex III are required for the activation of both p38α MAPK and HIF-1. Previous studies have suggested that mitochondrial complex III can serve as an oxygen sensor and oxidant production from this complex serves as the major signaling molecule to initiate activation of HIF-1 during hypoxia (Chandel et al., 1998; Chandel et al., 2000; Schroedl et al., 2002). However, the mechanisms by which mitochondrial ROS would activate HIF-1 remains unknown. The current finding that p38 MAPK signaling is required for HIF-1 activation provides the first known link between mitochondrial oxidant production during hypoxia and HIF-1 activation. This premise is consistent with the observations that nonhypoxic stimuli such as chromium or arsenite generate oxidative stress resulting in a p38α MAPK-dependent activation of HIF-1. The physiological implication of ROS as a positive regulator of HIF-1 has been recently highlighted by the observation that cells deficient in JunD, a member of the AP-1 family of transcription factors, have increased ROS levels under normoxic conditions resulting in the accumulation of HIF-1α

protein and induction of VEGF, a potent angiogenic factor (Gerald et al., 2004). Thus, JunD can reduce tumorigenesis by reducing oxidative stress and HIF-1 activation. Furthermore, radiation-induced reoxygenation of hypoxic tumor cells results in the production of ROS, which activates HIF-1 to prevent radiation-induced endothelial cell death (Moeller et al., 2004).

There are two important physiological implications of the present study. First, the requirement of p38 MAPK signaling for the hypoxic activation of HIF-1 may explain the resemblance seen in the phenotypes of the *p38α*, *Mkk3/6*, *Hif-1α*, and *Hif-1β* null mice. All these embryos die during midgestation due to multiple defects, including abnormal vascularization of the placenta (Adams et al., 2000; Adelman et al., 2000; Allen et al., 2000; Brancho et al., 2003; Carmeliet et al., 1998; Iyer et al., 1998; Maltepe et al., 1997; Mudgett et al., 2000; Ryan et al., 1998; Tamura et al., 2000). In particular, both the *Hif-1β* knockout and *p38α* null mice display a complete loss of the labyrinth layer and significant reduction of the spongiotrophoblast layer in the developing placenta. Secondly, HIF-1 is up-regulated in most human cancers and is required for tumor progression by up-regulating its target genes, which are involved in angiogenesis, anaerobic metabolism, cell survival, cell invasion, and drug resistance (Semenza, 2003). Here, we identify the p38 MAPK signaling pathway as a key upstream regulator of HIF-1 activity. Consequently, therapies targeted towards modulating the p38 MAPK signaling pathway may provide a basis for the development of new cancer therapies.

CHAPTER 3: Hypoxic Activation of HIF-1 is independent of SRC, ASK1, and AMPK

Introduction

Oxidative signaling has been implicated in a number of experimental interventions that lead to the initiation of gene transcription or other adaptive responses (Palmer and Paulson, 1997; Schulze-Osthoff et al., 1997; Suzuki et al., 1997). However, the direct molecular target of oxidants that activates signaling pathways leading to the stabilization of the HIF-1α protein during hypoxia remains unknown. The generation of mitochondrial reactive oxygen species (ROS) is required and sufficient for HIF-1 activation during hypoxia (Brunelle et al., 2005; Chandel et al., 2000; Guzy et al., 2005; Mansfield et al., 2005). The downstream signaling pathways that link mitochondrial ROS to HIF-1 activation still need to be elucidated. The p38 MAPK signaling pathway is required for the hypoxic activation of HIF-1 (Emerling et al., 2005), although, the mechanism underlying this activation of p38α MAPK or its relationship to mitochondrial ROS signals has not been described. Therefore, a candidate approach has been taken here in order to investigate what is/are the direct targets of the mitochondrial ROS that activate the p38 MAPK pathway during hypoxia and that eventually lead to the activation of HIF-1.

One possible mechanism is that the hydrogen peroxide generated during hypoxia in the cytosol activates the SRC family of kinases which activates the Rho family small GTPase Rac1 through focal adhesion kinase (FAK). The activation of Rac1 would result in the stimulation of p38α MAPK activity to prevent hydroxylation of the HIF-1α protein. The SRC family is a family of nonreceptor protein tyrosine kinases, which include SRC, LYN, FYN, LCK, HCK, FGR, BLK, and YES. SRC, FYN, YES are ubiquitously expressed and display redundancy in function. By

contrast, other members of the family such as HCK, BLK, or LCK exhibit restricted tissue expression. SRC is a nonreceptor tyrosine kinase and is a direct target of ROS, specifically hydrogen peroxide (Abe et al., 1997). Hydrogen peroxide activates SRC by causing SRC to autophosphorylate itself. Recently, it has also been shown that mitochondrial generated ROS during hypoxia activates SRC and that SRC activation induces HIF-1α protein and the expression of plasminogen activator inhibitor-1 (PAI-1), a HIF-1 target gene (Sato et al., 2005). The downstream SRC family target FAK is known to activate Rac1 (Hsia et al., 2003). FAK can be activated by hypoxia and by ROS, specifically hydrogen peroxide (Seko et al., 1999). Hypoxia causes an increased association of FAK with SRC; concomitantly SRC then phosphorylates FAK at a number of tyrosine residues (Seko et al., 1999). It is well established that Rac1 can activate the p38α MAPK pathway through the upstream MAPKKs, MKK3 and MKK6 (Minden et al., 1995). Hypoxia has been shown to activate p38α MAPK in a Rac1 dependent manner (Hirota and Semenza, 2001). One report has even shown that Rac1 activity is required for the activation of HIF-1 (Hirota and Semenza, 2001). Moreover, by targeting Rac1 with small interference RNA, VEGF and HIF-1α can be downregulated (Xue et al., 2004). These findings suggest that Rac1 may be involved in the hypoxic response and make Rac1 an attractive upstream candidate to investigate. Thus, the first pathway tested was the Src-FAK-Rac1-MKK3/6-p38 MAPK pathway. Interestingly, the mice deficient in SRC, FAK, Rac1, MKK3/MKK6, p38α MAPK, HIF-1α, and HIF-1β are all embryonic lethal at days 8.5-10.5 due to multiple defects including abnormal vascularization (Adams et al., 2000; Adelman et al., 2000; Allen et al., 2000; Brancho et al., 2003; Carmeliet et al., 1998; Ilic et al., 2003; Iyer et al., 1998; Klinghoffer et al., 1999; Maltepe et al., 1997; Mudgett et al., 2000; Ryan et al., 1998; Sugihara et al., 1998; Tamura et al., 2000).

The next candidate to study was the MAPKKK, apoptosis signal-regulating kinase 1 (ASK1). The kinase ASK1 has been shown to be directly activated by ROS and it in turn activates the downstream kinases MKK3 and MKK6-p38 MAPK signalinig cascades (Kyriakis and Avruch, 2001; Roux and Blenis, 2004). ASK1 functions as an important molecular sensor of internal and/or external environmental stresses to determine cell fate, such as survival, differentiation, and apoptosis (Sayama et al., 2001; Takeda et al., 2000). Thioredoxin (TRX) has been identified as a negative regulator of the ASK1-p38 pathway (Saitoh et al., 1998). In resting cells, ASK1 constantly forms a complex with TRX, but upon treatment of hydrogen peroxide, ASK1 is disassociated from TRX and activated by subsequent modifications, including oligomerization auto- and/or cross-phosphorylation at the site Thr845 within the activation loop of ASK1 (Fujino et al., 2006; Saitoh et al., 1998). TRX is a redox-regulatory protein that has two redox-sensitive cysteine residues within the conserved active center. Only a reduced form of TRX is associated with the N-terminal regulatory domain of ASK1 and thereby silences the activity of ASK1. Oxidation of TRX results in the disassociation of ASK1 from TRX and thereby switches an inactive form of ASK1 to active. Thus, the ASK1-TRX complex serves as a molecular switch that converts redox signals evoked by ROS to signaling through kinase cascades. Also, there is evidence that ASK1 may play a role in the hypoxic activation of HIF-1 (Kwon et al., 2005).

The third candidate investigated was AMP-activated kinase (AMPK). AMPK is a heterotrimeric serine/threonine consisting of a catalytic α subunit and two regulatory β and γ subunits (Carling, 2004; Hardie et al., 2003; Rutter et al., 2003). AMPK is ubiquitously expressed and functions as an intracellular fuel sensor by maintaining energy balance. Conditions that elevate intracellular AMP or decrease ATP levels activate AMPK through the allosteric binding of AMP, which

allows AMPK to sense cellular [AMP]/[ATP] ratios. ROS generated by hypoxia and ischemia is also a potent activator of AMPK independent of increased AMP levels (Laderoute et al., 2006; Quintero et al., 2006). Whether or not AMPK is a direct target of ROS is yet to be determined, but none the less AMPK is highly activated by oxidative stress. Full activation of AMPK requires the phosphorylation within the activation loop of the catalytic α subunit at Thr172 by LKB1, a serine/threonine kinase and tumor suppressor (Lizcano et al., 2004; Shaw et al., 2004). Recently, mammalian Ca^{2+}/calmodulin-dependent kinase kinase (CaMKK) has been identified as an AMPK kinase (Birnbaum, 2005). AMPK phosphorylates diverse targets, many that are directly involved in controlling cellular energy metabolism (Hue et al., 2003; Leff, 2003). It has been reported that the MKK3 dependent p38 MAPK cascade is a downstream component of AMPK signaling (Xi et al., 2001). Recently, it has also been shown that AMPK activates p38 MAPK in ischemic heart (Li et al., 2005).

The focus of this chapter is to study the potential upstream regulators of p38 MAPK that are required for the hypoxic activation of HIF-1. The three candidates that are investigated here are SRC, ASK1, and AMPK. All these kinases are activated by ROS and are known upstream regulators of the p38 MAPK signaling pathway.

Results

SRC is not required for hypoxic activation of HIF-1.

To investigate the first candidate SRC, fibroblasts isolated from *Src*$^{+/+}$ cells and cells deficient for the three SRC family members *Src, Yes*, and *Fyn* (SYF cells) were used. This triple mutation of the ubiquitously expressed SRC family members leads to embryonic lethality (Klinghoffer et al., 1999). *Src*$^{+/+}$ and SYF cells were exposed to hypoxia (1.5% O_2) or normoxia (21% O_2) in the presence or absence of the iron chelator desferrioxamine (DFO; 100 μM) to examine whether SRC is required for the stabilization of the HIF-1α protein. The HIF-1α protein was stabilized in both the *Src*$^{+/+}$ and SYF cells exposed to hypoxia or to the hypoxic mimetic, DFO (Figure 3.1). To examine HIF-1 activity, the luciferase reporter assay under the control of a promoter containing three hypoxic response element sites (HRE-Luciferase) was used. Hypoxia and DFO both increased HRE-dependent luciferase induction regardless of SRC status (Figure 3.2). These results indicate that the hypoxic activation of HIF-1 is independent of SRC. To further confirm these results the two downstream SRC targets, FAK and Rac1 were tested. FAK is phosphorylated by SRC at a number of tyrosine residues and then FAK can activate Rac1. As shown in Figure 3.3, FAK and Rac1 are not required for the stability of the HIF-1α protein. In order to demonstrate the requirement of FAK, *Fak*$^{+/+}$ and *Fak*$^{-/-}$ cells were used. To demonstrate the requirement of Rac1, WT cells were infected with a dominant negative Rac1 (N17) adenovirus. These results demonstrate that the SRC mediated pathway is not required for hypoxic activation of HIF-1.

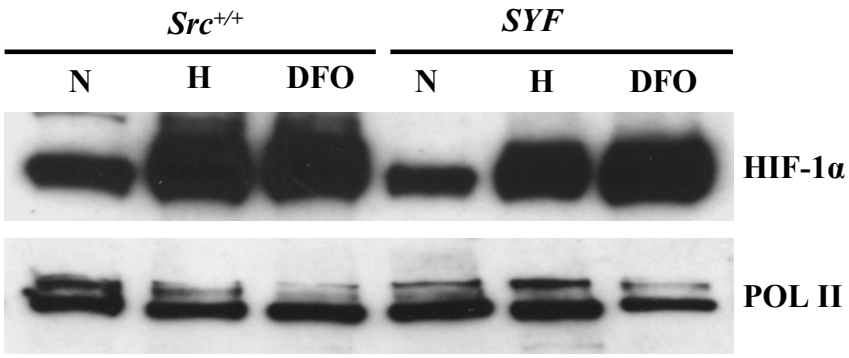

Figure 3.1. SRC is not required for HIF-1α protein stability.

HIF-1α protein levels in *Src*$^{+/+}$ and *SYF* cells exposed to 21% O_2 (N) ± 100 μM DFO or to 1.5% O_2 (H) for 2 h.

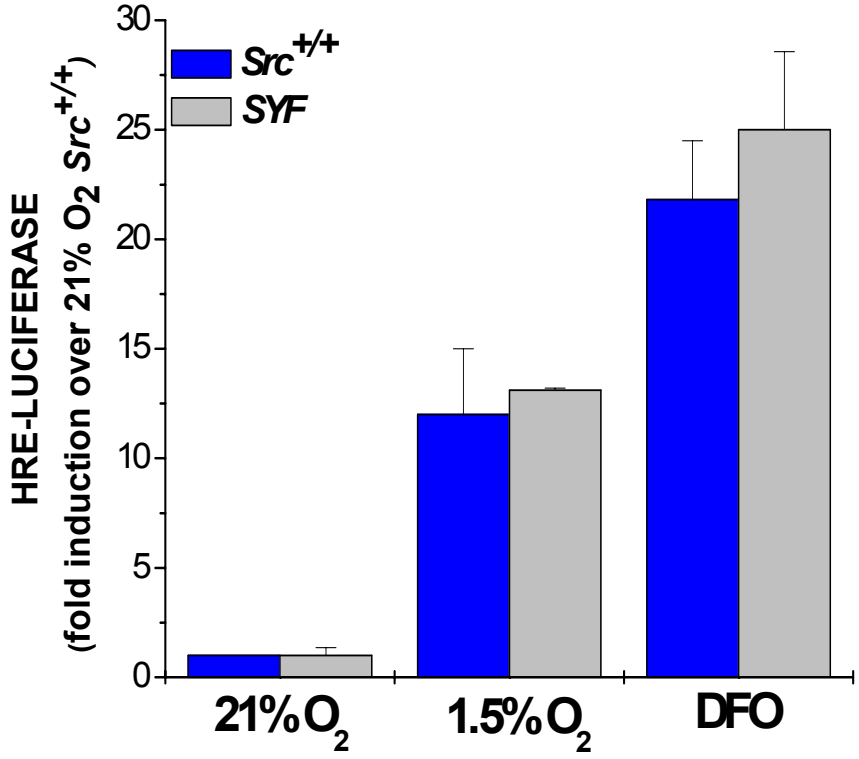

Figure 3.2. SRC is not required for HIF-1 activation.

Src$^{+/+}$ and *SYF* cells transfected with the HRE-Luciferase reporter gene construct and exposed to 21% O_2 ± 100 μM DFO or to 1.5% O_2 for 16 h. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to *Src*$^{+/+}$ cells. The data presented are the mean (±SEM) of four independent transfections.

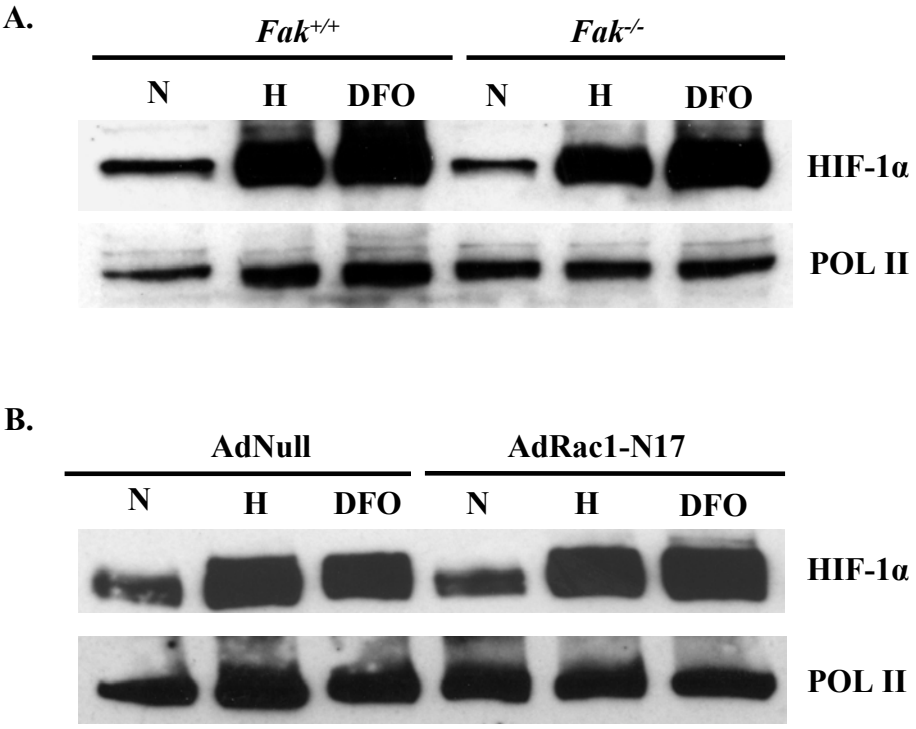

Figure 3.3. FAK and Rac1 are not required for HIF-1α protein stability.

(A). HIF-1α protein levels in *Fak*$^{+/+}$ and *Fak*$^{-/-}$ cells exposed to 21% O_2 (N) ± 100 μM DFO or to 1.5% O_2 (H) for 2 h. **(B).** HIF-1α protein levels in WT cells infected with null adenovirus (control) or adenovirus expressing a dominant negative Rac1 and subsequently exposed to 21% O_2 (N) ± 100 μM DFO or 1.5% O_2 (H) for 2 h.

ASK1 is not required for hypoxic activation of HIF-1.

The next candidate to be investigated was the MAPKKK, ASK1. To first test the requirement of ASK1 for HIF-1α protein stability, *.Ask1*$^{+/+}$ and *Ask1*$^{-/-}$ cells were exposed to hypoxia (1.5% O_2) or normoxia (21% O_2) in the presence or absence of the iron chelator desferrioxamine (DFO; 100 μM). Loss of *Ask1* did not affect the stabilization of the HIF-1α protein under hypoxia (Figure 3.4). Furthermore, hypoxia increased HRE-dependent luciferase to similar levels in both *Ask1*$^{+/+}$ and *Ask1*$^{-/-}$ cells (Figure 3.5). These results indicate that the hypoxic activation of HIF-1 is not dependent on ASK1.

AMPK is not required for hypoxic activation of HIF-1.

To test the requirement of AMPK for the hypoxic activation of HIF-1, the known AMPK inhibitor, Compound C was used. Compound C is an AMPK inhibitor that functions an ATP-competitive inhibitor of AMPK (Calbiochem).WT cells were first exposed to hypoxia (1.5% O_2) or normoxia (21% O_2) in the presence or absence of the PHD inhibitor dimethyloxaloylglycine (DMOG; 100 μM) ± 20μM Compound C. DMOG is a cell penetrant oxoglutarate analogue, expected to inhibit all enzymes of the oxoglutarate-dependent dioxygenase class, including collagen prolyl hydroxylases, PHD 1–3 and FIH. Therefore, DMOG serves a hypoxic mimetic under normal oxygen conditions, just as DFO did in the above experiments. Treatment with Compound C completely suppressed the stabilization of the HIF-1α protein under hypoxia, whereas HIF-1α protein stability was unaffected with DMOG (Figure 3.6). These results were further confirmed by examining HIF-1 activity using the HRE-Luciferase assay. Hypoxia and DMOG increased HRE-dependent luciferase induction in the untreated WT cells (Figure 3.7). In contrast, cells treated with Compound C displayed a marked

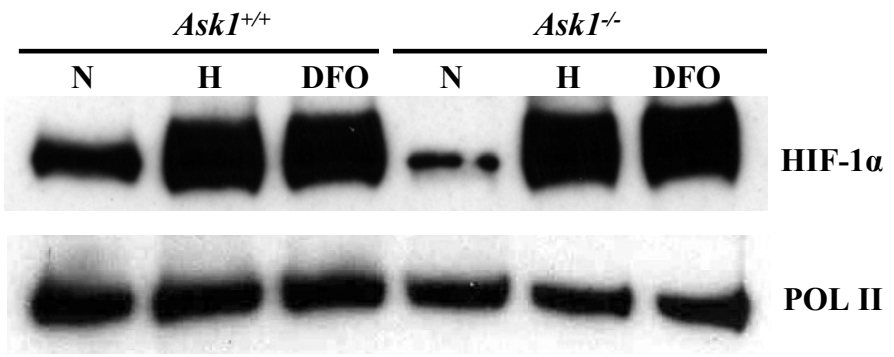

Figure 3.4. ASK1 is not required for HIF-1α protein stability.

HIF-1α protein levels in *Ask1*$^{+/+}$ and *Ask1*$^{-/-}$ cells exposed to 21% O_2 (N) ± 100 μM DFO or to 1.5% O_2 (H) for 2 h.

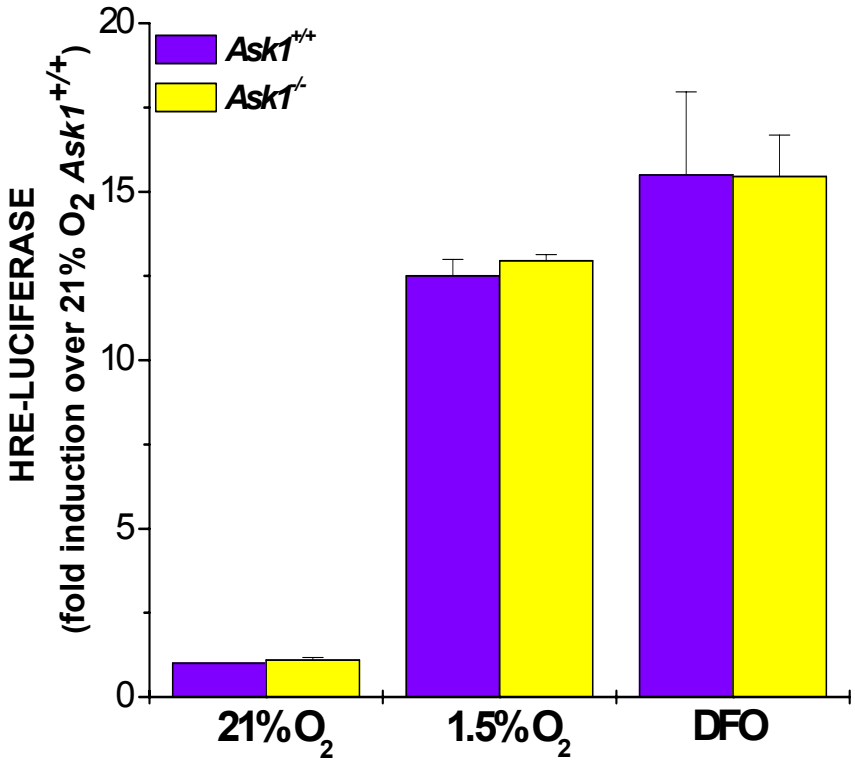

Figure 3.5. ASK1 is not required for the hypoxic activation of HIF-1.

