A Paradoxical Role for PTEN in the Cellular Response to Hypoxia

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
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ABSTRACT

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Abstract

Regulation of cell growth is controlled by a variety of factors, including a number of oncogenes and tumor suppressors. PTEN is an inositol phosphatase that regulates cell growth by hydrolyzing the phospholipid products of PI3K. PTEN is mutated in a number of cancers, leading to its characterization as an important tumor suppressor. Recent data indicate that PTEN may also perform important functions that are independent of its phosphatase activity, most notably within the nucleus. Studies in this thesis addressed a novel role for PTEN in the regulation of the cellular response to hypoxia.

PTEN overexpression significantly increased hypoxic gene expression independent of its catalytic activity, while shRNA-mediated silencing of PTEN significantly inhibited hypoxia-mediated HRE-luciferase activity. Nuclear-localized PTEN was more effective in promoting HRE activity than nuclear-excluded PTEN. These results suggested a scaffolding function of PTEN in the hypoxic nucleus. To identify specific gene targets regulated by PTEN in hypoxia, a custom oligo-array consisting of 46 hypoxia-responsive genes was utilized following both gain- and loss-of- PTEN function. Based on real-time quantitative results, PTEN positively regulated genes involved in metabolism (PFKFB3, PFKFB4, ALDOA, PGK-1), oxygen supply (VEGFA, EPO), cell growth (Tgf-α, TERT, cyclin D1, BNIP3), motility (E-cadherin) and transcription (DEC2). A single missense mutation at isoleucine 224 (I224M) of PTEN, however, abrogated the ability of PTEN to regulate the hypoxia response without affecting its lipid

phosphatase activity. PTEN has previously been shown to bind to the co-activator p300 and to affect p53 acetylation and stabilization. As p300 is also a co-activator for the HIF proteins, we hypothesized that PTEN's association with p300 would promote the HIF/p300 complex to positively regulate hypoxic gene transcription. Overexpression of PTEN-WT extended the half-life of p300 and histone acetyltransferase activity of p300 in hypoxia, while overexpression of PTEN-I224M or PTEN silencing decreased both. *In vivo*, these effects resulted in a significant increase in hypoxic area in PTEN-null tumors compared to tumors expressing endogenous levels of PTEN, suggesting an inability to mount a hypoxia response necessary for revascularization of the tissue. PTEN's effect on p300 extended to other functions of p300 outside of the hypoxia response, most notably p300's role in p53 stability and p53-mediated gene transcription. Overexpression of PTEN resulted in an increase in p53 reporter activity following DNA damage (mitomycin C treatment). PTEN silencing or overexpression of PTEN-I224M resulted in abrogation of these effects. Taken together, these findings demonstrate that PTEN is required for the hypoxia response and they suggest that PTEN acts as a scaffold for p300 and the HIF machinery in the hypoxic nucleus independent of its canonical lipid phosphatase activity. These results may have important implications for the treatment of tumors in which PTEN is lost or mutated. The potential use of PTEN-I224M as a therapeutic is also discussed.

Dedication

To my parents and my husband, Steven, and all other family members and friends for their unending love and support. Their encouragement has meant so much to me.

Contents

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List of Abbreviations

293-PTEN$^{C/S-R}$	293^{low} cells transiently overexpressing a non-targeted catalytically inactive PTEN rescue construct
293-PTEN^{low}	HEK-293 cells stably expressing PTEN-targeting shRNA
293-PTEN^{WT-R}	293^{low} cells transiently overexpressing a non-targeted wild-type PTEN construct
293^{scr}	HEK-293 cells stably expressing scrambled shRNA
bHLH	basic helix-loop-helix
C/S	Cysteine 1224 to serine mutation (C124S)
CBP	CREB-binding protein
Cdk	cyclin-dependent kinase
ChIP	chromosomal immunoprecipitation
Ct	cycle threshold
DMEM	Dulbecco's modified Eagle media
EBM	endothelial basal medium
EC	endothelial cell
ECRF	EC-RF24; immortalized HUVECs
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EPAS1	endothelial PAS-domain containing protein 1 (HIF-2α)
EPO	erythropoietin

F/V	Phenylalanine 341 to valine mutation (F341V)
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FIH-1	factor inhibiting HIF-1
FISH	fluorescence *in situ* hybridization
GAP	GTP-ase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GLUT	glucose transporter
GPCR	G protein-coupled receptor
GTP	guanine triphosphate
HAT	histone acetyltransferase
HIF-1α	hypoxia-inducible factor-1α
HIF-2α	hypoxia-inducible factor-2α
HIF-3α	hypoxia-inducible factor-3α
HRE	hypoxia response element
HUVEC	human umbilical vein endothelial cell
I/M	Isoleucine 224 to methionine mutation (I224M)
IP	immunoprecipitation
L/V	Leucine 182 to valine mutation (L182V)
MAPK	mitogen-activated protein kinase