Ask1$^{+/+}$ and *Ask1*$^{-/-}$ cells transfected with the HRE-Luciferase reporter gene construct and exposed to 21% O_2 ± 100 μM DFO or to 1.5% O_2 for 16 h. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to *Ask1*$^{+/+}$ cells. The data presented are the mean (±SEM) of four independent transfections.

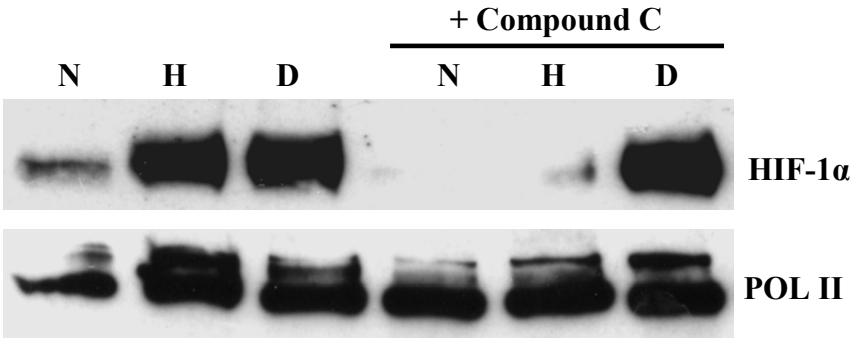

Figure 3.6. AMPK inhibitor prevents HIF-1α protein stability.

HIF-1α protein levels in WT cells exposed to 21% O_2 (N) ± 100 μM DMOG (D) or to 1.5% O_2 (H) ± 20μM Compound C, an AMPK inhibitor, for 2 h.

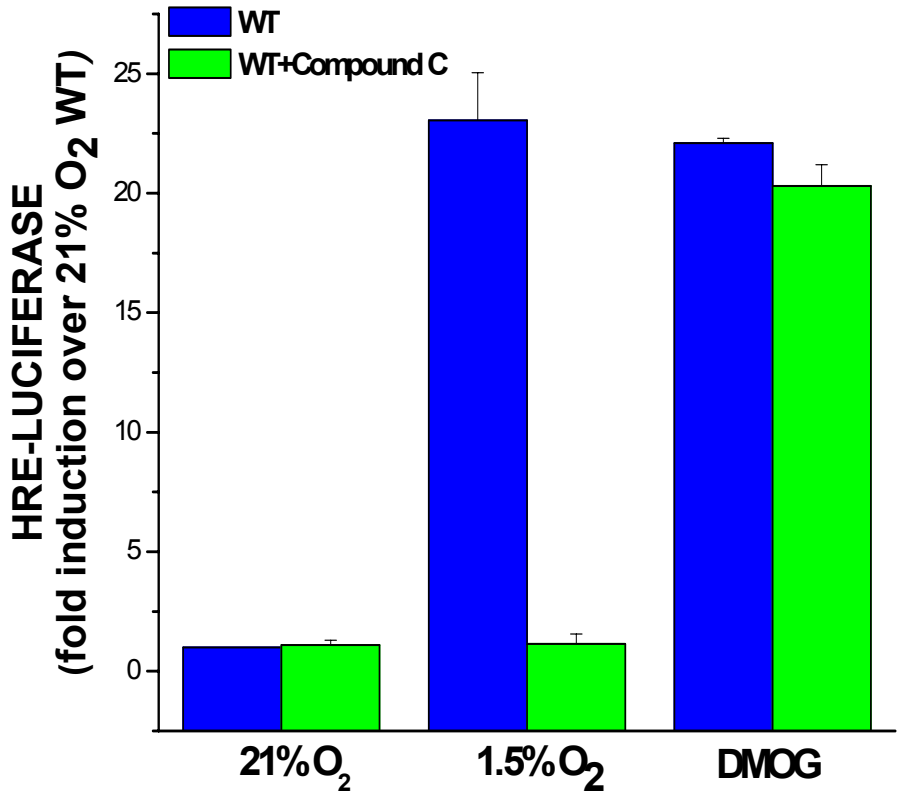

Figure 3.7. AMPK inhibitor prevents HIF-1 activation.

WT cells transfected with the HRE-Luciferase reporter gene construct and exposed to 21% O_2 ± 100 μM DMOG or to 1.5% O_2 ± 20μM Compound C, an AMPK inhibitor, for 16 h. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to untreated normoxic WT cells. The data presented are the mean (±SEM) of four independent transfections.

attenuation of luciferase induction under hypoxia but not in the presence of DMOG during normoxia (Figure 3.7). These data are consistent with the results observed in both the *Mkk3/6*$^{-/-}$ and *p38α*$^{-/-}$ cells, thereby suggesting that AMPK could be the upstream regulator of the MKK3/6-p38α-HIF-1 hypoxic pathway. Usage of chemical inhibitors is not the ideal method to prove the requirement of a protein of interest, as many chemicals have off targets and are not always specific for their target. Therefore, the double knockout cells for AMPKα1 and AMPKα2 were used in order to genetically test the requirement of AMPK in the hypoxic activation of HIF-1. *Ampkα WT* and the *Ampkα1*$^{-/-}$*2*$^{-/-}$ cells were exposed to hypoxia (1.5% O_2) or normoxia (21% O_2) in the presence or absence of the PHD inhibitor dimethyloxaloylglycine (DMOG; 100 μM). Interestingly, the loss of bothe AMPKα1 and AMPKα2 did not effect HIF-1α protein stability under hypoxia (Figure 3.8). Moreover, the HRE-Luciferase assay displayed no difference between the *Ampkα WT* and the *Ampkα1*$^{-/-}$*2*$^{-/-}$ cells under hypoxia (Figure 3.9), proving that the hypoxic activation of HIF-1 is not dependent on AMPK.

These data were unexpected since Compound C, an AMPK inhibitor, prevented the hypoxic stabilization of the HIF-1α protein. Therefore, to further validate these results the HRE-Luciferase assay was used to examine HIF-1 activity in the *Ampkα1*$^{-/-}$*2*$^{-/-}$ (AMPKα null) in the presence or absence of Compound C. As shown by immunoblotting, these cells are null for AMPKα (Figure 3.10A). Confirming the results shown in Figure 3.7, WT cells treated with Compound C displayed a severe reduction in HRE- Luciferase expression when exposed to hypoxia in comparison to untreated WT cells (Figure 3.10B). Interestingly, untreated AMPKα null cells showed an ever greater increase in HRE-Luciferase expression than WT cells and treated AMPKα null cells displayed no induction of HRE-Luciferase under hypoxia (Figure

3.10B). From these results, it can be concluded through the use of genetics that the hypoxic activation is independent of AMPK. Furthermore, these data show that the AMPK chemical inhibitor, Compound C, is not specific to the one target, AMPK. Here we show that Compound C does have alternate targets. What those targets are or how Compound C is preventing the hypoxic activation of HIF-1 remains unknown. Compound C may inhibit other kinase pathways, such as the p38 MAPK pathway, thereby suppressing HIF-1 activity. Or it may function as an antioxidant in order to scavenge ROS generated by the mitochondria during hypoxia to inhibit HIF-1. Or it may function as a respiratory or mitochondrial inhibitor in order to prevent the hypoxic activation of HIF-1. There are many possible scenarios as for the mechanisms of action for Compound C. It is a bonafide AMPK inhibitor, although as the data presented here indicates, it is definitely not specific to AMPK.

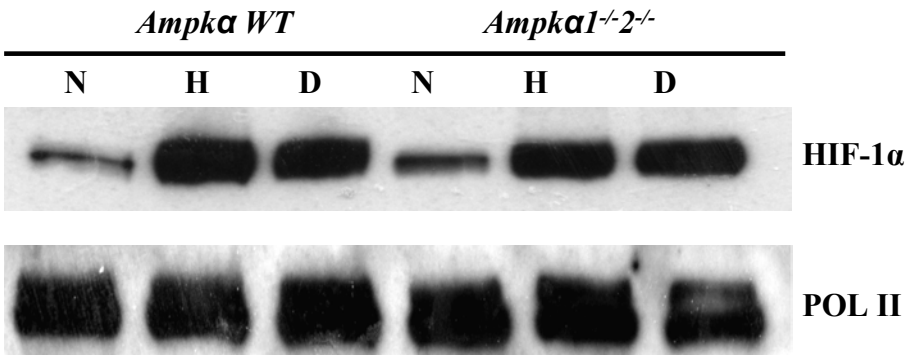

Figure 3.8. AMPK is not required for HIF-1α protein stability.

HIF-1α protein levels in *Ampkα WT* and *Ampkα1*$^{-/-}$*2*$^{-/-}$ cells exposed to 21% O_2 (N) ± 100 μM DMOG (D) or to 1.5% O_2 (H) for 2 h.

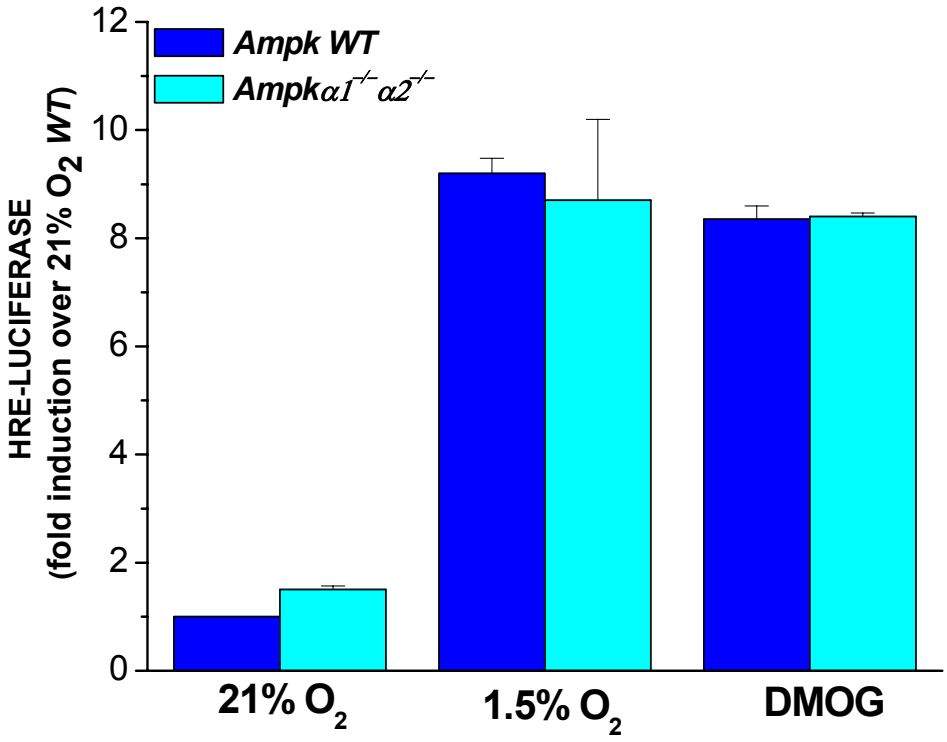

Figure 3.9. AMPK is not required for HIF-1 activition.

Ampkα WT and *Ampkα1*$^{-/-}$*2*$^{-/-}$ transfected with the HRE-Luciferase reporter gene construct and exposed to 21% O_2 ± 100 μM DMOG or to 1.5% O_2 for 16 h. Relative luciferase expression is the ratio of luciferase/tnormalized to *Ampkα WT* cells. The data presented are the mean (±SEM) of four independent transfections.

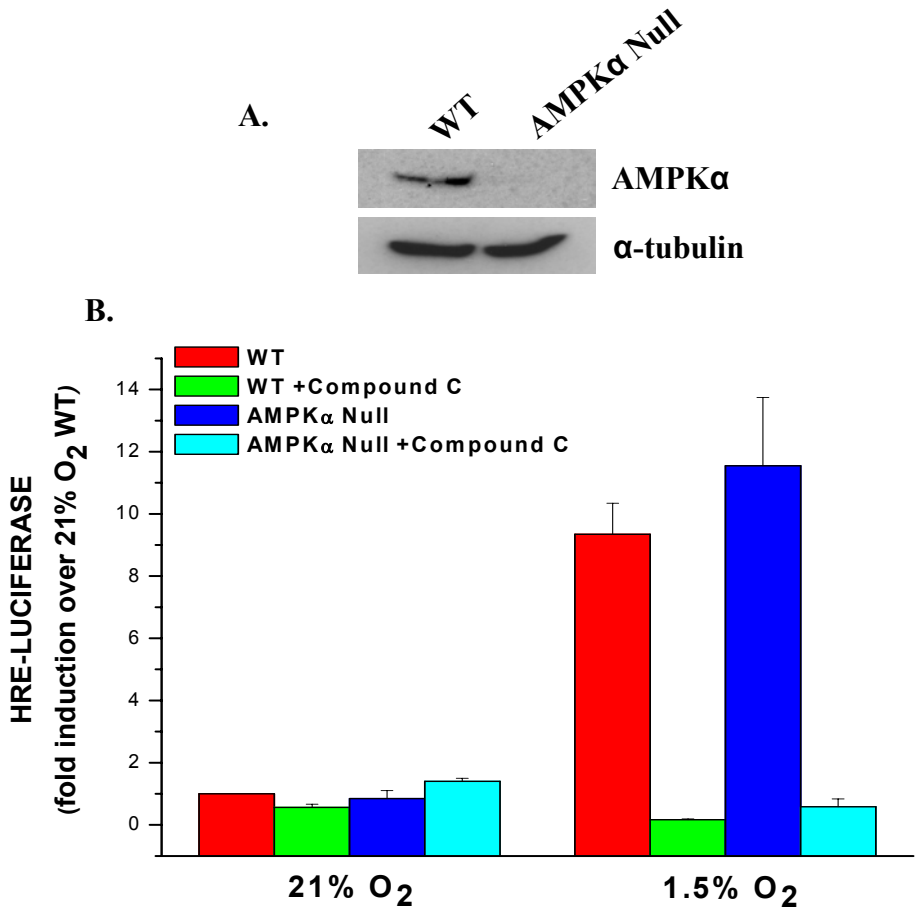

Figure 3.10. AMPK inhibitor prevents HIF-1 activation in AMPKα null cells.

(A). AMPKα protein levels in *Ampkα WT* cells (WT) and *Ampkα1*$^{-/-}$*2*$^{-/-}$cells (AMPKα null). **(B).** *Ampkα1*$^{-/-}$*2*$^{-/-}$ cells transfected with the HRE-Luciferase reporter gene construct and exposed to 21% O2 ± 100 μM DMOG or to 1.5% O2 ± 20μM Compound C, an AMPK inhibitor, for 16 h. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to untreated normoxic WT cells. The data presented are the mean (±SEM) of four independent transfections.

Discussion

Understanding how hypoxia activates HIF-1 is important for understanding the pathophysiology of vascular disease, pulmonary disease, heart disease, and cancer. HIF-1 is overexpressed in many human cancers and plays a crucial role in altering the transcriptional repertoire of tissues as oxygen levels drop. To date the upstream regulators of HIF-1 are poorly understood. Mitochondria have been implicated as oxygen sensors by increasing the generation of reactive oxygen species (ROS) within complex III. Furthermore, it has been demonstrated that the ROS generated within complex III during hypoxia is required and sufficient to induce the stabilization of HIF-1α (Brunelle et al., 2005; Chandel et al., 2000; Guzy et al., 2005; Mansfield et al., 2005). The p38 MAPK signaling pathway provides a mechanistic link between the generation of oxidant production and the activation of HIF-1 during hypoxia. The p38 MAPK signaling cascade is necessary for the hypoxic activation of HIF-1 (Emerling et al., 2005). However, the direct targets of the mitochondrial ROS or the upstream regulators of the p38 MAPK pathway to activate HIF-1 remain unknown. To investigate potential upstream regulators of HIF-1, a candidate approach was used here. Three known targets of ROS were chosen on the basis that they could also activate MKK3/MKK6 dependent p38 MAPK signaling. In this study SRC, ASK1, and AMPK were tested and the data demonstrates that all three of these kinases do not play a role in regulating the hypoxic activation of HIF-1 (Figure 3.11).

Currently, the upstream regulators still need to be identified. Interestingly, Compound C, a known AMPK inhibitor, prevents the hypoxic activation of HIF-1 independent of AMPK. Obvious alternate targets of the chemical inhibitor Compound C could be p38 MAPK or antioxidants in order to inhibit HIF-1 activation, yet other unknown proteins may prove to be

targets, thereby uncovering upstream regulators in the hypoxic signaling pathway for HIF-1 activation. Identifying the targets of Compound C may prove to be important in deciphering the upstream regulators of HIF-1. Nevertheless, having a blueprint of an oxygen sensing pathway emanating from the mitochondrial electron transport chain, to the p38 MAPK pathway, and ultimately to HIF-1 will allow investigators to fill the critical details of signaling molecules linking mitochondria to HIF-1 activation. Moreover, having eliminated SRC, ASK1, and AMPK as upstream regulators of HIF-1 will be vital information for investigators to have.

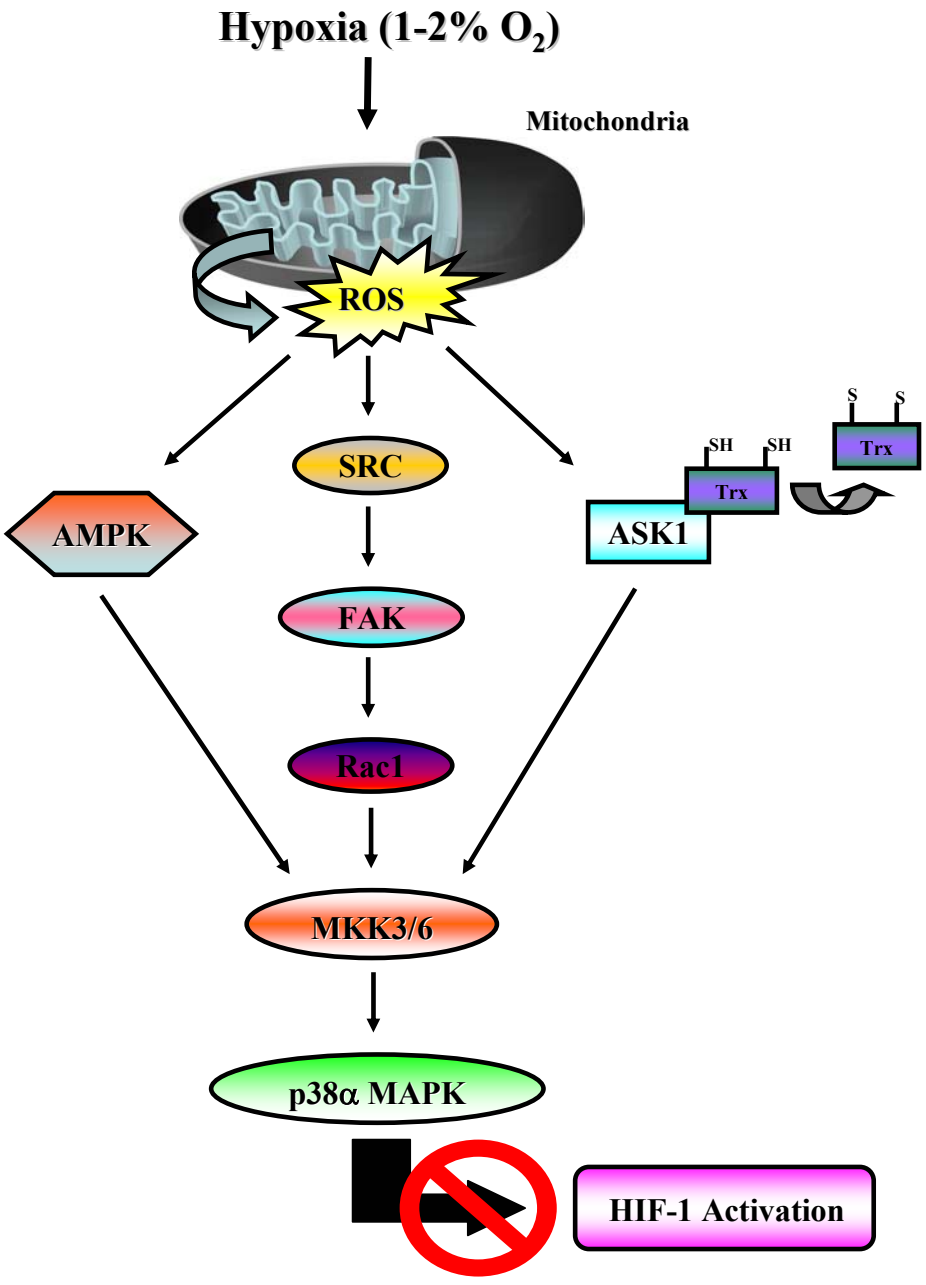
Hypoxia (1-2% O_2)
Mitochondria
ROS
SRC
AMPK
ASK1
SH
SH
Trx
S
S
Trx
FAK
Rac1
MKK3/6
p38α MAPK
HIF-1 Activation

Figure 3.11. Hypoxic regulation of HIF-1.

Hypoxia stimulates the generation of reactive oxygen species (ROS) from the mitochondria, thereby initiating the MKK3/6 dependent p38α MAPK pathway to activate HIF-1. SRC, ASK1, and AMPK are activated by ROS and hypoxia and are known activators of the p38α MAPK signaling pathway. Data presented here indicate that SRC, ASK1, and AMPK are not required for the hypoxic activation of HIF-1.

Chapter 4: PTEN Represses HIF-1 Transcriptional Activity through FOXO3a

Introduction

The PTEN tumor suppressor was first identified as a gene mutated or deleted in multiple primary malignant tumors, including prostate cancers and glioblastomas (Cairns et al., 1997; Li and Sun, 1997; Li et al., 1997; Liu et al., 1997; Steck et al., 1997; Teng et al., 1997; Wang et al., 1997). PTEN is a lipid phosphatase that functions as a tumor suppressor by antagonizing the PI3K/AKT dependent signaling pathway (Di Cristofano and Pandolfi, 2000; Maehama et al., 2001; Simpson and Parsons, 2001). Loss of PTEN, in both murine embryonic stem cells or in human cancer cell lines, allows the accumulation of PIP3 and thus the activation of downstream effectors, one major factor being AKT (Franke et al., 1997; Stambolic et al., 1998; Sun et al., 1999; Wu et al., 1998). AKT is a serine/threonine kinase that phosphorylates many critical signaling molecules, which are specifically involved in cell growth, cell survival, glucose metabolism, and cell invasiveness. Several indirect and direct phosphorylation targets of AKT have been identified, including the mammalian target of rapamycin (mTOR) and the Forkhead (FOXO) transcription factors respectively (Brunet et al., 1999; Gao et al., 2002b; Inoki et al., 2002; Kops et al., 1999; Tee et al., 2003). A hallmark of PTEN null cancers is an increase in mTOR activity and a decrease in FOXO activity (Di Cristofano and Pandolifi 2000).

Mammalian members of the Forkhead transcription factors include FOXO1a, FOXO3a, and FOXO4 (also referred as FKHR, FKHRL1, and AFX, respectively). FOXO factors function as key regulators of cell cycle progression and cell survival. Phosphorylation of FOXO factors by AKT prevents their transcriptional activity by inducing the binding of 14-3-3 proteins, thereby promoting export to the cytoplasm (Biggs et al., 1999; Brownawell et al., 2001; Brunet et al.,

1999; Rena et al., 1999; Takaishi et al., 1999; Tang et al., 1999; Tomizawa et al., 2000). In PTEN null cells, FOXO factors are constitutively phosphorylated and consequently predominantly cytoplasmic. Therefore, when wild-type FOXO is expressed exogenously in PTEN null cells, they are still not capable of activating FOXO responsive promoters (Nakamura et al., 2000). However, mutations in the three AKT phosphorylation sites to alanine (FOXO-AAA) allows the accumulation in the nucleus, thus restoring FOXO transcriptional activity in PTEN null cells (Nakamura et al., 2000; Ramaswamy et al., 1999). An alternate method of sequestering FOXO in the nucleus is treatment with the nuclear export inhibitor, Leptomycin B (LMB). Interestingly, it has been shown that forcible localization of FOXO1a to the nucleus can reverse tumorigenicity of PTEN null cells (Ramaswamy et al., 2002).

The majority of solid tumors require angiogenesis for tumor expansion. Many PTEN null tumors are highly vascularized, including prostate cancers and glioblastomas. In particular, glioblastomas are one of the most vascularized tumors and display increased expression of VEGF (Wesseling et al., 1997). Hypoxia is a potent stimulus for triggering the 'angiogenic switch'. The master transcription factor that regulates the cellular responses to hypoxia is hypoxia-inducible-factor-1 (HIF-1) (Semenza, 2000a). HIF-1 is composed of two subunits, an oxygen-sensitive HIF-1α subunit, and a constitutively expressed HIF-1β subunit. HIF-1 activity is dependent on the availability of the HIF-1α subunit. Under normoxic (21% O_2) conditions, HIF-1α is targeted for ubiquitin-mediated degradation by an E3 ubiquitin ligase complex that contains the von Hippel-Lindau tumor suppressor protein (pVHL), elongin B, elongin C, Cul2, and Rbx (Maxwell et al., 1999). This process is dependent on the hydroxylation of two proline residues by a family of prolyl hydroxylase (PHD) enzymes (Ivan et al., 2001; Jaakkola et al.,

2001; Masson et al., 2001). Under hypoxic conditions, hydroxylation is presumed to be inhibited and HIF-1α is stabilized, thereby allowing HIF-1α to localize in the nucleus where it can dimerize with HIF-1β, recruit p300 and CBP, allowing the transcriptional activation of HIF-1 target genes. HIF-1 target genes include angiogenic factors, glucose transporters, glycolytic enzymes, survival factors, and invasion factors- all crucial trademarks of cancer. Previous data shows that loss of PTEN can increase HIF-1 activity in glioma and prostate cancer cell lines (Jiang et al., 2001; Zhong et al., 2000; Zundel et al., 2000). Therefore, exaggerated HIF-1 activity in PTEN null cancers may explain the aggressiveness of these tumors. Taken together, these studies suggest a link between the loss of PTEN and the hypoxic activation of HIF-1. However, the mechanism by which loss of PTEN increases HIF activity is not fully understood.

To investigate whether the loss of PTEN regulates HIF-1, we used PTEN null murine embryonic fibroblasts (MEFs), prostate cancer and glioma cells. Our results indicate that nuclear PTEN represses HIF-1 transcriptional activity via FOXO activation, and independent of mTOR signaling. Here, we show by co-immunoprecipitation that endogenous FOXO3a can associate with HIF-1α and p300 in the nucleus. Moreover, when FOXO3a is sequestered in the nucleus, by treatment with LMB, we demonstrate by chromatin immunoprecipitation (ChIP) that FOXO3a is found in complex with HIF-1α and p300 on the hypoxia response element (HRE) of the HIF-1 target gene, *Glut-1*. Nuclear FOXO3a appears to interfere with p300's ability to function as a co-activator for HIF-1. Overall, these data suggest by inhibiting FOXO nuclear export in PTEN null cancers may decrease tumor growth by decreasing HIF-1 transcriptional activity.

Results

PTEN modulates HIF-1 transcriptional activity.

To determine whether PTEN regulates HIF-1 activity, *Pten*$^{+/-}$ and *Pten*$^{-/-}$ murine embryonic fibroblasts (MEFs) were exposed to normoxia (21%O_2), hypoxia (1.5%O_2), or anoxia (0%O_2). The loss of PTEN had no effect on HIF-1α stability (Figure 4.1). However, the *Pten*$^{-/-}$ cells displayed a robust increase in HIF-1 dependent luciferase (HRE-Luciferase) induction in comparison to the *Pten*$^{+/-}$ cells under both hypoxic (1.5%O_2) and anoxic (0%O_2) conditions (Figure 4.2). Loss of PTEN also markedly increased luciferase induction in the presence of the hypoxia mimetic agent dimethyloxaloylglycine. (Figure 4.3). HIF-1 transcriptional activity was further assessed by examining the induction of HIF-1 target genes, vascular endothelial growth factor (*Vegf A*) and phosphoglycerate kinase 1 (*Pgk1*), using real-time quantitative RT-PCR. *Pten*$^{-/-}$ cells exposed to hypoxia and anoxia displayed a dramatic increase in both *Vegf A* and *Pgk1* levels in comparison to the *Pten*$^{+/-}$ cells (Figure 4.4). In order to test whether the increase in HIF-1 transcriptional activity, in the *Pten*$^{-/-}$ cells, is independent on the β subunit, a specific GAL4 DNA binding domain (amino acids 1 to 147) fused to HIF-1α (531-826) fusion construct was utilized. HIF-1α contains two oxygen regulated transactivation domains (TADs), which are termed N-TAD (amino acids 531 to 575) and C-TAD (amino acids 786 to 826) (Jiang et al., 1997). The C-TAD is regulated by the hydroxylation of an asparagine residue by FIH to prevent the interaction of the HIF-1α protein with transcriptional co-activators, such as p300. Transactivation by GAL4-HIF-1α (531 to 826) increased by five-fold in the *Pten*$^{-/-}$ cells compared to the normal transactivation seen in the *Pten*$^{+/-}$ cells (Figure 4.5). These results indicate that the increase in HIF-1 transcriptional activity caused by the loss of PTEN can be assigned to the transactivation domain within the HIF-1α subunit. Interestingly, the C-TAD

region of HIF-1α is where p300 interacts with the α-subunit in order to fully activate the HIF-1 transcription factor. Collectively, these data demonstrate that the loss of PTEN increases HIF-1 transcriptional activity, but has no effect on HIF-1α protein stability.

To confirm that the increase in transcriptional activity of HIF-1 was indeed due to the loss of PTEN, we reintroduced PTEN into the *Pten*$^{-/-}$ cells using an adenovirus. Immunoblot analysis demonstrates that the reconstituted cells express PTEN (Figure 4.6A). Corroborating the results in Figure 4.1, the reintroduction of PTEN had no effect on the stability of the HIF-1α subunit (Figure 4.6B). In contrast, the transcriptional activity of HIF-1, as assessed by HRE-Luciferase, was significantly decreased by the reintroduction of PTEN (Figure 4.7). These results indicate that HIF-1 transcriptional activity can be suppressed by the presence of PTEN in *Pten*$^{-/-}$ cells.

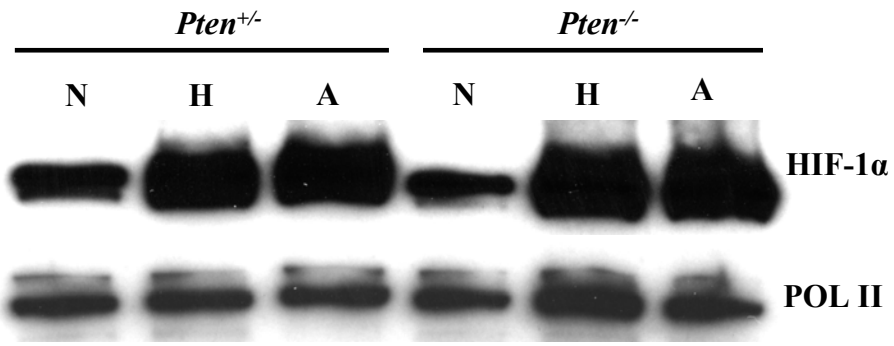

Figure 4.1. Loss of PTEN has no effect on HIF-1α protein stability.

HIF-1α protein levels were analyzed in *Pten*$^{+/-}$ and *Pten*$^{-/-}$ cells were exposed to 21% O_2 (N), 1.5% O_2 (H), or 0% O_2 for 2 hours.

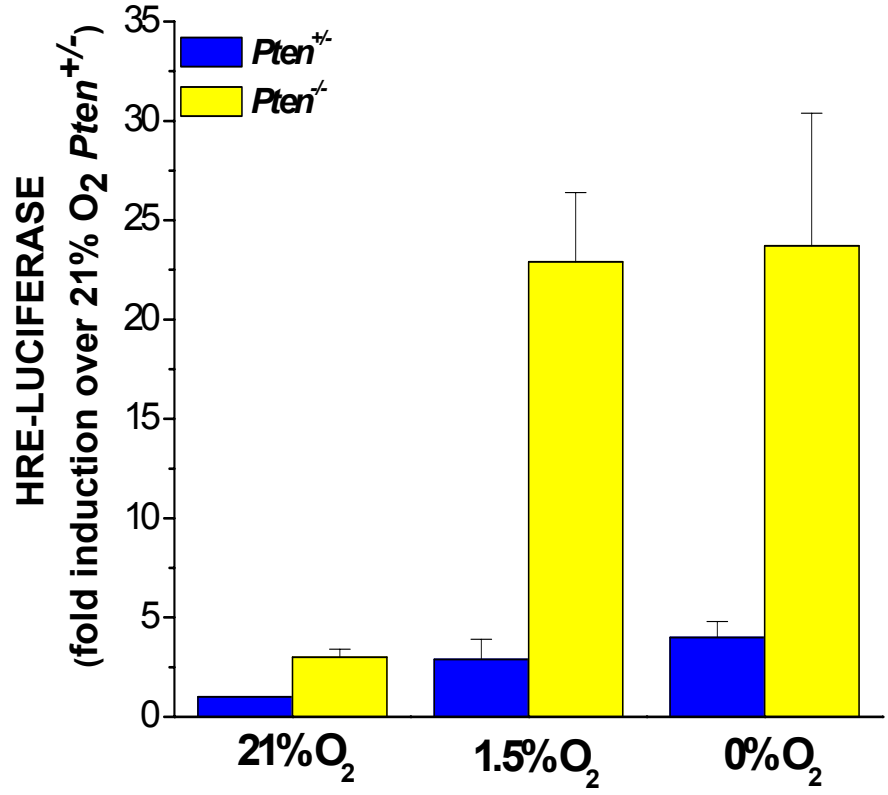

Figure 4.2. Loss of PTEN increases transcriptional activity of HIF-1.

Pten$^{+/-}$ and *Pten*$^{-/-}$ cells were transfected with a HRE-Luciferase reporter gene construct and exposed to 21% O_2, 1.5% O_2, or 0% O_2 for 16 hours before being harvested. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to *Pten*$^{+/-}$ cells. The data presented are the mean (±SEM) of four independent transfections.

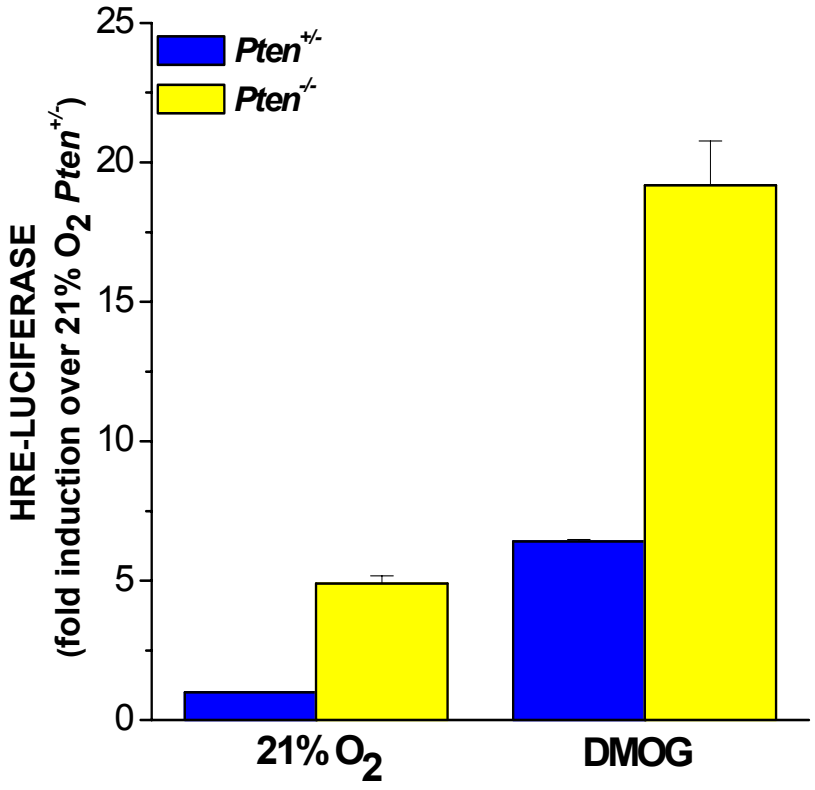

Figure 4.3. Effect of HIF-1 transcriptional activity using hypoxic mimetic agent. *Pten*$^{+/-}$ and *Pten*$^{-/-}$ cells were transfected with a HRE-Luciferase reporter gene construct and exposed to 21% O_2 ± the hypoxia mimetic agent dimethyloxaloylglycine. (DMOG) for 16 hours before being harvested. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to *Pten*$^{+/-}$ cells. The data presented are the mean (±SEM) of four independent transfections.

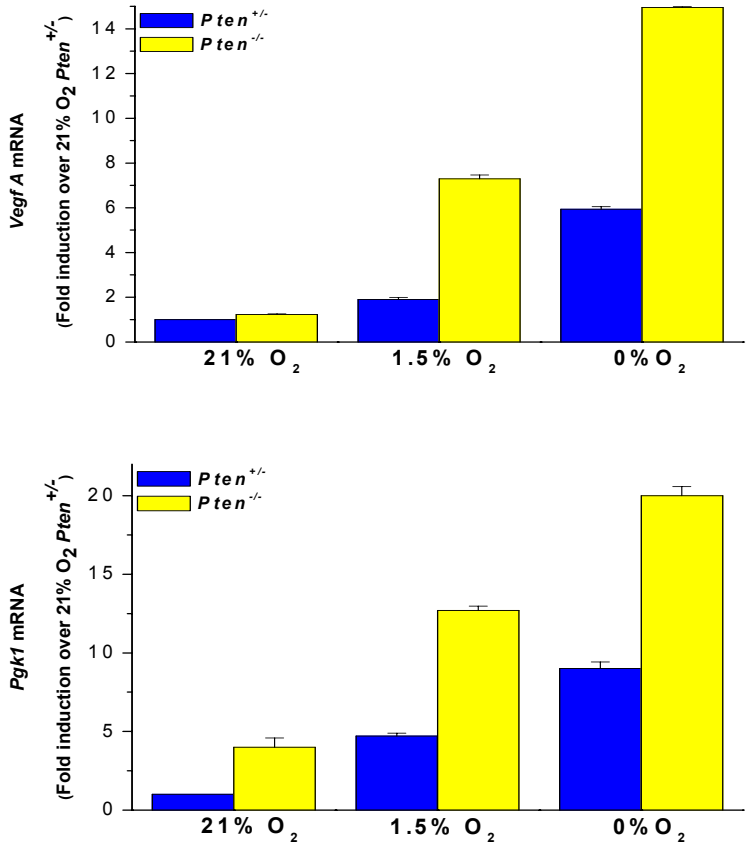

Figure 4.4 . Loss of PTEN induces HIF-1 target genes.

Pten$^{+/-}$ and *Pten*$^{-/-}$ cells were cultured for 16 hours under 21% O_2, 1.5% O_2, or 0% O_2, harvested, and transcription levels of the target genes *Vegf A* and *Pgk1* were determined by quantitative real-time RT-PCR analysis. Cycle Threshold (Ct) values were normalized for amplification of the mitochondrial ribosomal protein *L19*. The data presented are the result of triplicate analyses and the error bars indicate SEM.

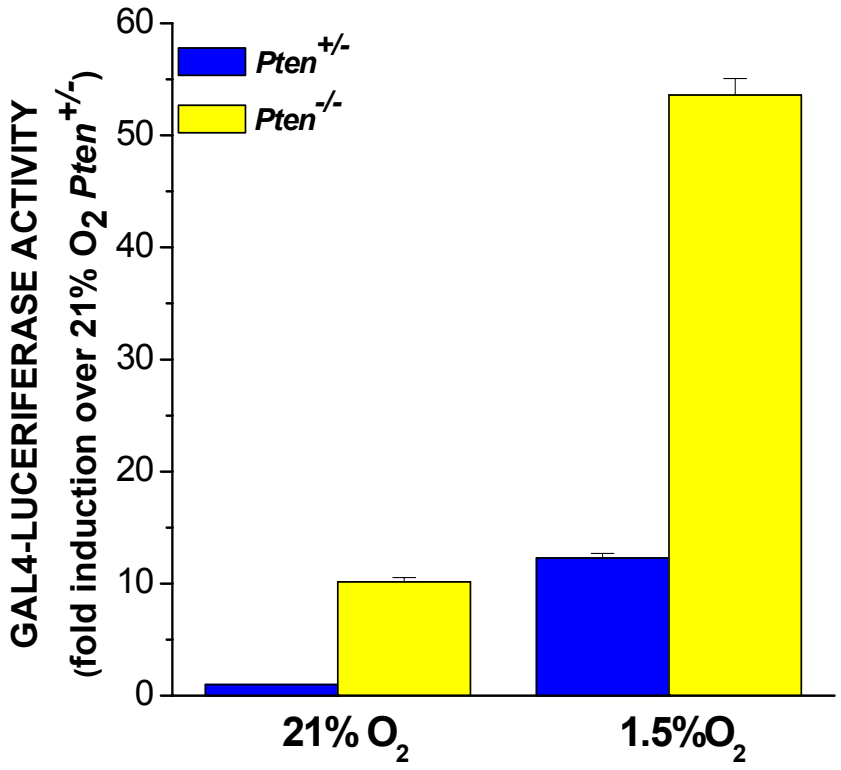

Figure 4.5. Loss of PTEN increases HIF-1α transactivation.

Pten$^{+/-}$ and *Pten*$^{-/-}$ cells were transfected with a GAL4 (1-147) DNA-binding domain fused to HIF-1α (531-826) construct and a reporter gene construct encoding five GAL4-binding sites. Cells were incubated at 21% O_2 for 20 hours, followed by 36 hours at 21% O_2 or 1.5% O_2. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to *Pten*$^{+/-}$ cells. The data presented are the mean (±SEM) of four independent transfections.

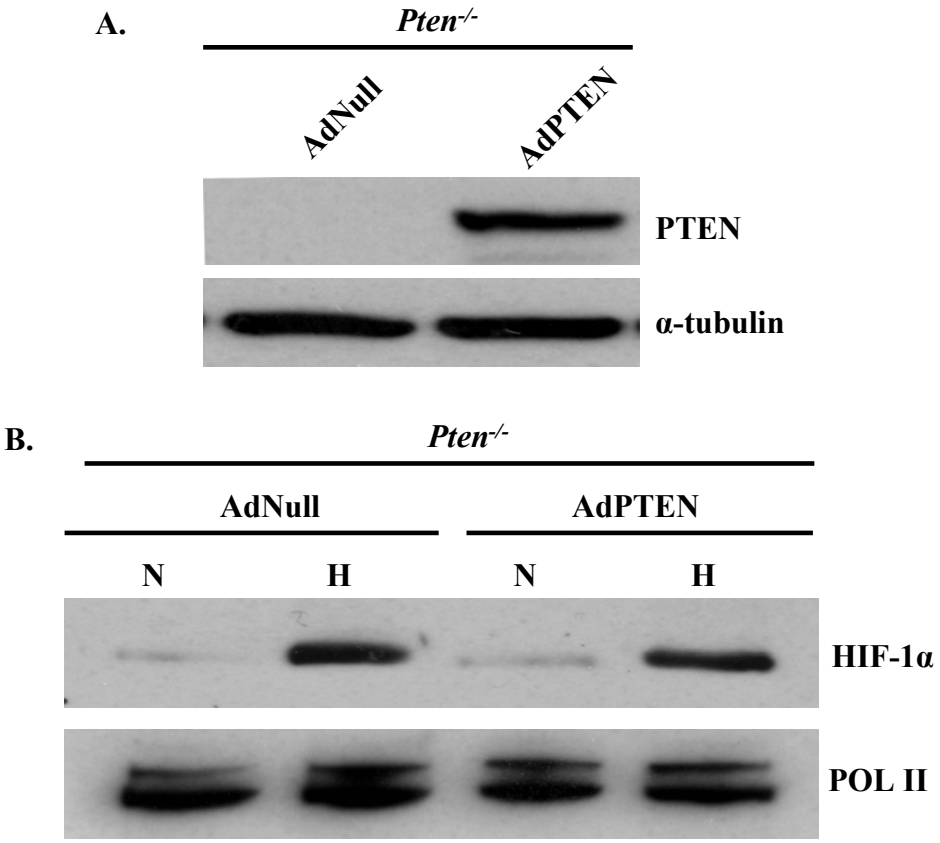

Figure 4.6. Reintroduction of PTEN has no effect on HIF-1α protein levels.

(A). *Pten*$^{-/-}$ cells infected with null adenovirus (control) or adenovirus expressing PTEN (50pfu/μl) for 3 hours in serum/antibiotic-free media and subsequently incubated for 16 hours in serum-free media before harvesting. Cell lysates were analyzed by immunoblotting with an anti-PTEN antibody. **(B).** HIF-1α protein levels were analyzed in *Pten*$^{-/-}$ cells infected with adenoviruses and were subsequently exposed to 21% O_2 (N) or 1.5% O_2 (H) for 2 hours.

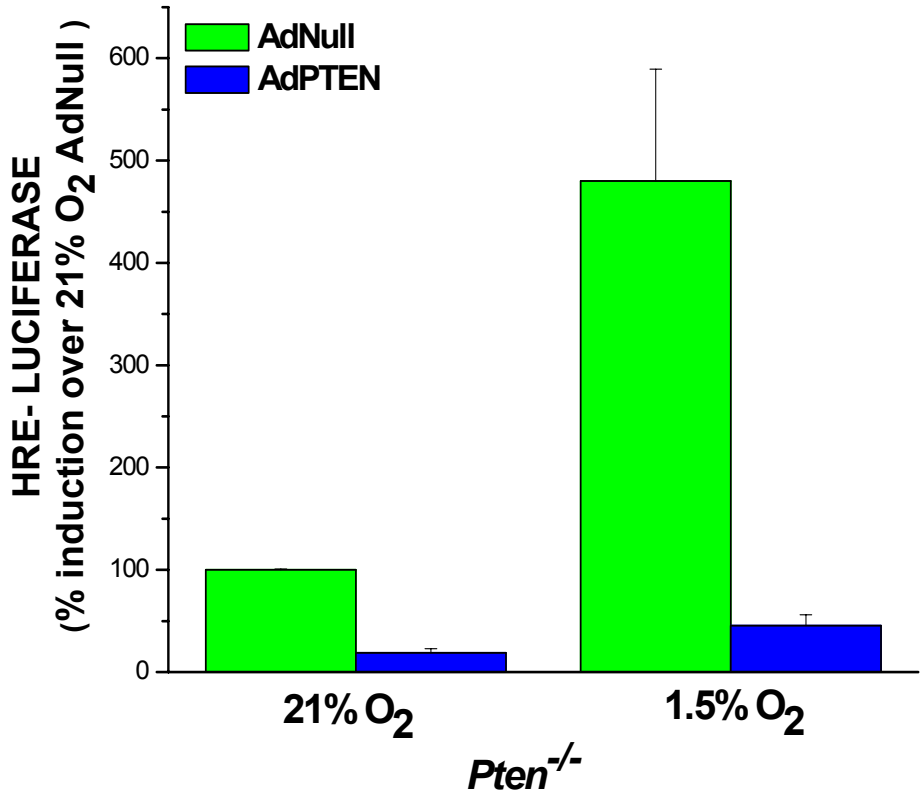

Figure 4.7. Reintroduction of PTEN decreases HIF-1 transcriptional activity. *Pten*$^{-/-}$ cells were transfected with a HRE-Luciferase reporter gene construct. 24 hours after transfection, cells were infected with adenoviruses, and subsequently exposed to 21% O_2 or 1.5% O_2 for 16 hours. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to cells expressing AdNull. The data presented are the mean (±SEM) of four independent transfections.

Upregulation of HIF-1 transcriptional activity is independent of mTOR pathway.

A key downstream target of the PI3K/PTEN/AKT pathway is the mammalian target of rapamycin (mTOR). To investigate if mTOR is required for HIF-1 activation, *Pten*$^{+/-}$ and *Pten*$^{-/-}$ cells were treated with the mTOR inhibitor, rapamycin. Treatment of rapamycin in both cell types inhibited the phosphorylation of the S6 ribosomal protein (Figure 4.8). Therefore, rapamycin was indeed repressing mTOR signaling in both cell types. As expected, the phosphorylation is more robust in the PTEN null cells, due to the increase in PI3K/AKT/mTOR signaling. Next, we examined whether inhibiting mTOR has any effect on the stability of the HIF-1α subunit. The HIF-1α subunit was stabilized under hypoxia or anoxia in both *Pten*$^{+/-}$ and *Pten*$^{-/-}$ cells that were treated with rapamycin (Figure 4.9). Similar to the immunoblot analysis, rapamycin had no effect on HIF-1 activity, as shown by HRE-luciferase (Figure 4.10). These results demonstrate that the increase in HIF-1 transcriptional activity in the PTEN null cells is not dependent on mTOR signaling.

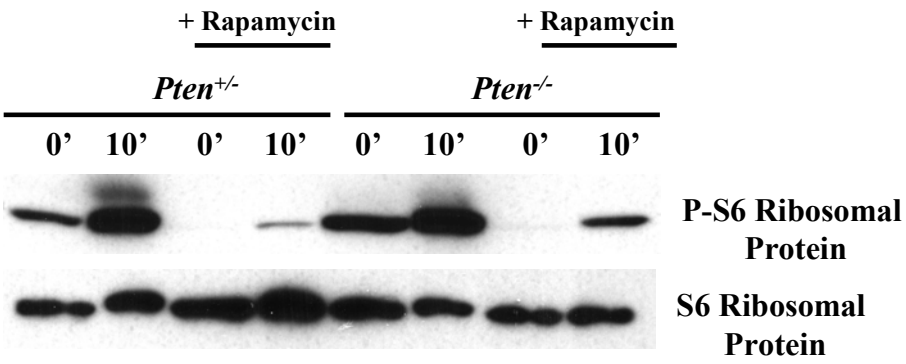

Figure 4.8. Inhibition of mTOR signaling by rapamycin.

mTOR activation as assessed by phospho-S6 Ribosomal protein in *Pten*$^{+/-}$ and *Pten*$^{-/-}$ cells ± 100nM of the mTOR inhibitor rapamycin. Cells were serum starved overnight and then placed in serum complete media for 10 minutes before harvesting.

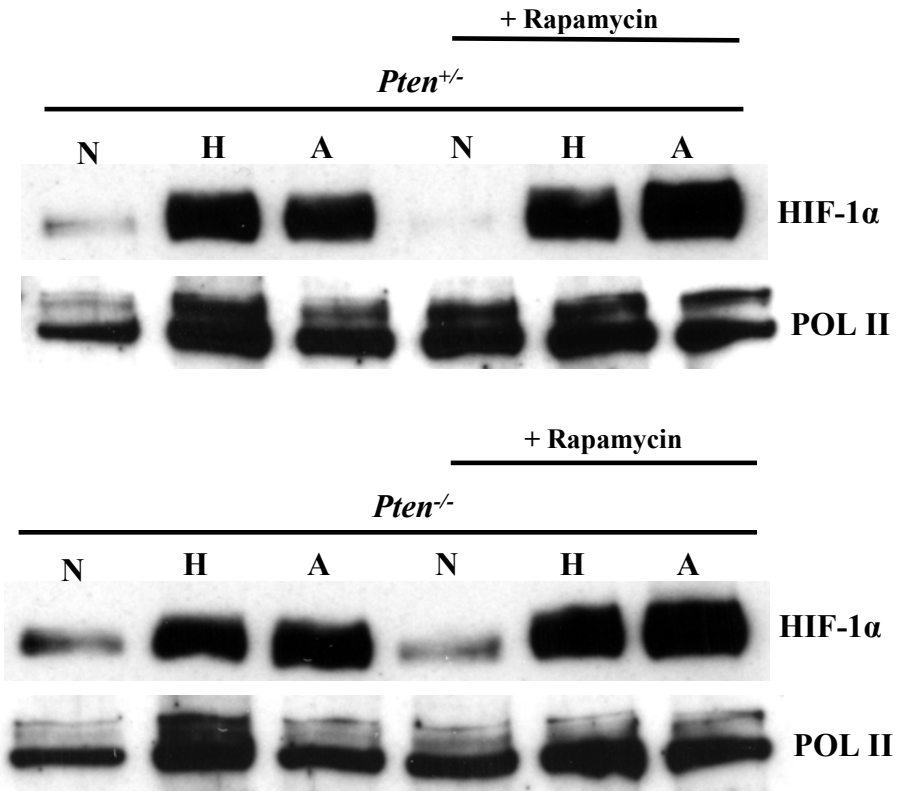

Figure 4.9. Inhibition of mTOR has no effect on HIF-1α protein stability.

HIF-1α protein levels in *Pten*$^{+/-}$ *and Pten*$^{-/-}$ cells exposed to 21% O_2 (N), 1.5% O_2 (H), or 0% O_2 (A) for 2 hours ± 100nM rapamycin.

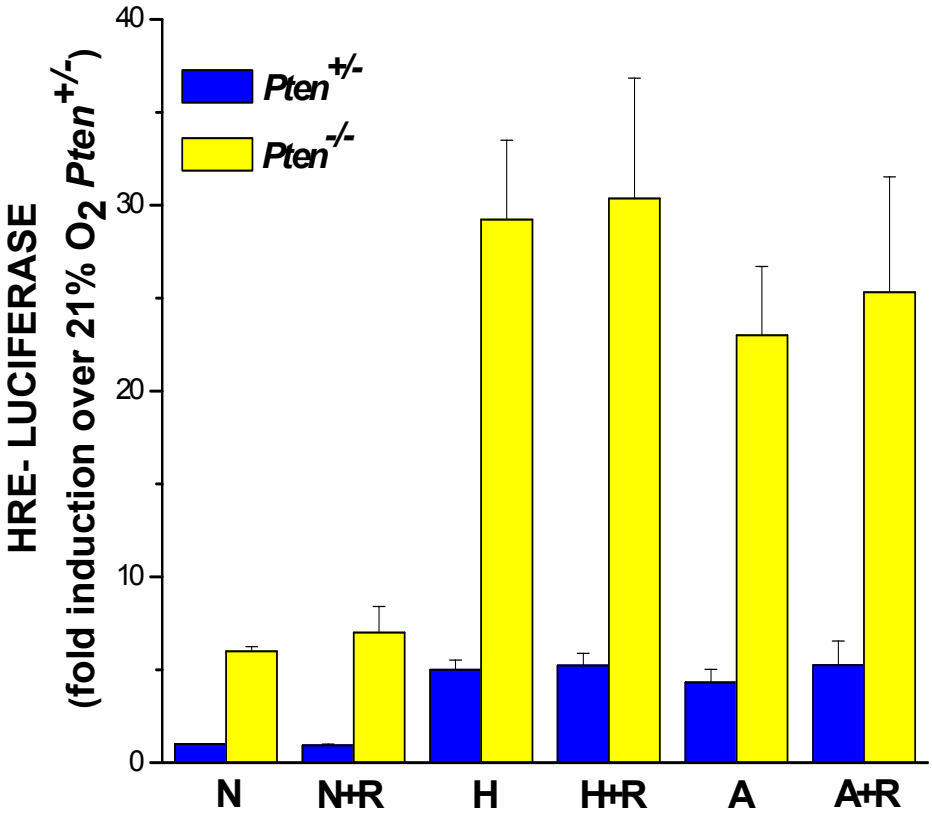

Figure 4.10. Loss of PTEN increases HIF-1 transcriptional activity independent of mTOR signaling.

Pten$^{+/-}$ and *Pten*$^{-/-}$ cells transfected with the HRE-Luciferase reporter gene construct and exposed to 21% O_2 (N), 1.5% O_2 (H), or 0% O_2 (A) for 16 hours ± 100nM rapamycin (R). Relative luciferase expression is the ratio of luciferase/total protein levels normalized to *Pten*$^{+/-}$ cells. The data presented are the mean (±SEM) of four independent transfections.

Nuclear PTEN regulates HIF-1 transcriptional activity.

Recently, a growing body of work has implicated that the tumor suppressor PTEN is not only found in the cytoplasm, but also localizes to the nucleus. Therefore, since the loss of PTEN had no effect on the stability of the α-subunit, and increased the transcriptional activity of HIF-1via the transactivation domain of the α-subunit and not the β-subunit, we examined whether nuclear PTEN regulates HIF-1 transcriptional activity. Both the wild-type PTEN and the phosphatase mutant PTEN (G129R) were expressed in the cytoplasm, as well as the nucleus (Figure 4.11). In contrast, PTEN targeted to the nucleus (NLS-PTEN) was primarily found in the nucleus (Figure 4.11). This result is consistent with previously reported data (Liu et al., 2005). As shown in Figure 4.11, wild-type PTEN, as well as the NLS-PTEN repressed HIF-1 activity, as measured by HRE-Luciferase. In contrast, the PTEN-G129R mutant had no effect on HIF-1 activity (Figure 4.12). These data suggest a role for PTEN in the nucleus and that the phosphatase function of PTEN is required for the repression of HIF-1 transcriptional activity.

FOXO3a controls HIF-1α transactivation in the nucleus.

In PTEN null cells, FOXO transcription factors are exported from the nucleus (Biggs et al., 1999; Brownawell et al., 2001; Brunet et al., 1999; Rena et al., 1999). The three known phosphorylation sites on FOXO3a can be mutated to alanine [FOXO3a (AAA)] and this mutant therefore can not be phosphorylated and accumulates in the nucleus, allowing the constitutive activity of FOXO3a in PTEN null cells (Nakamura et al., 2000; Ramaswamy et al., 1999). Cells expressing the constitutively active FOXO3a (AAA) mutant repressed HIF-1 transcriptional activity, whereas expression of the wild-type FOXO3a had no effect on HIF-1 (Figure 4.13). Furthermore, transactivation of HIF-1α was also severely impaired by nuclear FOXO3a in

comparison to wild-type FOXO3a (Figure 4.14). The expression of the FOXO3a WT was seen predominantly in the cytoplasm and the FOXO3a (AAA) triple mutant was almost exclusively found in the nucleus of *Pten*$^{-/-}$ cells (Figure 4.29).

Leptomycin B (LMB) inhibits the nuclear export of proteins that are escorted to the cytoplasm by the nuclear export receptor CRM1 (Kudo et al., 1998). It has been shown that FOXO transcription factors are sequestered in the nucleus upon treatment with LMB (Biggs et al., 1999; Brownawell et al., 2001; Brunet et al., 1999; Kau et al., 2003). To determine whether HIF-1 transcriptional activity could be inhibited by preventing endogenous FOXO3a from exiting the nucleus, PTEN null cells were treated with LMB. Figure 4.15 illustrates that LMB indeed keeps endogenous FOXO3a in the nucleus in *Pten*$^{-/-}$ cells, independent of oxygen tension. LMB represses HIF-1 transcriptional activity, as assessed by the HRE-Luciferase (Figure 4.16). As expected, LMB had no effect on HIF-1α protein stability (Figure 4.30). Taken together, these date indicate that either by overexpressing a non-phosphorylatable FOXO3a, which accumulates in the nucleus, or by inhibiting the nuclear export of FOXO3a by LMB, HIF activation can be prevented in PTEN null cells.

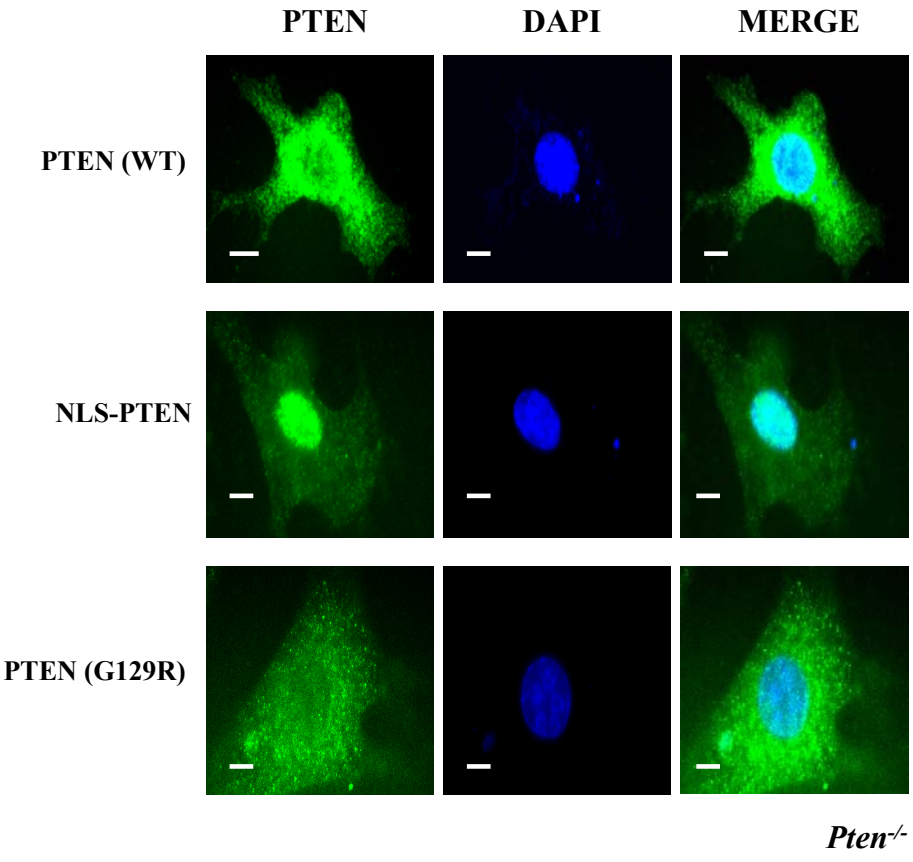

Figure 4.11. Expression and localization of PTEN mutants.

To examine subcellular localization of PTEN mutants, *Pten*$^{-/-}$ cells were transfected with PTEN expression constructs and visualized by immunofluorescence.

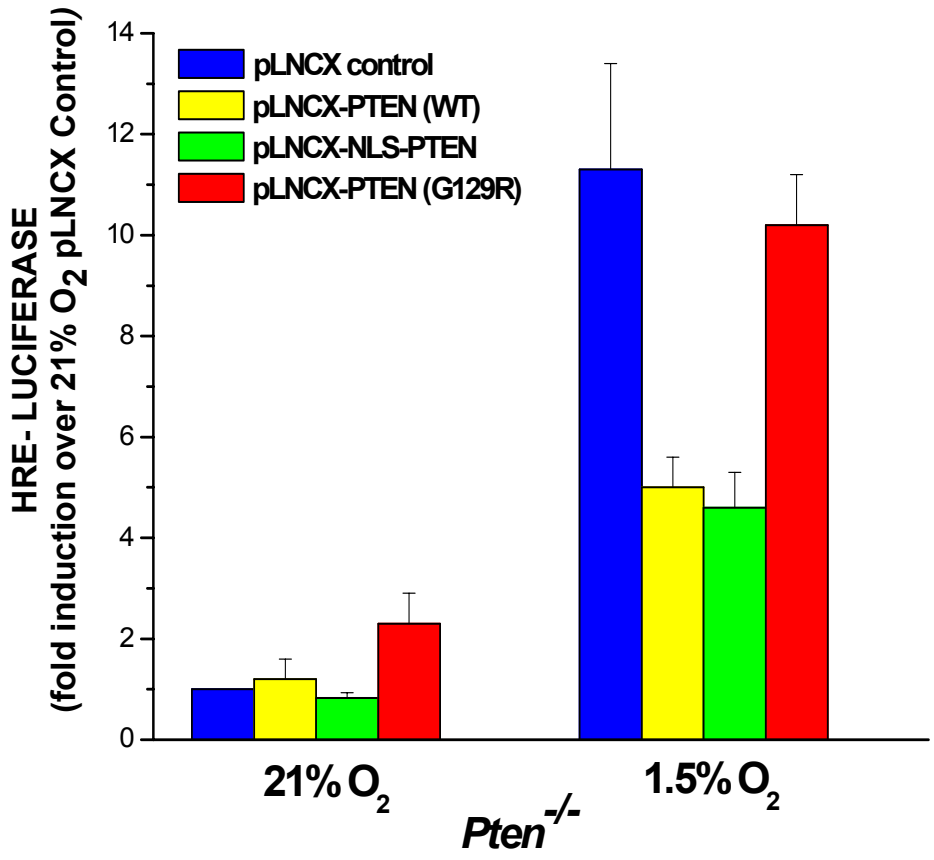

Figure 4.12. Nuclear PTEN represses HIF-1 transcriptional activity

Pten$^{-/-}$ cells were co-transfected with the HRE-Luciferase reporter gene construct and either pLNCX vector control, pLNCX-PTEN (WT), pLNCX-NLS-PTEN, or pLNCX-PTEN (G129R). 24 hours after transfections cells were exposed to 21% O_2 or 1.5% O_2 for 16 hours. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to pLNCX vector control. The data presented are the mean (±SEM) of four independent transfections.

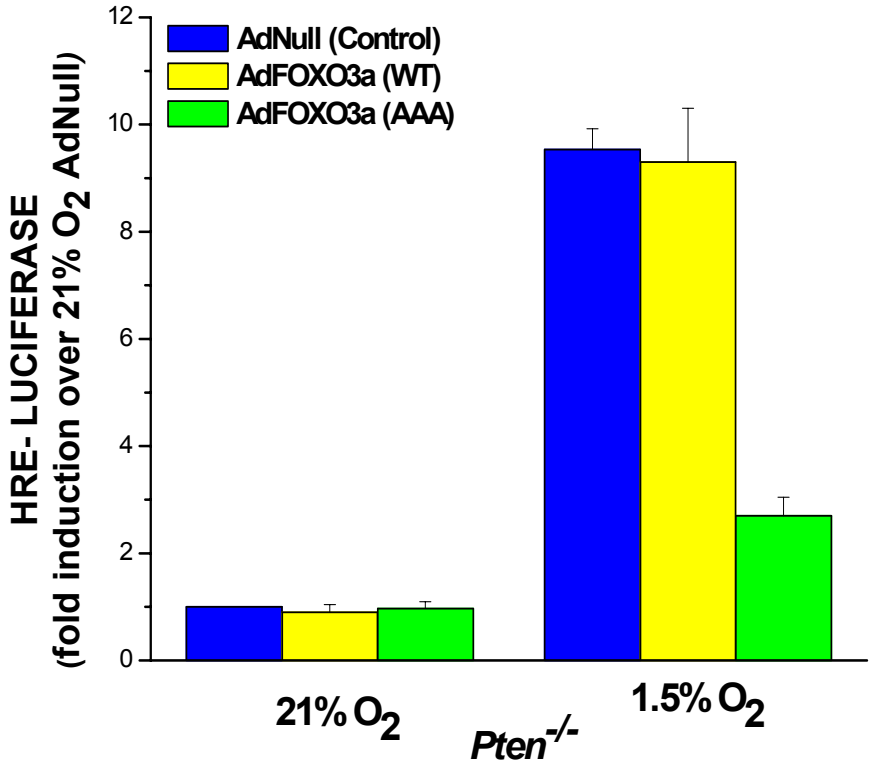

Figure 4.13. Nuclear FOXO3a represses HIF-1 transcriptional activity.

Pten$^{-/-}$ cells were transfected with a HRE-Luciferase reporter gene construct. 24 hours after transfection, cells were infected with null adenovirus or adenovirus expressing wild-type FOXO3a or the constitutively active FOXO3a (AAA) (100pfu/µl), and subsequently exposed to 21% O_2 or 1.5% O_2 for 16 hours. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to cells expressing AdNull. The data presented are the mean (±SEM) of four independent transfections.

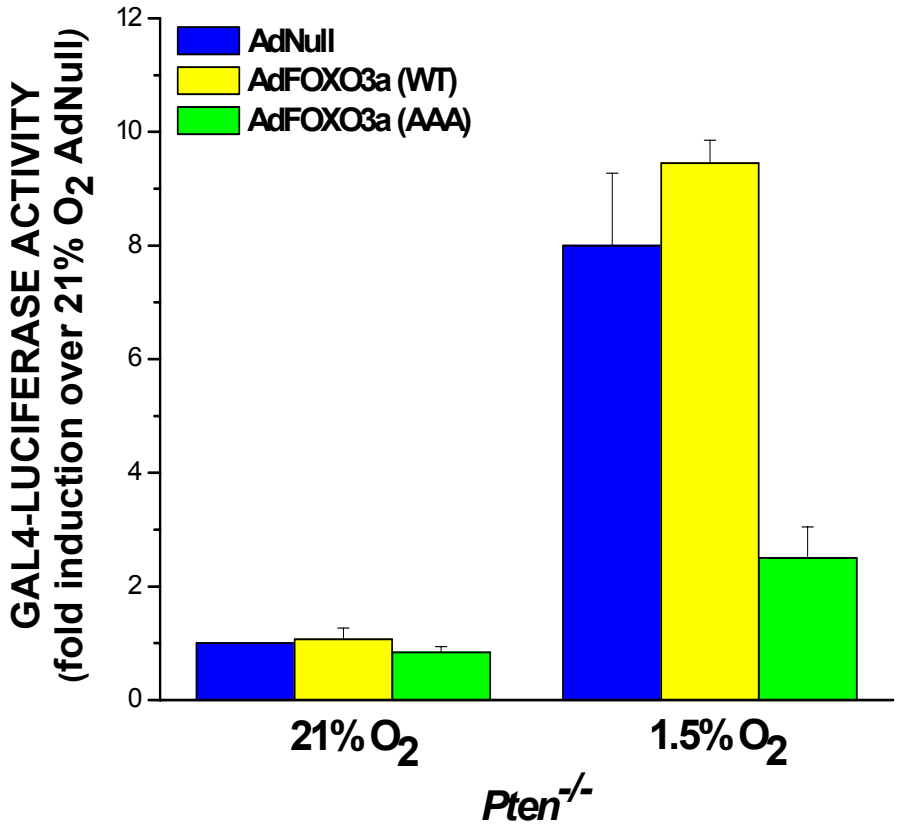

Figure 4.14. Nuclear FOXO3a represses HIF-1α transactivation.

Pten$^{-/-}$ cells were transfected with a GAL4 (1-147) DNA-binding domain fused to HIF-1α (531-826) construct and a reporter gene construct encoding five GAL4-binding sites. Following transfection, cells were infected with adenoviruses. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to AdNull infected cells. The data presented are the mean (±SEM) of four independent transfections.

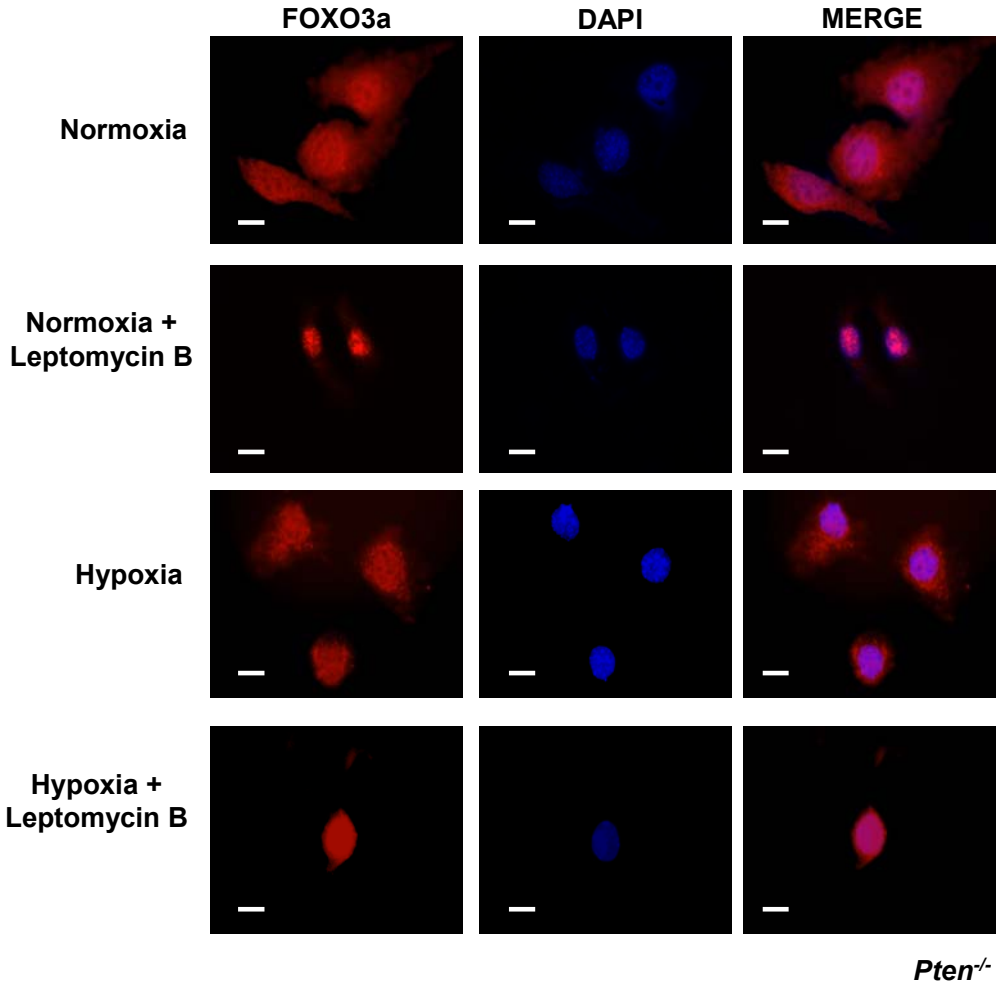

Figure 4.15. Inhibition of FOXO3a nuclear export by Leptomycin B.

Immunofluorescence of FOXO3a in *Pten*$^{-/-}$ cells exposed to 21% O_2 or 1.5% O_2 for 16 hours ± 20nM Leptomycin B.

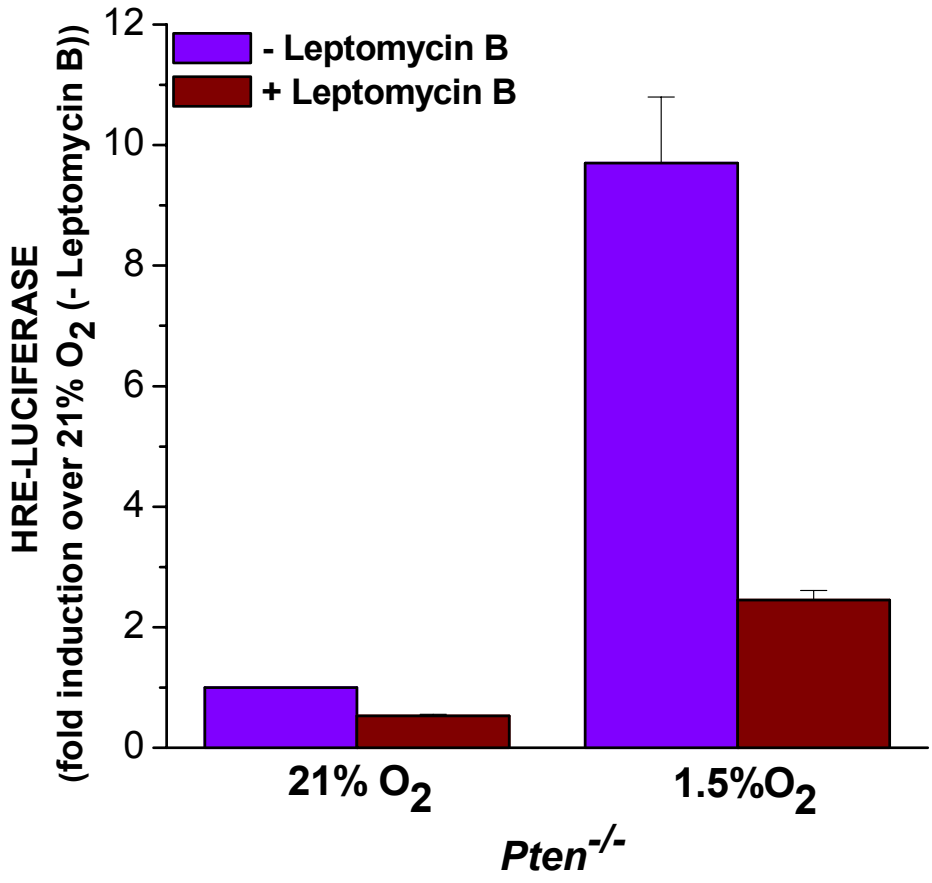

Figure 4.16. Inhibition of FOXO3a nuclear export represses HIF-1 activity.

Pten$^{-/-}$ cells were transfected with the HRE-Luciferase reporter gene construct and exposed to 21% O_2 or 1.5% O_2 for 16 hours ± 20nM Leptomycin B. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to untreated normoxic cells. The data presented are the mean (±SEM) of four independent transfections.

Nuclear FOXO3a forms a complex with HIF-1α and p300 to impede HIF-1 transcriptional activity.

First, to test whether endogenous FOXO3a could associate with HIF-1α and p300 in the nucleus we performed nuclear co-immunoprecipitation (Co-IPs) assays at 1.5%O_2 ± LMB in PTEN null cancer cells (U251 cells). These Co-IPs with antibodies to HIF-1α, FOXO3a, and p300 revealed that FOXO3a complexed with HIF-1α and p300 when sequestered in the nucleus with LMB (Figure 4.17). Interestingly, less HIF-1α is pulled down with the anti-p300 antibody when FOXO3a is kept in the nucleus and is able to associate with HIF-1α.

To further investigate whether endogenous FOXO3a is in a complex with HIF-1 and p300 on HREs of HIF-1 dependent target genes we performed chromatin immuonprecipitation (ChIP) on hypoxic *Pten*$^{-/-}$ cells treated with LMB, in order to sequester FOXO3a in the nucleus. *Glut-1* is a common HIF-1α target gene with a well characterized HRE. Therefore, an anti-HIF-1α, an anti-p300, and an anti-FOXO3a antibody were used to precipitate HRE-containing genomic DNA fragments from the *Glut-1* promoter of hypoxic *Pten*$^{-/-}$ cells. The immunoprecipitated samples were then assessed by quantitative SYBR green-based real-time PCR, as well as regular PCR. As expected, immunoprecipitation (IP) with the anti- HIF-1α antibody generated a strong signal under hypoxic conditions regardless of LMB treatment (Figures 4.18 and 4.19). IP with an anti-p300 also generated a signal under hypoxia regardless of LMB treatment. Whereas, IP with the FOXO3a antibody only generated a signal in the hypoxic cells that were treated with LMB (Figures 4.18 and 4.19). Both mock IgG antibodies that were used did not detect the *Glut-1* promoter. These ChIP results indicate that HIF-1α, p300, and FOXO3a are all in a complex on the HRE of *Glut-1* under hypoxic conditions in PTEN null cells when FOXO3a is kept in the

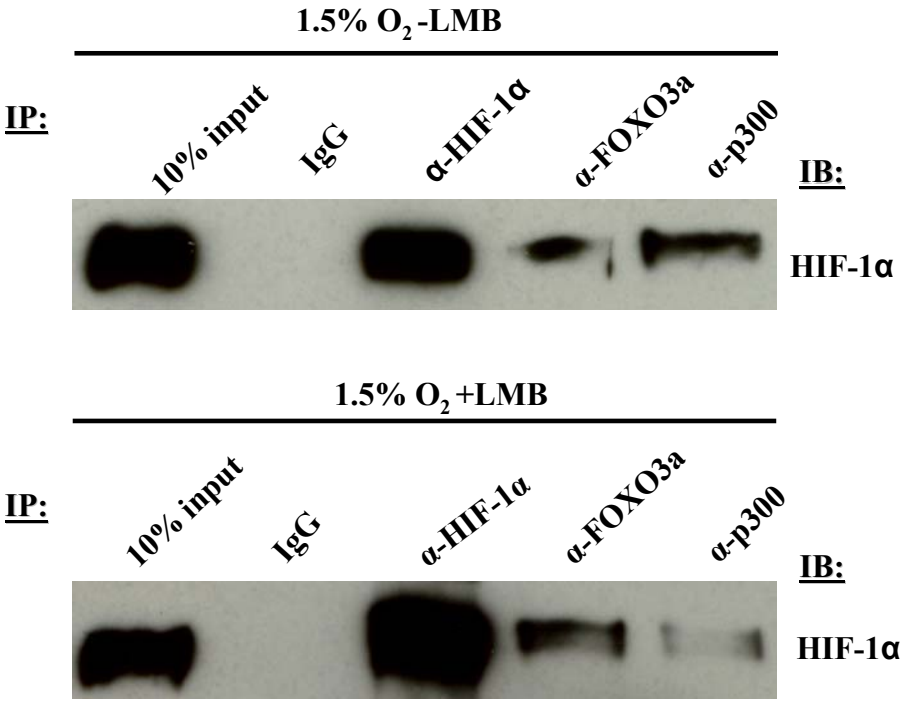

Figure 4.17. Endogenous HIF-1α, FOXO3a, and p300 form complex in nucleus. U251 cells were exposed to 1.5%O_2 ± 20nM Leptomycin B for 16 hours and subsequently nuclear fractions were collected. Immunoprecipitations were carried out on the U251 nuclear lysates using anti-HIF-1α, anti-FOXO3a, anti-p300, or an IgG control antibody followed by immunoblotting for HIF-1α.

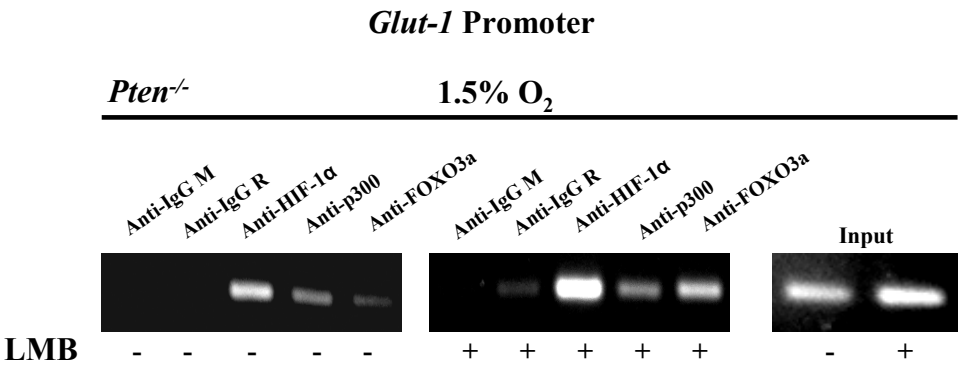

Figure 4.18. Endogenous FOXO3a complexes with HIF-1α and p300 on the HRE of the *Glut-1* promoter.

Chromatin fragments were immunoprecipitated with anti-HIF-1α, anti-p300, anti-FOXO3a, or control antibodies (mouse IgG for the anti-HIF-1α and rabbit IgG for both the anti-p300 and anti-FOXO3a) in cross-linked hypoxic *Pten*$^{-/-}$ cells ± 20nM Leptomycin B (LMB). DNA from input and immunoprecipitated samples were detected using PCR and run on a 2% agarose gel. Primers specific for the HRE of the common HIF-1α target gene *Glut-1* were used. A representative gel is shown above of three independent experiments.

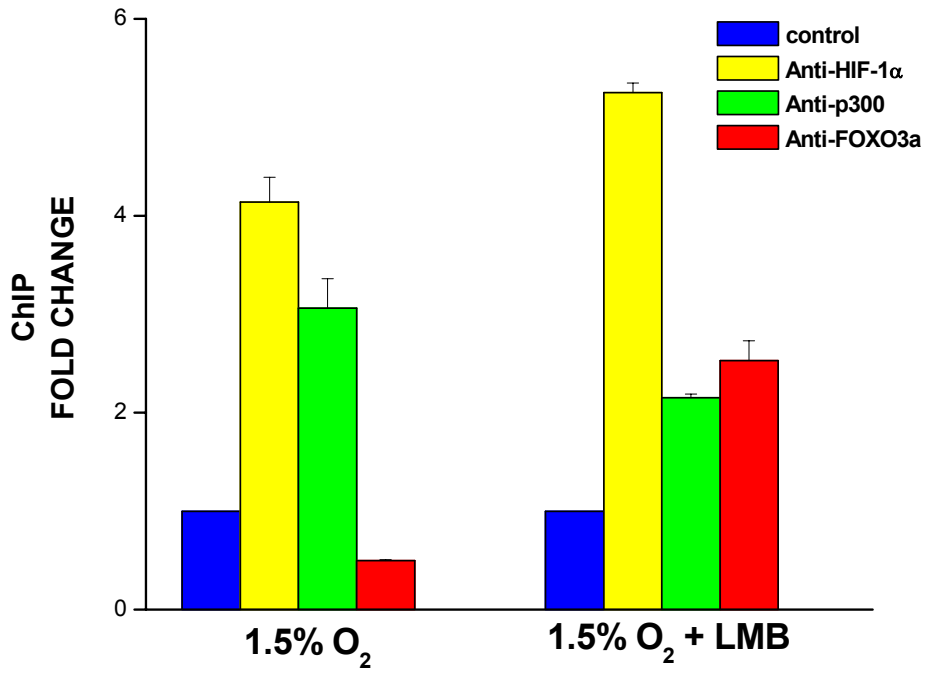

Figure 4.19. Endogenous FOXO3a complexes with HIF-1α and p300 on the HRE of *Glut1* promoter.

ChIP was performed in hypoxic *Pten*$^{-/-}$ cells as in Figure 17. DNA from input and immunoprecipitated samples were detected using SYBR green real-time PCR. Primers specific for the HRE of the common HIF-1α target gene *Glut-1* were used. The data presented are the result of triplicate analyses and the error bars indicate SEM.

nucleus. During hypoxic conditions in PTEN null cells FOXO3a is not found on the HRE of *Glut-1*. This is because FOXO3a is continually being exported out of the nucleus and into the cytoplasm. These data argue that under hypoxia when FOXO3a is sequestered in the nucleus of PTEN null cells, that FOXO3a can complex with p300 and HIF-1α on the HRE to suppress HIF-1 target genes. Moreover, the results suggest that FOXO3a may interfere with p300's ability to bind to HIF-1α and thereby prevent transactivation of HIF-1α.

To determine whether FOXO3a interferes with p300's ability to function as a co-activator by forming a complex on HREs with p300 and HIF-1α, we overexpressed p300 in *Pten*$^{-/-}$ cells in the presence or absence of LMB. Consistent with data shown in Figure 4.16, treatment of LMB in the *Pten*$^{-/-}$ cells decreased HIF-1 transcriptional activity (Figure 4.20). Co-transfection of p300 increased HRE-luciferase activity under both normoxia and hypoxia (Figure 4.20). Interestingly, the overexpression of p300 in the LMB treated cells reversed the inhibition of HIF-1 transcriptional activity seen by the treatment of LMB in PTEN null cells (Figure 4.20). We conclude that even though FOXO3a is sequestered in the nucleus by LMB treatment, HIF-1 activity can be rescued by increasing the amount of p300. These results suggest that FOXO3a may interfere with p300's ability to function as a co-factor on the HREs on HIF-1 target genes.

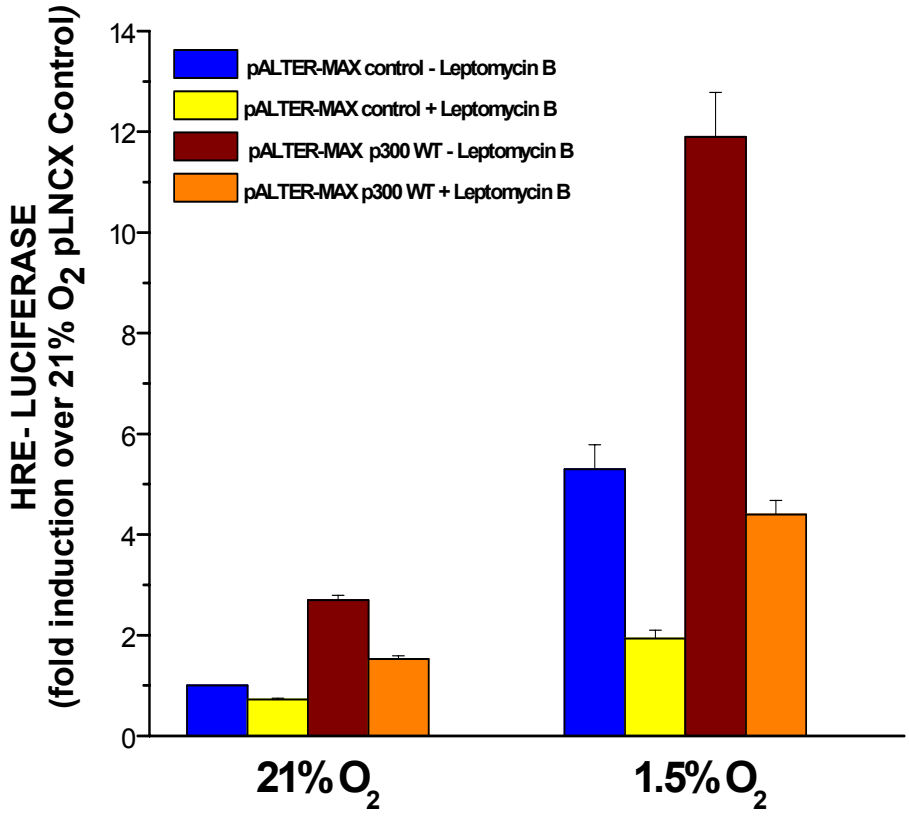

Figure 4.20. Overexpression of p300 rescues HIF-1 activity.

Pten$^{-/-}$ cells were co-transfected with the HRE-Luciferase reporter gene and either the pALTER-MAX vector control or pALTER-MAX p300 wt. 24 hours after transfection cells were exposed to 21% O_2 or 1.5% O_2 for 16 hours ± 20nM Leptomycin B. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to the pALTER-MAX vector control untreated normoxic cells. The data presented are the mean (±SEM) of four independent transfections.

HIF-1α transactivation is regulated by nuclear PTEN/FOXO signaling in PTEN null cancer cells.

PTEN is commonly mutated or lost in prostate cancers and in glioblastomas. LnCaP prostate carcinoma cells are PTEN null (Figure 4.21A). Reintroduction of PTEN in LnCaP cells had no effect on the protein stability of HIF-1α (Figure 4.21B). In contrast, the presence of PTEN decreased HIF-1 transcriptional activity in the LnCaP cells, as measured by HRE-Luciferase (Figure 4.22). These results coincide with the above data in the *Pten*$^{-/-}$ MEFs. Next, to investigate whether nuclear PTEN could regulate HIF-1 activity, PTEN mutants were used just as in *Pten*$^{-/-}$ MEFs. As shown in Figure 4.23, wild-type PTEN, as well as, the NLS-PTEN repressed HIF-1 activity. In contrast, the PTEN-G129R mutant actually increased HIF-1 activity in comparison to the vector control cells. Wild-type PTEN, as well as, NLS-PTEN also decreased HIF-1 transcriptional activity in human malignant glioma cells, U251 cells (Figure 4.27). U251 cells are also PTEN null cancer cells and reintroduction of PTEN using an adenovirus had no effect on HIF-1α protein stability (Figure 4.26A and B).

To determine whether FOXO can suppress HIF-1 transcriptional activity by in the PTEN null cancer cells, the FOXO3a WT and FOXO3a (AAA) adenoviruses were used, as well as, treated cells with LMB. Both the expression of the triple mutant FOXO3a (AAA), which predominately is nuclear; and treatment of LMB, which prevents nuclear export of FOXO3a, significantly decreased HIF-1 transcriptional activity in the LnCaP cells (Figures 4.24 and 4.25). Moreover, treatment of LMB also decreased HIF-1 transcriptional activity in the U251 cells (Figure 4.28). These data indicate that by inhibiting FOXO nuclear export in PTEN null cancers cells, HIF-1 activity can be repressed.

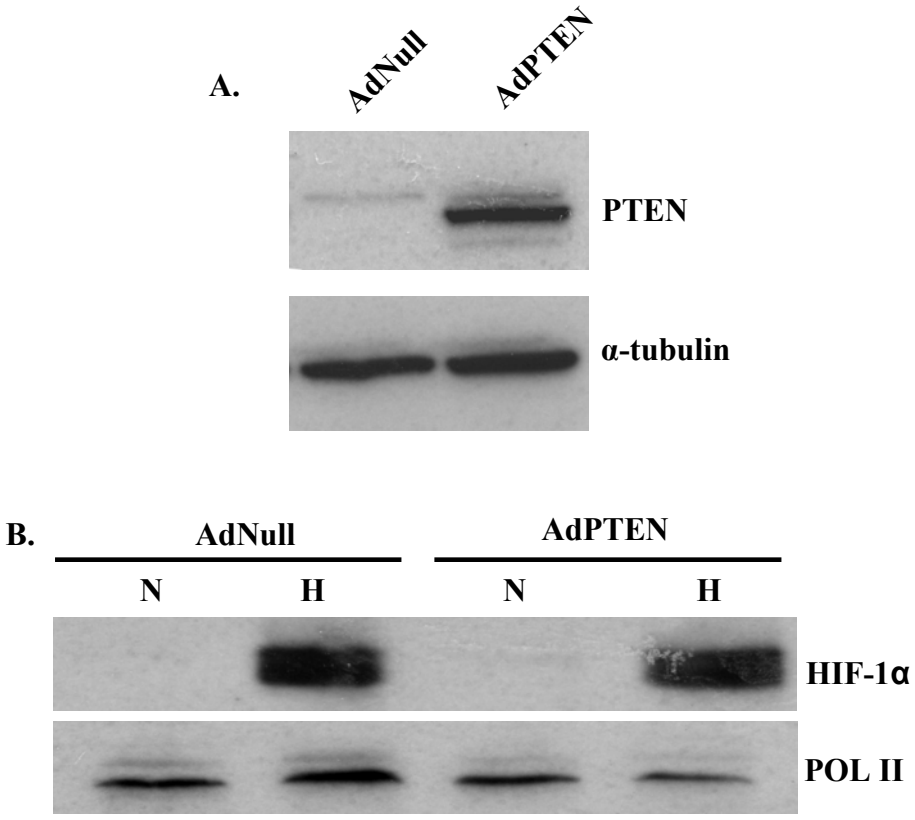

Figure 4.21. Reintroduction of PTEN has no effect on HIF-1α protein levels PTEN null cancer cells .

(A). LnCaP cells infected with adenoviruses. Cell lysates were analyzed by immunoblotting with an anti-PTEN antibody. **(B).** HIF-1α protein levels in LnCaP cells infected with adenoviruses and were subsequently exposed to 21% O_2 (N) or 1.5% O_2 (H) for 2 hours.

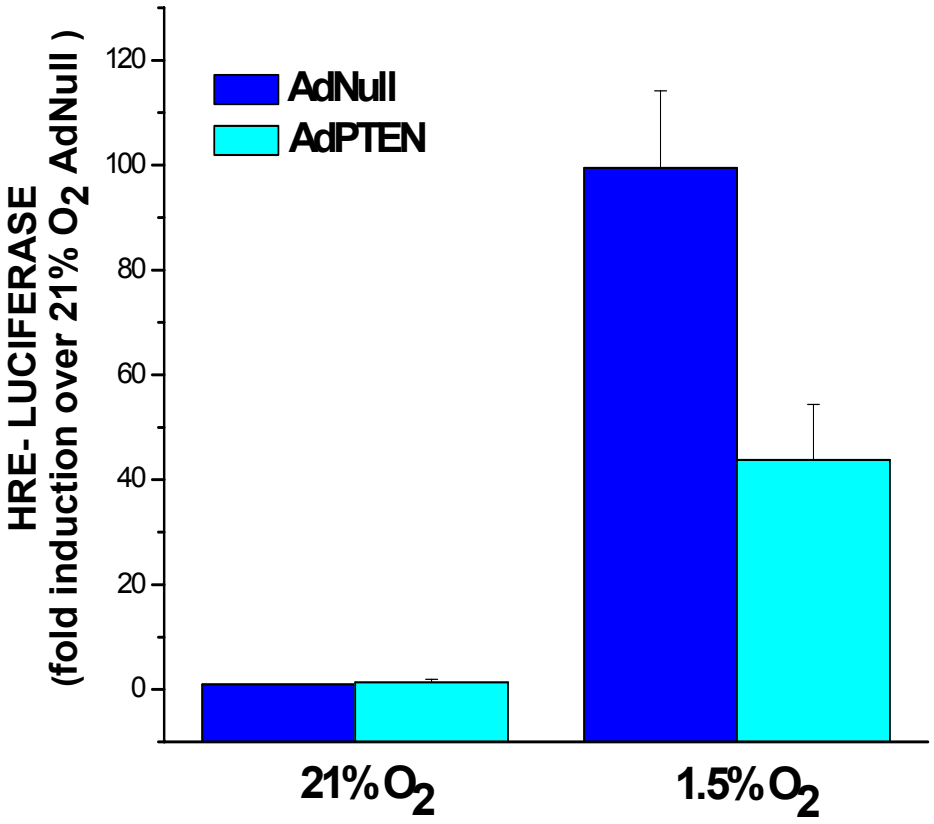

Figure 4.22. Reintroduction of PTEN decreases HIF-1 transcriptional activity in PTEN null cancer cells.

LnCaP cells were transfected with a HRE-Luciferase reporter gene construct. 24 hours after transfection, cells were infected with adenoviruses, and subsequently exposed to 21% O_2 or 1.5% O_2 for 16 hours. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to cells expressing AdNull. The data presented are the mean (±SEM) of four independent transfections.

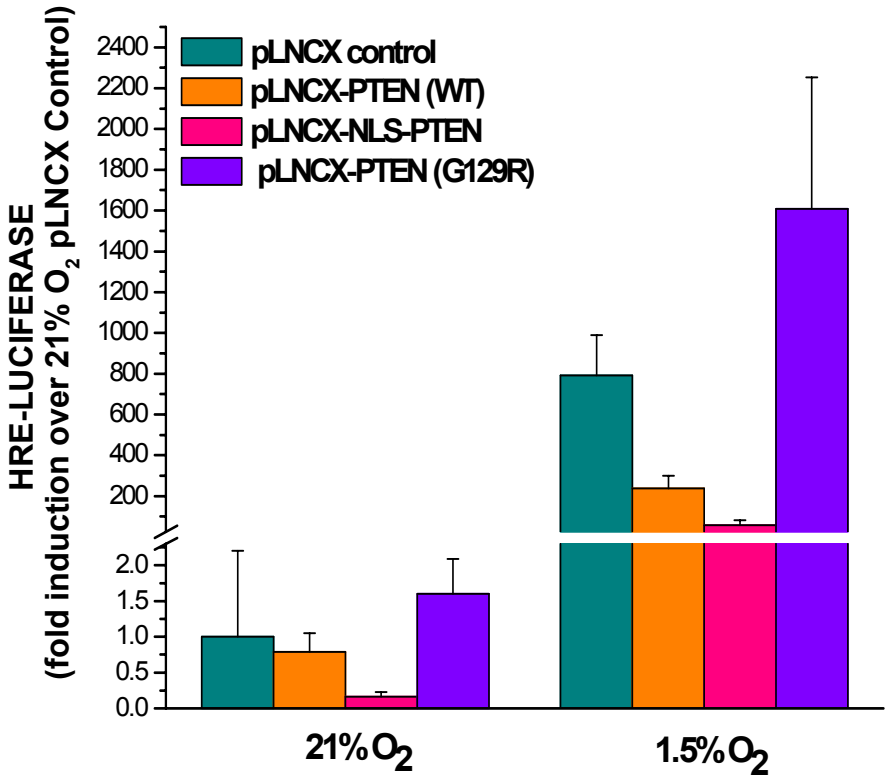

Figure 4.23. Nuclear PTEN represses HIF-1 transcriptional activity in PTEN null cancer cells.

LnCaP cells were co-transfected with the HRE-Luciferase reporter gene construct and either pLNCX vector control, pLNCX-PTEN (WT), pLNCX-NLS-PTEN, or pLNCX-PTEN (G129R). 24 hours after transfections cells were exposed to 21% O_2 or 1.5% O_2 for 16 hours. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to pLNCX vector control. The data presented are the mean (±SEM) of four independent transfections.

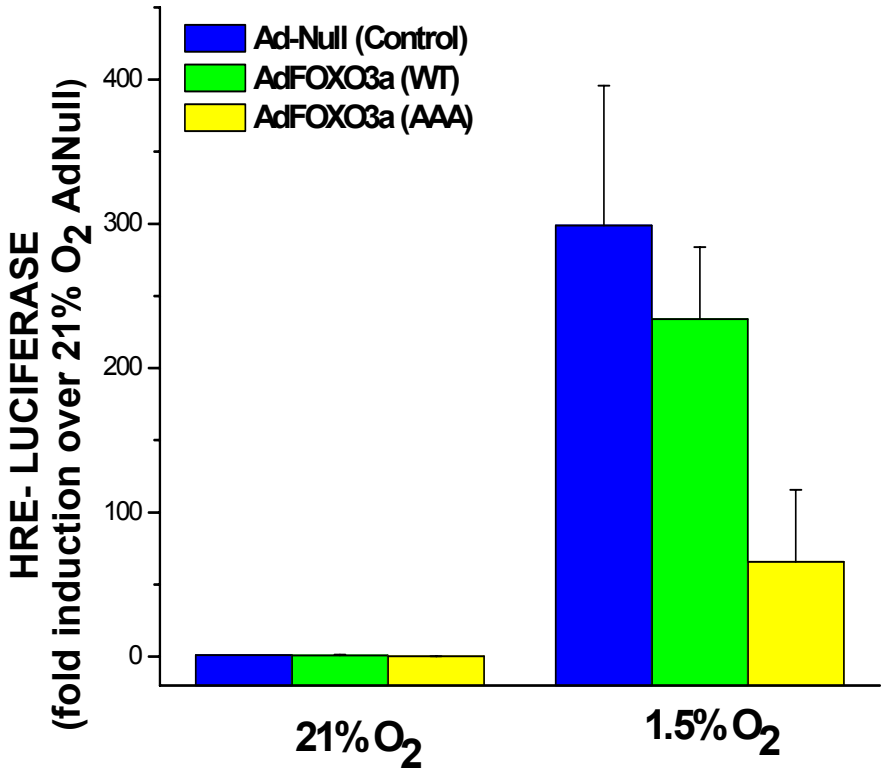

Figure 4.24. Nuclear FOXO3a represses HIF-1 transcriptional activity in PTEN null cancer cells.

LnCaP cells were transfected with a HRE-Luciferase reporter gene construct. 24 hours after transfection, cells were infected with adenoviruses, and subsequently exposed to 21% O_2 or 1.5% O_2 for 16 hours. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to cells expressing AdNull. The data presented are the mean (±SEM) of four independent transfections.

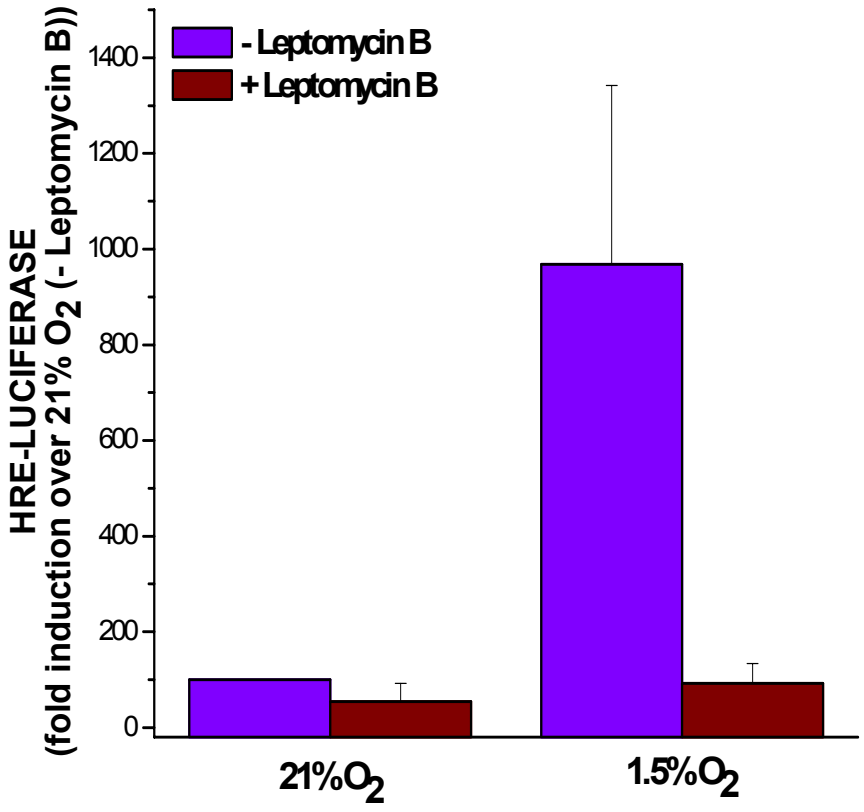

Figure 4.25. Inhibition of FOXO3a nuclear export represses HIF-1 activity in PTEN null cancer cells.

LnCaP cells were transfected with the HRE-Luciferase reporter gene construct and exposed to 21% O_2 or 1.5% O_2 for 16 hours ± 20nM Leptomycin B. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to untreated normoxic cells. The data presented are the mean (±SEM) of four independent transfections.

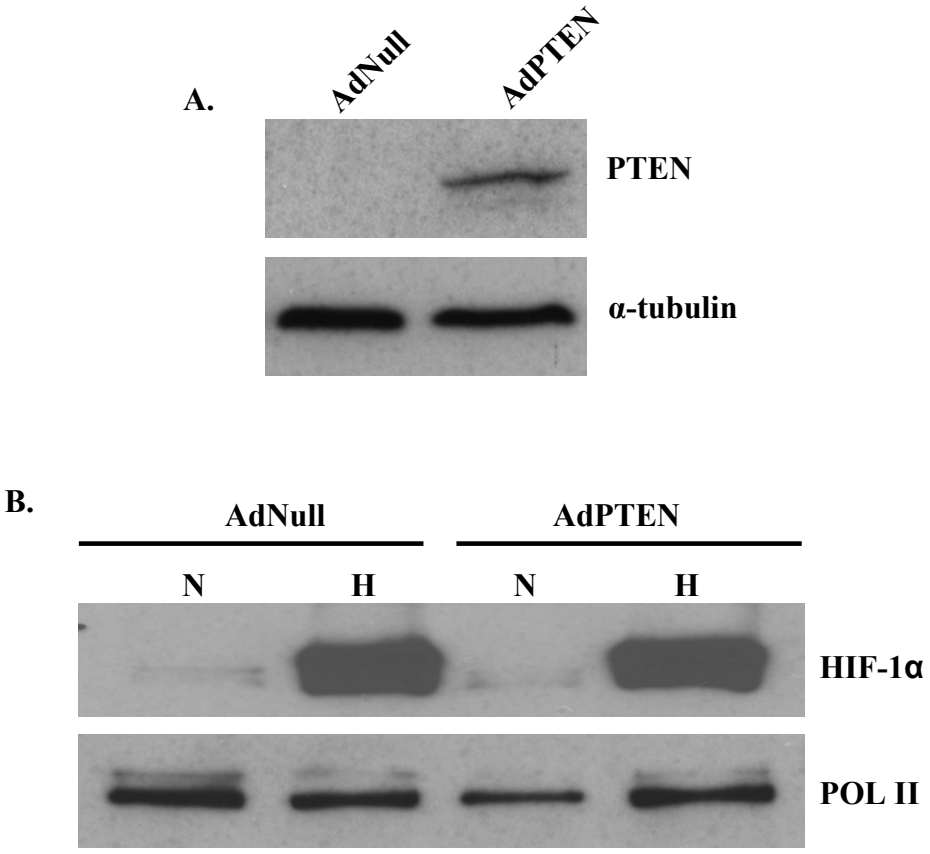

Figure 4.26. Reintroduction of PTEN has no effect on HIF-1α protein levels PTEN null cancer cells .

(A). U251 cells infected with adenoviruses. Cell lysates were analyzed by immunoblotting with an anti-PTEN antibody. **(B).** HIF-1α protein levels in U251 cells infected with adenoviruses and were subsequently exposed to 21% O_2 (N) or 1.5% O_2 (H) for 2 hours.

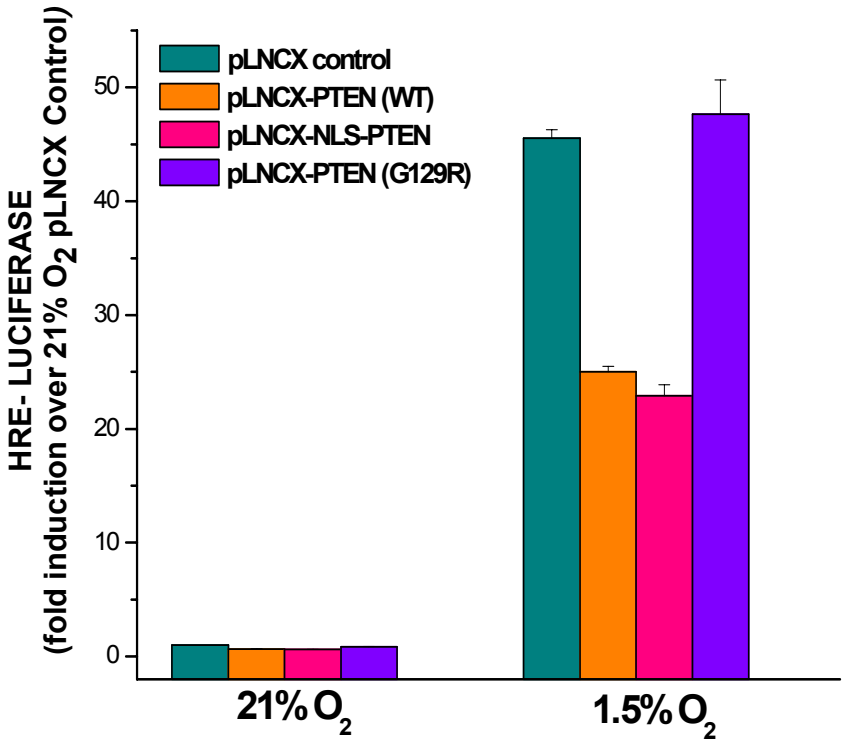

Figure 4.27. Nuclear PTEN represses HIF-1 transcriptional activity in PTEN null cancer cells.

U251 cells were co-transfected with the HRE-Luciferase reporter gene construct and either pLNCX vector control, pLNCX-PTEN (WT), pLNCX-NLS-PTEN, or pLNCX-PTEN (G129R). 24 hours after transfections cells were exposed to 21% O_2 or 1.5% O_2 for 16 hours. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to pLNCX vector control. The data presented are the mean (±SEM) of four independent transfections.

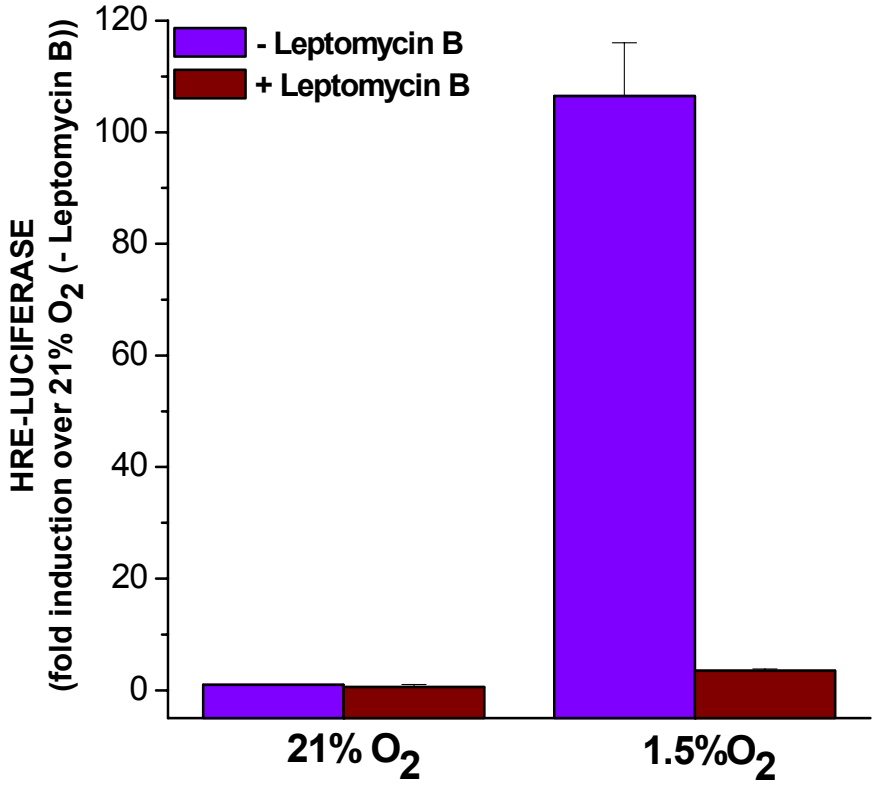

Figure 4.28. Inhibition of FOXO3a nuclear export represses HIF-1 activity in PTEN null cancer cells.

U251 cells were transfected with the HRE-Luciferase reporter gene construct and exposed to 21% O_2 or 1.5% O_2 for 16 hours ± 20nM Leptomycin B. Relative luciferase expression is the ratio of luciferase/total protein levels normalized to untreated normoxic cells. The data presented are the mean (±SEM) of four independent transfections.

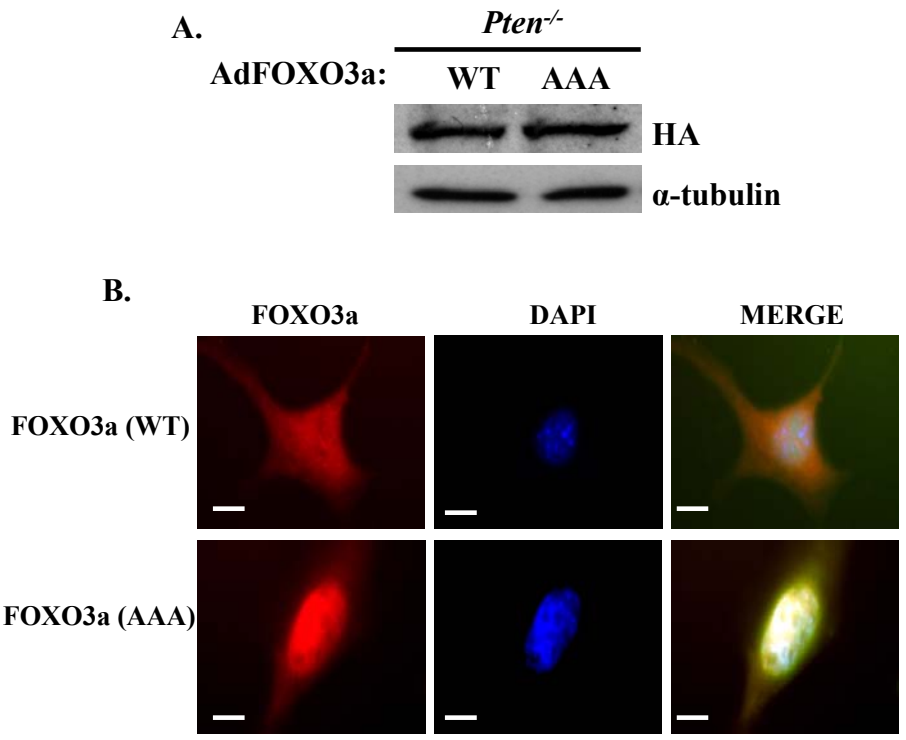

Figure 4.29. Expression and localization of FOXO3a Adenoviruses.

(A). *Pten*$^{-/-}$ cells were infected with the HA-tagged adenoviruses expressing the wild-type FOXO3a or the constitutively active FOXO3a (AAA) (100pfu/μl) for 3 hours in serum/antibiotic-free media and subsequently incubated for 16 hours in serum-free media before harvesting. Cell lysates were analyzed by immunoblotting with an anti-HA antibody. The same blot was stripped and reprobed with an anti-α-tubulin antibody to control for loading. **(B).** To examine subcellular localization of the FOXO3a adenoviruses, *Pten*$^{-/-}$ cells were infected, as in Panel A, and visualized by immunofluorescence.

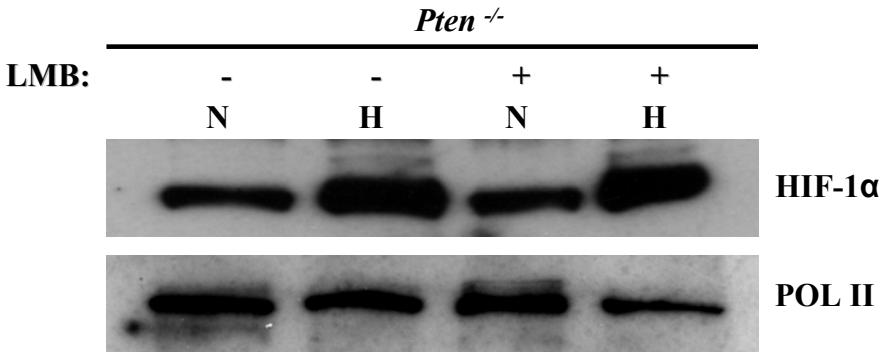

Figure 4.30. Leptomycin B (LMB) has no effect on HIF-1α protein stability.

Pten$^{-/-}$ cells were exposed to 21% O_2 (N) or 1.5% O_2 (H) ± 20nM LMB for four hours. Nuclear extracts were prepared, analyzed by SDS-PAGE and immunoblotted with an anti-HIF-1α antibody. The same blot was stripped and re-probed with an anti-POL II antibody to control for loading.

Discussion

Hypoxia occurs during tumorigenesis due to the metabolic demands of cancer cells exceeding the development of angiogenesis. It induces a transcriptional program that promotes angiogenesis and is associated with resistance to radiation therapy, chemotherapy, and poor treatment outcome (Harris, 2002). The transcription factor HIF-1 mediates the adaptive responses, including the induction of angiogenesis and a switch to anaerobic metabolism, during low oxygen tensions, making HIF-1 or upstream regulators of HIF-1 ideal targets for anticancer therapeutics. HIF-1 levels are increased in many human primary tumors (Blancher et al., 2000; Bos et al., 2001; Semenza, 2000c; Zhong et al., 1998; Zhong et al., 1999). PTEN is one of the most commonly mutated tumor suppressor genes in human cancers (Cantley and Neel, 1999). The loss of PTEN activity is associated with the induction of HIF-1 activity and an increase in the expression of HIF-1 inducible genes (Zhong et al., 2000). We show here that loss of PTEN does increase HIF-1 transcriptional activity coinciding with previous work. Furthermore, PTEN regulates the HIF-1α transactivation domain, which is known to bind p300. However in contrast to previous work we observed no difference in the stability of the HIF-1α protein in comparison to *Pten*$^{+/-}$ cells. Furthermore, our findings show that by treating *Pten*$^{-/-}$ cells with rapamycin we can effectively inhibit mTOR signaling, without having any effect on HIF-1α protein or the HIF-1 transcriptional activity. Our results are in contrast with previous reports indicating that mTOR inhibition decreases HIF-1α protein levels in a mouse model of AKT-dependent prostate intraepithelial neoplasia (Majumder et al., 2004). However, previous reports do indicate that activation of AKT is not sufficient to activate HIF-1 and that AKT and HIF-1 both independently increase tumorigenesis ((Arsham et al., 2004).

PTEN, once considered a strictly cytoplasmic protein, has been shown by multiple investigators to be present and functional in the nucleus. In fact, PI3K and AKT have been shown to be in the nucleus (Ahn et al., 2004; Trotman et al., 2006). Recent findings also suggest that PTEN could mediate tumor suppressive activities independent of AKT in the nucleus (Liu et al., 2005). Furthermore, nuclear PTEN has been shown to regulate p53 protein levels independent of its phosphatase activity (Freeman et al., 2003; Li et al 2006). Together, these studies highlight supporting evidence of how PTEN can have dual roles in the cytoplasm and in the nucleus. Here we provide data that nuclear PTEN also plays a significant role in regulating HIF-1 transcriptional activity. Additionally, by using the phosphatase mutant PTEN we show that PTEN's phoshatase activity is required for the repression of HIF-1 activity. Consequently, our study emphasizes the importance of the subcellular localization of PTEN.

Aberrant growth signaling associated with PTEN null cancers causes FOXO proteins to become phosphorylated and inactivated through the relocalization from the nucleus to the cytoplasm. FOXO proteins regulate genes that are involved in key cellular processes downstream of PTEN, such as cell cycle arrest and apoptosis (Nakamura et al., 2000). Small molecules that would enforce the nuclear relocalization of FOXO proteins would be useful tools to investigate for cancer therapy. Silver and colleagues elegantly identified a series of novel inhibitors that regulated nuclear export of FOXO1a in PTEN null cancer cells by using a chemical genetic screen (Kau et al., 2003). Here, we show that by using the nuclear export inhibitor LMB or by forcible localization of FOXO3a in the nucleus we can repress HIF-1 transcriptional activity (Figure 4.31). IP assays reveal that FOXO3a can associate with HIF-1α in the nucleus. ChIP assays further indicate that nuclear FOXO3a complexes with HIF-1α and p300 or actually binds

to the HRE of the *Glut-1* promoter, a HIF-1 target gene, in order to physically interfere with p300's ability to activate HIF-1 transcriptional activity. In support of this premise, the FOXO3a-mediated HIF-1α transactivation repression can be reversed by overexpression of p300 in PTEN null cells. An additional mechanism by which FOXO3a could negatively regulate HIF-1 transcriptional activity is through upregulation of Cited2 mRNA (Bakker et al. 2007). Hypoxia induces transcription of FOXO3a through HIF-1α (Bakker et al. 2007). Hypoxia induced FOXO3a increases the transcription of Cited2. Previous reports have indicated that Cited2 competes with HIF-1α for p300/CBP binding (Bhattacharya et al. 1999). Thus, FOXO3a increase in Cited2 would interfere with HIF-1 transcriptional activity during hypoxia by blocking the interaction between HIF-1α and p300/CBP. Collectively data of Bakker et al. and our findings argue that FOXO3a is a negative regulator of HIF-1 transcriptional activity by interfering with p300's ability to serve a transcriptional co-activator.

The physiological implication of FOXO3a negatively regulating HIF-1 has important implications. HIF-1 is required for angiogenesis and the shift to glycolysis during hypoxia. In rapidly growing tumors where the cell proliferation exceeds blood supply the activation of HIF-1 is important for adaptation. FOXO3a could inhibit HIF-1's ability to promote angiogenesis and the shift to glycolysis resulting in impairment of tumorigenesis. However, HIF-1 does have other functions, notably in regulating apoptosis as cells become anoxic. Hypoxia does not result in apoptosis. HIF-1 activation during hypoxia promotes tumorigenesis but as cells approach anoxia, HIF-1's function may impair tumorigenesis by inducing apoptosis. There are multiple targets of HIF-1 including BNIP3, NOXA, and RTP801 that have been implicated in the induction of apoptosis (Guo et al. 2001; Kim et al. 2004; Shoshani et al. 2002). In this context,

FOXO3a would promote survival of cells by inhibiting HIF-1's ability to induce apoptosis. Indeed, FOXO3a inhibits HIF-1's ability to induce apoptosis during anoxia in normal and breast cancer cells (Bakker et al. 2007). Thus, depending on oxygen level, FOXO3a could have different outcomes with respect to HIF-1 dependent tumorigenesis. HIF-1 has been shown to both promote as well as inhibit tumorigenesis (Carmeliet et al., 1998; Ryan et al., 1998). Similarly we predict that FOXO3a may either promote or impair tumorigenesis by interfering with HIF-1 transcriptional activity.

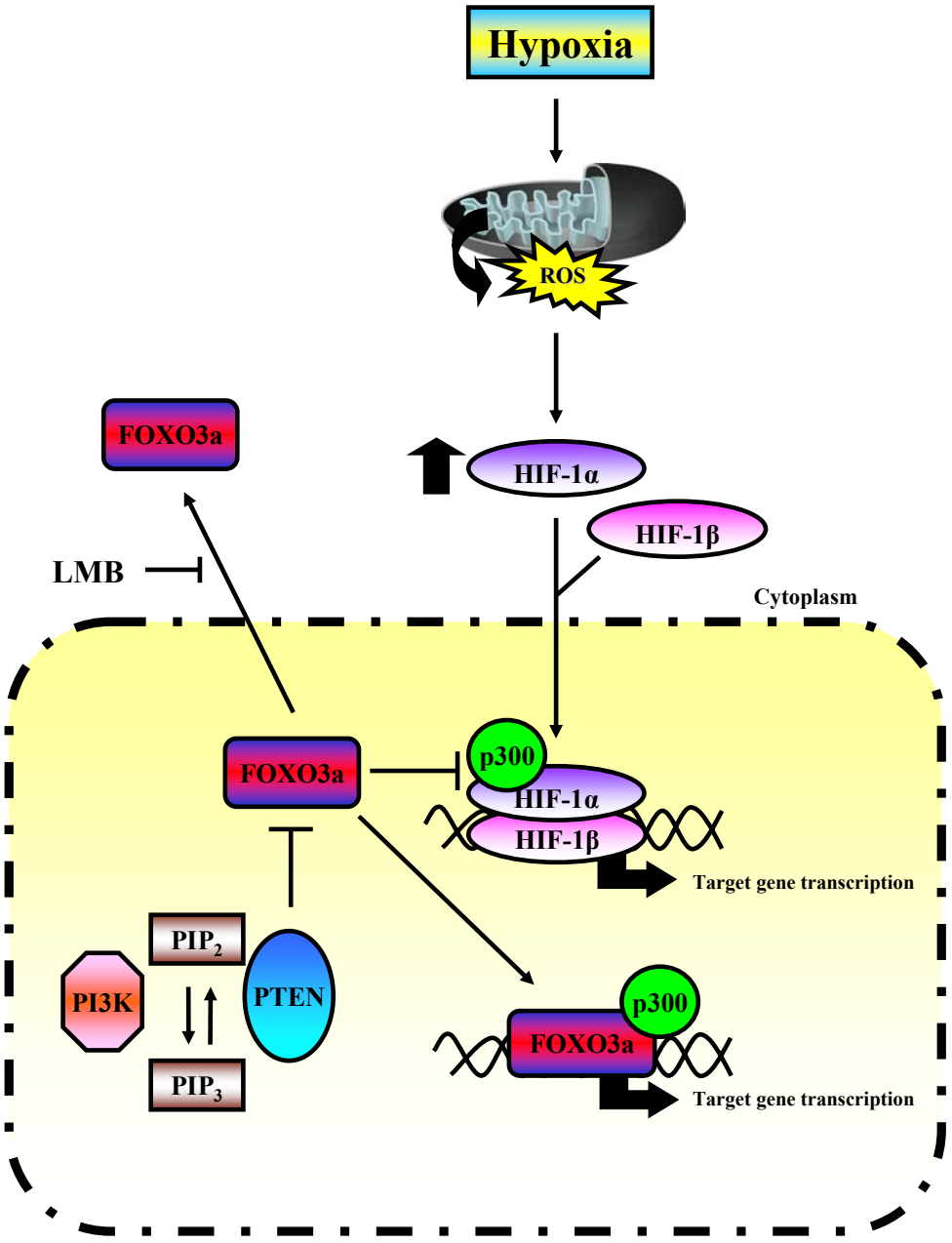
Hypoxia
ROS
FOXO3a
HIF-1α
HIF-1β
LMB
Cytoplasm
FOXO3a
p300
HIF-1α
HIF-1β
Target gene transcription
PIP2
PI3K
PTEN
PIP3
p300
FOXO3a
Target gene transcription

Figure 4.31. Proposed model for HIF-1 regulation in PTEN null cells.

Hypoxia stimulates the generation of reactive oxygen species (ROS) within the mitochondria, which activate HIF-1. In PTEN null cells, HIF-1 activity is further increased during hypoxia due to FOXO being exported out of the nucleus, thus allowing more p300 to be available for HIF-1α on the HREs of HIF-1 target genes. Nuclear PTEN, forcible localization of Foxo3a in the nucleus, or inhibition of nuclear export of endogenous FOXO3a by leptomycin B (LMB) treatment all repress HIF-1 activity in PTEN null cells. This repression may be due to FOXO3a interfering with p300 to function as a co-factor for HIF-1 since overexpression of p300 rescues the FOXO3a repression of HIF-1 transcriptional activity.

Chapter 5: Conclusions

Hypoxia occurs in most solid tumors in regions where tumor growth outstrips new blood vessel formation. Hypoxic cancer cells are resistant to both chemotherapy and radiation, thereby making them the major reason for the failure of cancer therapy (Harris, 2002). The transcription factor hypoxia inducible factor-1 (HIF-1) is critically important for tumor progression and angiogenesis. In fact, HIF-1α is overexpressed in 70% of human cancers and their metastases (Harris, 2002; Semenza, 2003). Therefore, understanding how hypoxia activates HIF-1 is important for understanding the biology of tumor growth.

The molecular mechanisms by which cells detect hypoxia, resulting in the stabilization of HIF-1α remain unclear. The increased generation of reactive oxygen species (ROS) at complex III of the mitochondrial electron transport chain is required and sufficient for HIF-1α protein stabilization during hypoxia (Brunelle et al., 2005; Chandel et al., 2000; Guzy et al., 2005; Mansfield et al., 2005). The downstream signaling pathways that connect the mitochondrial ROS to HIF-1α stabilization were previously unknown. My thesis research has shown that the p38 MAPK signaling pathway is required for the hypoxic activation of HIF-1, thereby linking mitochondrial ROS to HIF-1 activation for the first time (Emerling et al., 2005) (Figure 5.1). Furthermore, I have discovered that the hypoxic activation of p38α MAPK and its upstream MAPKKs MKK3 and MKK6, as well as HIF-1, is dependent on the generation of mitochondrial ROS. These findings have significantly advanced the field and have set forth a model of ROS-dependent signaling under hypoxia. Moreover, my work has validated the requirement of intercellular signaling for the regulation of HIF-1. Although, much remains to be learned about how cells actually sense low oxygen. Currently, there is confusion in defining the oxygen levels

that investigators utilize to study the biology of low oxygen. Clearly, the biology of cells exposed to oxygen levels close to 0% O_2 (anoxia) is different from cells exposed to 1-3% O_2 (hypoxia). Thus, there are completely different oxygen sensing mechanisms under hypoxia as compared to anoxia. My work has corroborated this by demonstrating that p38 MAPK signaling initiating from the mitochondrial generated ROS during hypoxia is required for HIF-1 activation. In contrast, I have shown that neither a functional electron transport chain nor p38 MAPK signaling are required for the anoxic activation of HIF-1. Anoxia directly limits the oxygen availability for hydroxylation to occur; thus, the PHDs and FIH-1 are likely to serve as direct oxygen sensors regulating the anoxic HIF-1 dependent gene expression. It is still not clear how the mitochondrial ROS-dependent signaling prevents PHD or FIH-1 from hydroxylating the HIF-1α protein during hypoxia. I hypothesize that PHD2 may be a target of p38α MAPK. Of the four known HIF hydroxylases, PHD2 has been shown to be primarily responsible for determining HIF-1α stability as a function of oxygen availability (Berra et al., 2003). Therefore, by inhibiting PHD2 activity, HIF-1 can be activated during hypoxia. I have identified four consensus sequences within the PHD2 protein that are specific for p38α MAPK phosphorylation. Determining if PHD2 is a direct substrate of the p38α MAPK signaling pathway will be crucial and the control of PHD2 by the p38 MAPK pathway would define a mechanism in which cells can detect low oxygen levels.

In the past few years, the role of mitochondria as a potential oxygen sensor has been highly controversial. However, it is difficult to ignore the premise that mitochondrial ROS are likely sensors necessary for hypoxic gene expression mediated by the transcription factor HIF-1 based on current genetic evidence (Brunelle et al., 2005; Guzy et al., 2005; Mansfield et al., 2005).

However, the source of ROS within the electron transport chain and the mechanism by which the decrease in oxygen levels elicits an increase in ROS remains to be established. Furthermore, the direct target of the mitochondrial generated oxidants in the cytosol that trigger signaling pathways has not been identified. I used a candidate approach in order to investigate what is/are the direct targets of the mitochondrial ROS that activate the p38 MAPK pathway during hypoxia and that lead to the activation of HIF-1. Chapter 3 of my thesis demonstrates that the kinases SRC, ASK1, and AMPK do not function as upstream regulators of HIF-1 during hypoxia. In the end, the advent of genetic tools such as, RNA interference and gene knockouts in mice will yield new insights into the upstream regulators of HIF-1. Moreover, there may prove to be numerous upstream regulators and in different mammalian cell types different signaling kinases may be necessary for the activation of HIF-1. Nonetheless, having a map of an oxygen sensing pathway emanating from the mitochondrial electron transport chain to the p38 MAPK signaling pathway and ultimately to HIF-1 will allow investigators to fill in the crucial components that will complete the hypoxic signaling pathway.

Two crucial physiological implications have emerged from my thesis work presented here. First, the requirement of p38 MAPK signaling for the hypoxic activation of HIF-1 may explain the resemblance seen in the phenotypes of the *p38α*, *Mkk3/6*, *Hif-1α*, and *Hif-1β* null mice. All these embryos die during midgestation due to multiple defects, including abnormal vascularization of the placenta (Adams et al., 2000; Adelman et al., 2000; Allen et al., 2000; Brancho et al., 2003; Carmeliet et al., 1998; Iyer et al., 1998; Maltepe et al., 1997; Mudgett et al., 2000; Ryan et al., 1998; Tamura et al., 2000). In particular, both the *Hif-1β* knockout and *p38α* null mice display a complete loss of the labyrinth layer and significant reduction of the spongiotrophoblast layer in

the developing placenta. Alternations in oxygen tensions have profound effects on development and HIF-1 is essential for life. The mammalian embryo resides in a physiologically hypoxic environment during gestation, therefore understanding the signaling pathways that regulate the responses to oxygen deprivation are fundamentally important at a cellular level. My work has shed light by identifying the p38 MAPK signaling pathway as an upstream regulator of the transcription factor HIF-1.

Secondly, HIF-1 is up-regulated in most human cancers and is required for tumor progression by up-regulating its target genes, which are involved in angiogenesis, anaerobic metabolism, cell survival, cell invasion, and drug resistance (Semenza, 2003). I have showed that the p38 MAPK signaling pathway is a key upstream regulator of HIF-1 activity. By understanding the complex molecular mechanisms that regulate HIF-1, we can ultimately target HIF-1 to selectively inhibit hypoxic tumor cells. Moreover, with the correlations between increased HIF-1 protein levels and the poor prognosis of cancer, deciphering the upstream regulators of HIF-1 is vital for cancer research. Thereby, altering the p38 MAPK signaling pathway may prove to hinder HIF-1 dependent tumor growth.

Understanding the connection between the loss of the tumor suppressor PTEN and HIF-1 activation is also fundamental in understanding the biology of tumor growth. Many PTEN null tumors are highly vascularized and display increased HIF-1 activity. Numerous studies have suggested a link between the loss of PTEN and the hypoxic activation of HIF-1. However, the mechanism by which loss of PTEN increases HIF-1 activity has never been described. Therefore, I set out to investigate whether the loss of PTEN regulates HIF-1. Here I have

described a model in which a novel nuclear PTEN-FOXO3a signaling pathway regulates the activation of HIF-1 (Figure 5.1). These findings have significant implications for cancer biology. First, they highlight the importance of subcellular localization. PTEN can participate in several cellular processes relevant to tumorigenesis including regulation of proliferation and survival, cell migration and invasion, angiogenesis, genomic instability, induction of cell-cycle checkpoints in response to DNA damage, and stem cell self-renewal (Chow and Baker, 2006). This wide range of activities spans both the nucleus and the cytoplasm. Despite its well-defined role in signaling at the plasma membrane, PTEN has recently been shown by many groups to be found in the nucleus in a number of different normal and tumor cell types. Interestingly, nuclear localization of PTEN may play a role in its tumor suppressor activity (Lian and Di Cristofano, 2005). There is increasing evidence now that many of the main components of the PI3K signaling pathway are found in the nucleus, including PIP2, PIP3, PI3K, PDK1, and AKT (Deleris et al., 2006), therefore, it is possible that PTEN is a PIP3 phosphatase in the nucleus as well. However, a recent study demonstrates that nuclear PTEN does not dephosphorylate the nuclear pool of PIP3 (Lindsay et al., 2006). Other studies have implicated phosphatase-independent functions of PTEN within the nucleus including protein-protein interactions that modulate the activity and stability of p53 (Li et al., 2006; Lian and Di Cristofano, 2005). My data implicates yet another role for nuclear PTEN as a repressor of HIF-1 activity through FOXO3a activation and further corroborates that nuclear localization of PTEN adds to its tumor suppressor functions. Moreover, I have shown that the repressing function of nuclear PTEN is dependent on its phosphatase activity.

Secondly, my findings propose that by inhibiting FOXO3a nuclear export in PTEN null cancers may serve as a potential treatment in HIF-1 dependent tumors. FOXO transcription factors function as tumor-suppressor proteins by inhibiting cell proliferation, promoting apoptotic cell death and protecting cells from DNA damage and oxidative stress (Biggs et al., 1999; Brownawell et al., 2001; Brunet et al., 1999; Rena et al., 1999; Takaishi et al., 1999; Tang et al., 1999; Tomizawa et al., 2000). Recently, two papers have showed that FOXO factors are bona fide tumor suppressors *in vivo* (Paik et al., 2007; Tothova et al., 2007). Moreover, the tumorigenicity of PTEN null cancer cells can be reversed by forcible localization of FOXO1a in the nucleus (Kau et al., 2003). My study here further suggests that by inhibiting FOXO3a nuclear export in PTEN null cancers may decrease tumorigenicity by repressing HIF-1 transcriptional activity. In support of this implication I have shown that FOXO3a is a negative regulator of HIF-1 transcriptional activity. More specifically, that FOXO3a stifles HIF-1 transcriptional activity by interfering with p300's ability to activate HIF-1. However, the actual mechanism of how FOXO3a represses HIF-1 transcriptional activity still needs to be elucidated. IP assays reveal that FOXO3a interacts with HIF-1α and p300 in the nucleus. ChIP assays show the nuclear FOXO3a complexes with HIF-1α and p300 on the promoter of HIF-1 target genes. Therefore, we have proposed a model in which FOXO3a physically interferes with p300's ability to induce HIF-1 transcriptional activity. In support of this model, my work shows that the FOXO3a-mediated HIF-1α transactivation repression can be reversed by overexpression of p300 in PTEN null cells.

How nuclear FOXO3a interferes with p300's ability to function as a co-activator for HIF-1 is also not entirely defined. It is know that FOXO3a and p300 directly interact (Mahmud et al.,

2002; Motta et al., 2004); therefore FOXO3a may bind to p300 on the HRE's of HIF-1 target genes, thereby repressing HIF-1α transactivation. Or FOXO3a may directly bind to HIF-1α. The actual physical interactions need to be solved. Furthermore, does inhibiting FOXO3a nuclear export in PTEN null cancers decrease tumor growth by decreasing HIF-1 transcriptional activity. This can be tested by designing a stable PTEN null cell line that expresses the constitutively active FOXO3a (FOXO3a-AAA). FOXO3a would be sequestered in the nucleus, thereby repressing HIF-1 transcriptional activity. By injecting these cells into nude mice one could monitor whether the presence of nuclear FOXO3a decreases tumor formation or growth in comparison to control PTEN null cancer cells. From my work thus far, I would predict that nuclear FOXO3a would decrease tumorigenesis, consequently identifying FOXO3a as a tumor suppressor and a bona fide negative regulator of HIF-1 *in vivo*.

In conclusion, my work has uncovered two novel signaling pathways that regulate HIF-1. Both these pathways have increased our understanding of how HIF-1 is regulated. Furthermore, I have identified key molecular targets that could be manipulated in order to decrease HIF-1 dependent tumor growth by repressing HIF-1 activity.

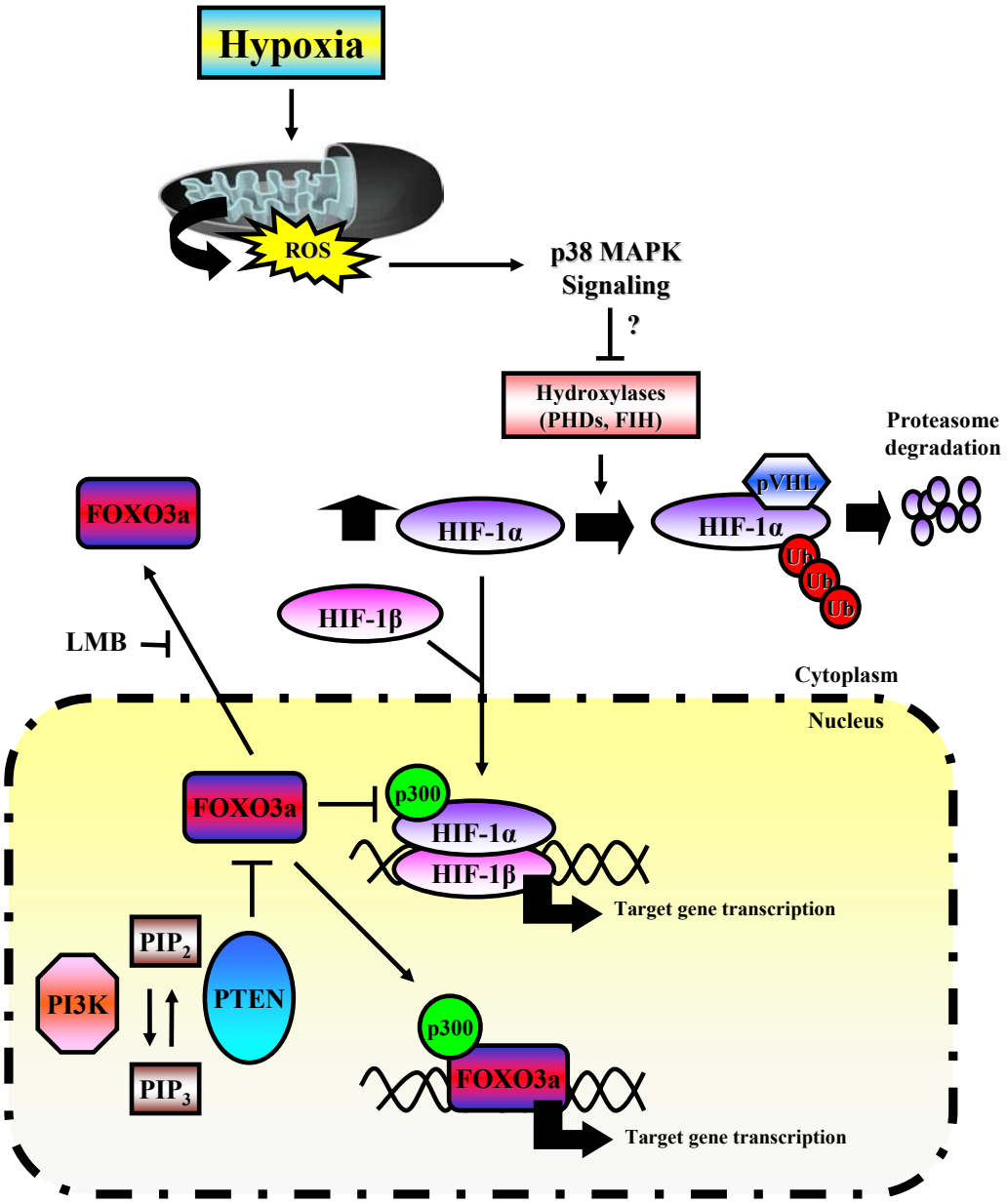

Figure 5.1. Signaling pathways regulating HIF-1.

Chapter 6: Materials and Methods

Cell Culture:

All mouse embryonic fibroblasts (MEFs) and U251 glioma 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 (Gibco), 100U/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericin B, and 20mM HEPES. LnCaP prostate cancer cells were grown in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (Gibco), 1% sodium pyruvate, 100U/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericin B, and 20mM HEPES. All cells were maintained at 37^{o}C in 5% CO_2 humidified incubators and plated at a confluency near 30-50% for experimentation.

Stable cell lines were established by retroviral infection. Briefly, retroviral vectors were transiently transfected using the Mirus *Trans*IT Transfection reagent (Mirus Bio Corporation) into the RetroPack PT67 packaging cell line (BD Biosciences). Media containing the virus was collected, filtered through a .45μM membrane, and 8μg/ml polybrene was added. This was placed on desired cell line for 24 hours and media containing the appropriate antibiotic was used for selection. *p38α*$^{-/-}$ cells were retrovirally infected with a p38α cDNA or vector control. These cells were selected in Dulbecco's modified Eagle media (DMEM) containing hygromycin.

Null adenovirus (control) and adenoviruses encoding a myc-tagged GPX-1 (25pfu/μl), Rac1-N17 (50pfu/μl), PTEN (50pfu/μl), a HA-tagged FOXO3a (WT) (100pfu/μl), and a HA-tagged constitutively active FOXO3a (AAA) (100pfu/μl) were used to infect the desired cell line for 3

hours in serum/antibiotic-free media and subsequently incubated for 16 hours in serum-free media before experimentation.

Oxygen Conditions:

Hypoxic conditions (1.5% O_2 and 5% CO_2 balanced with N_2) were achieved in a humidified variable aerobic workstation (INVIVO O_2, BioTrace), which contains an oxygen sensor that continuously monitors the chamber oxygen tension.

Anoxic conditions (10% H_2 and 5% CO_2 balanced with N_2) were achieved using the Bugbox workstation (BioTrace). An anaerobic color indicator (Oxoid) confirms anaerobicity of the chamber.

Transfections and Reporter assays:

Transfections were done using the Mirus *Trans*IT Transfection reagent (Mirus Bio Corporation) according to the manufacturer's protocol. The HRE-Luciferase reporter gene construct (1μg), a pGL2 vector containing three hypoxia response elements from the *Pgk-1* gene upstream of firefly luciferase, was transfected into cells. After 24 hours, the media was replaced and cells were exposed to various conditions.

For GAL4-reporter assays, cells were transfected with 3μg of a GAL4 (1 to 147) DNA-binding domain fused to HIF-1α (531 to 826) construct and 2μg of a reporter gene construct encoding five GAL4-binding sites using the AMAXA Nucleofactor transfection system (AMAXA biosystems). For MEFs, 2 million cells are needed and for all other cell lines 1 million cells are

needed per transfection. Briefly, cells were pelleted and resuspended in 100µl of Nucleofactor solution. DNA was then added and the nucleofection sample was transferred into AMAXA cuvette using a sterile transfer pipette. The Nucleofector program was selected (for MEFs, A-23), the cuvette was inserted into holder, and the start button pressed. The cuvette is then removed and 500µl of media is added to cells and transferred to an eppendorf tube. The samples are then transferred to culture dishes for experimentation. Cells were incubated at 21% O_2 for 20 hours, followed by 36 hours at various conditions. Data were normalized by using total protein concentration as determined by the Bio-Rad protein assay (Bio-Rad Laboratories).

Measurement of Reactive Oxygen Species (ROS):

Intracellular ROS generation was assessed using 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes). ROS in the cells cause oxidation of DCFH, yielding the fluorescent product 2',7'-dichlorofluorescein (DCF). Cells were plated on Petri dishes and incubated with DCFH-DA (10 µM) under various conditions. The media was then removed, cells were lysed, centrifuged to remove debris, and the fluorescence in the supernatant was measured using a spectrofluorometer (excitation, 500 nm; emission, 530 nm). Data were normalized to values obtained from normoxic, untreated controls.

Immunoblot analysis:

HIF-1α protein was analyzed in nuclear extracts prepared from cells using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce). For whole cell lysates, cells were scraped and lysed using 1X Cell Lysis Buffer (Cell Signaling) supplemented with 1mM phenylmethylsulfonyl fluoride (PMSF). The BioRad protein assay (Bio-Rad Laboratories) was used to measure protein

concentration. Nuclear extracts (15-30 μg) and whole cell lysates (50-100μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond-ECL nitrocellulose membrane (Amersham) using a Semi-Dry Transblot blotter (Bio-Rad Laboratories). Blots were stained in Ponceau S to ensure equal loading. Membranes were blocked in 5% milk in Tris-buffed saline with Tween (TBST). Primary antibodies used included: anti-HIF-1α antibody (1:500; Novus Biological Sciences, 1:250; Cayman Chemical, 1:200; BD Biosciences), p38α MAPK (1:1,000; Cell Signaling Technology, Inc.), phospho-p38 MAPK (1:1,000; Cell Signaling Technology, Inc.), MKK3 (1:1,000; Cell Signaling Technology, Inc.), MKK6 (1:250; R&D Systems, Inc), myc (1:5,000; Invitrogen), S6 Ribosomal protein (1:1000; Cell Signaling Technology, Inc.), phospho-S6 Ribosomal protein (1:1000; Cell Signaling Technology, Inc.), PTEN (1:1000; Cell Signaling Technology, Inc.), p300 (1:200; Santa Cruz Biotechnology, Inc.), FOXO3a (1:1000; Santa Cruz Biotechnology, Inc.), and HA (1:1000; Covance Research Products, Inc.). To control for loading in nuclear extracts, membranes were stripped and reprobed with an anti-POL II antibody (1:200; Santa Cruz Biotechnology, Inc.). To control for loading in whole cell lysates, an anti-α-tubulin antibody (1:2,000; Sigma-Aldrich, Inc.) was used. Secondary antibodies used were an anti-Mouse IgG and an anti-Rabbit IgG, HRP-linked antibodies (1:1000; Cell Signaling Technology, Inc.). Membranes were stripped in stripping buffer (100mM β-mercaptoethanol, 2% Sodium Dodecyl Sulphate (SDS), 62.5 mM Tris-HCl pH 6.7) at 50°C for 30 minutes with occasional agitation, washed in TBST for 2 x 10 minutes, blocked, and reprobed. SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used to develop immunoblots.

Immunoprecipitation (IP) analysis:

IP assays were performed using the Nuclear Complex Co-IP kit (Active Motif). U251 cells were exposed to 1.5% O_2 ± 20nM Leptomycin B (Sigma) for 16 hours and nuclear extracts were prepared using the kit's extraction reagents (Active Motif). The protocol was adjusted for 150 mm plates and the low IP buffer was used without DTT, NaCl, or detergent. 100μg of nuclear protein was used per IP reaction and incubated with 2μg HIF-1α antibody (BD Biosciences), 2μg FOXO3a antibody (Santa Cruz Biotechnology, Inc.), 2μg p300 antibody (Santa Cruz Biotechnology, Inc.), or 2μg IgG control antibody (Sigma). 50μl of Protien A/G PLUS-Agarose (Santa Cruz Biotechnology, Inc.) was added to each IP reaction. Following the IP, 2x sample buffer was added to each IP reaction, samples were boiled and run on an SDS-PAGE gel. The samples were then subjected to immunoblotting as described above.

Chromatin-immunoprecipitation (ChIP) analysis:

ChIP assays were performed using the EZ ChIP Assay Kit and protocol (Upstate). *Pten*$^{-/-}$ cells were grown at 1.5% O_2 ± 20nM Leptomycin B (Sigma). 4.5 x 10^7cells were fixed in 1% formaldehyde at RT for 20 minutes. Isolated nuclei were lysed, followed by chromatin shearing using the Enzymatic Shearing Kit (Active Motif). An anti-HIF-1α monoclonal Ab (1μg; Novus Biological, Inc.), anti-FOXO3a polyclonal Ab (μg; Santa Cruz Biotechnology, Inc), and anti-p300 polyclonal Ab (μg; Santa Cruz Biotechnology, Inc.) were used. A mouse IgG Ab (μg; Upstate), and rabbit IgG Ab (μg; Sigma) were used as controls. After reverse crosslinking and DNA purification, DNA from Input (1:20 diluted) or immunoprecipitated samples were assayed using PCR and products were separated by agarose gel electrophoresis. The following were the

primers to detect HRE-containing *Glut-1*: *Glut-1* F, 5′-GGGCTGTGTTACTCACTCTTACTCC-3′; *Glut-1* R, 5′-CTCTTCCTGGGTTGTGTTCAAGCTG-3’. DNA from input and immunoprecipitated samples was also amplified using the Biorad iCycler iQ system (Biorad Laboratories) and quantified using the iQ SYBR Green SuperMix (BioRad Laboratories). The following primers were used to detect HRE-containing *Glut-1* using quantitative SYBR-green real-time PCR: *Glut-1* F, 5'-ATTTCTAAGGCCCTGGGTCC-3'; *Glut-1* R, 5'-CCTGCCTGATGCGTGTCA-3'. All Cycle threshold (Ct) values were compared to the input amounts to normalize for variations. The data was analyzed using the Pfaffl method (Pfaffl, 2001). The results were graphed as fold changes relative to the control IgG antibodies.

Real-time reverse transcription-PCR (RT-PCR) analysis:

Total RNA was isolated from cells exposed to various conditions using the RNAqueous-4PCR kit (Ambion, Inc.). First-strand cDNA was synthesized from 1 μg of total RNA using the RETROscript cDNA synthesis kit (Ambion, Inc.) with the random decamer primers. The diluted cDNAs were amplified using the Bio-Rad iCycler iQ system (Bio-Rad Laboratories). Quantitative real-time RT-PCR was carried out using gene-specific dual fluorescently labeled probes. The following primer and probe sequences were used: for *Pgk1*, the primer sequences were TCTGTTCTTGAAGGATTGTGTGG and CTCTACATGAAAGCGGAGGTTT and the probe sequence was 6-carboxyfluorescein (FAM)-CGAGAATGCCTGTGCCAACCCAGCGG-black hole quencher 1 (BHQ-1); for *Glut-1*, the primer sequences were AACATGGAACCACCGCATCG and CCGACAGAGAAGGAACCAATCAT and the probe sequence was 6-FAM-AGCCCATCCCATCCACCACACTCACCACGC-BHQ-1; for *L19*, the

primer sequences were CATCAAGCGATCAGGGAATG and GAGGATTATACAGTTCAAAGCAAAT and the probe sequence was Texas Red-CACCTTGTCCTTCAATCGTGTTCCTGAGGG-black hole quencher 2 (BHQ-2). For cDNA amplication using the iQ SYBR Green SuperMix (BioRad Laboratories), the following primer sequences were used: for *Vegf A*, 5'- GTACCCCGACGAGATAGAGT-3' (forward) and 5'-ATGATCTGCATGGTGATGTTG-3' (reverse); for *Pgk1*, 5'-TCTGTTCTTGAAGGATTGTGTGG-3' (forward) and 5'-CTCTACATGAAAGCGGAGGTTT-3' (reverse); for *L19*, 5'-CATCAAGCGATCAGGGAATG-3' (forward) and 5'-GAGGATTATACAGTTCAAAGCAAAT-3' (reverse). The specificity of primers and probes were first tested under normal PCR conditions before quantitation. Cycle threshold (Ct) values were normalized for amplification of the mitochondrial ribosomal protein *L19* and the data was analyzed using the Pfaffl method (Pfaffl, 2001).

Immunofluorescence microscopy:

Pten$^{+/-}$ or *Pten*$^{-/-}$ MEFs were cultured on glass coverslips. Cells were either transfected with PTEN constructs as indicated, infected with the adenoviruses, AdFOXO3a (WT) and AdFOXO3a (AAA), or subjected to conditions as indicated. Cells were washed three times with PBS and fixed with 3.7% paraformaldehyde (PFA) for 20 min at RT. PFA was quenched with PBS+glycine (10mM) for 10 min at RT, washed one more time with PBS and then permeabilized/blocked using 0.3% Triton X-100+ 0.3&BSA+Normal Goat serum (NGS) in PBS for 30 min at RT. First antibody incubation was carried out with either anti-FKHRL-1/FOXO3a (1:200; Upstate Biotechnology, Inc.) or anti-PTEN (1:2000; BD PharMingen) for 1 hour at RT,

followed by a secondary antibody incubation with either an Alexa®-Fluor488 goat anti-rabbit (1:1000; Molecular Probes) or a fluorescein goat anti-mouse (1:1000; Molecular Probes). The coverslips were then washed three times in PBS/BSA/TX-100; removed and mounted using Vectashield® mounting medium containing DAPI (Vector Laboratories). Cells were observed under the Axioplan 2 fluorescence microscope (Carl Ziess MicroImaging, Inc.; 63x objective) and imaged with AxioVision LE software (Carl Ziess MicroImaging, Inc.).

Reagents:

The following reagents were used: Desferrioxamine (DFO) (Sigma), Myxothiazol (Sigma), dimethyloxaloylglycine (DMOG) (Frontier Scientific, Inc.), Rapamycin (Sigma), Leptomycin B (Sigma).

The adenovirus expressing PTEN (AdPTEN) were purchased from the Vector Core Facility (Univ. of Pittsburgh). The adenoviruses, AdNull (control), AdFoxo3A (WT) and AdFoxo3A (AAA) were purchased from Vector Biolabs. The pLNCX-PTEN wild-type (WT), pLNCX-NLS-PTEN, and pLNCX-PTEN (G129R) constructs were provided by J.L. Liu. The pALTER-MAX p300 WT and vector control constructs were provided by T. Unterman.

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Candidate gene isolation and comparative analysis of a commonly deleted segment of 7q22 implicated in myeloid malignancies. *Genomics.* Oct; 77(3):171-80.

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3. **Emerling BM**, Bonifas J, Kratz CP, Donovan S, Taylor BR, Green ED, Le Beau MM, Shannon KM. (2002) MLL5, a homolog of Drosophila trithorax located within a segment of chromosome band 7q22 implicated in myeloid leukemia. *Oncogene*. Jul 18; 21(31):4849-54.
4. Curtiss, NP, Bonifas J, Lauchle, JO, Balkman, JD, Kratz CP, **Emerling BM,** Green ED, Le Beau MM, Shannon KM. (2005) Isolation and analysis of candidate myeloid tumor suppressor genes from a commonly deleted segment of 7q22. *Genomics*. May; 85(5):600-7.
5. **Emerling BM,** Platanias LC, Black E, Nebreda AR, Davis RJ, and Chandel NS. (2005) Mitochondrial ROS activation of p38 MAPK is required for hypoxia signaling. *Mol Cell Biol*. Jun; 25(12):4853-62.
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8. **Emerling BM**, Weinberg F, Liu JL, Mak TW, and Chandel, NS. (2007) PTEN represses HIF-1 transcriptional activity through FOXO3a. *Mol Cell (in review).*

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