MEF	murine embryonic fibroblast
mTOR	mammalian target of rapamycin
NES	nuclear exclusion signal
NLS	nuclear localization signal
ODDD	oxygen-dependent degradation domain
PCAF	p300/CBP-associated factor
PCR	polymerase chain reaction
PEST	proline (P) – glutamate (E) - serine (S) - threonine (T)
PET	positron emission tomography
PH	pleckstrin homology
PHD	prolyl hydroxylase
PI	phosphoinositide
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
pO_2	partial pressure of oxygen
PTEN	phosphatase and tensin homolog deleted on chromosome 10
PTEN-C/S-R	cDNA containing a non-targeted catalytically dead PTEN (C124S)
PTEN-I224M	PTEN cDNA containing I224M
PTEN-F341V	PTEN cDNA containing F341V
PTEN-L182V	PTEN cDNA containing L182V

PTEN-R234W	PTEN cDNA containing R234W
PTEN-WT	wild-type PTEN cDNA
PTEN-WT-R	cDNA containing a non-targeted wild-type PTEN
R/W	Arginine 234 to tryptophan mutation (R234W)
RCC	renal cell carcinoma
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
scr	scrambled
SH2	Src homology 2
shRNA	short hairpin RNA
TAD	terminal activation domain
$U87^{Tet\text{-}On:PTEN}$	U87 glioblastoma cells (PTEN-null) stably expressing Tet-inducible PTEN
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VHL	von Hippel-Lindau protein
VSMC	vascular smooth muscle cell
WT	wild-type

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1. Background and Overview

1.1 Cell Growth Pathways

Proper cellular responses to external stimuli are critical for maintaining organismal homeostasis by balancing cell division and death. At the most basic level, external growth or death signals are received by the cell through various transmembrane receptors that initiate signal transduction cascades, post-translational modifications and subsequent changes in gene expression. Failure to properly regulate cell division and cell death results in an immortal phenotype characteristic of cancer. Put simply, the development of cancer is a result of activation of oncogenes and loss-of-function of tumor suppressor proteins. Here I will discuss the roles of Ras and Myc oncogenes as well as the tumor suppressor proteins p53 and the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in tumorigenesis as a consequence of their biochemical function in two of the most prominent signaling pathways regulating cell growth: the mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide (PI) 3-kinase pathway.

One of the primary MAPK signaling pathways relays growth and proliferative extracellular signals to the cell nucleus, where specific genes are activated. As seen in Figure 1, receptor ligand-binding results in consecutive phosphorylation events that propagate the signal, culminating in nuclear localization of transcription factors and downstream gene transcription. Initially, RAF (a MAP-kinase-kinase-kinase) is activated at the membrane of stimulated cells through the action of activated Ras and receptor. Functional RAF adds

activating phosphate groups to the conserved Thr-Xxx-Tyr motif of MEK, a MAP-kinase-kinase(2). MEK, in turn, activates the MAPK ERK in the same manner, allowing ERK to translocate to the nucleus where it affects gene expression by phosphorylating key transcription factors and gene regulatory proteins(2-4). Different MAP kinases are activated in the cell specific to the external signals received. For example, the p38 MAPK is ubiquitously expressed and is activated following cellular stress (osmotic shock, hyperthermia, UV exposure, low oxygen conditions)(3-7). Although all of the MAP kinases are integral players in regulating MAPK signaling, it is the activation of RAF that couples the MAP-kinase pathway with the first key player in tumorigenesis discussed here, the monomeric GTPase Ras.

1.1.1 Ras Signaling

Ras is anchored to the plasma membrane through a covalently attached farnesyl lipid chain, which enables Ras to interact with the cytoplasmic domain of transmembrane receptors through the adapter proteins GRB2 and SOS(8). In the case of receptor tyrosine kinases (RTKs), extracellular ligand (i.e., growth factor) binding induces receptor multimerization and cross-phosphorylation. This phosphorylation of receptor allows binding of adapter proteins and activation of a guanine nucleotide exchange factor (GEF), which then activates membrane-associated Ras(9). Like other GTPases, Ras is cycled between active and inactive conformations depending on its association with GTP or GDP, respectively. Inactive Ras is converted to active Ras by the action of GEFs that exchange GDP for GTP. Once bound to GTP, Ras is a potent signaling molecule that can activate

many signaling pathways and molecules, including the MAPK pathway through RAF. Active Ras is inactivated following hydrolysis of GTP through the action of GTPase-activating proteins (GAPs).

Specificity of the Ras/MAPK pathway is achieved through the coordinated events of multiple isoforms of each component. There are at least 12 MAP-kinases, 7 MAP-kinase-kinases and 7 MAP-kinase-kinase-kinases. In addition, there are 4 isoforms of Ras, as well as various adapter proteins, GEFs and GAPs that interact with specific cell receptors. As the Ras/MAPK pathway is ubiquitous among all mammalian cells, multiple parallel pathways utilizing different MAP-kinases may be active in the same cell, specific to the extracellular signals received (UV stress, cytokine exposure, growth factor binding, oxidative stress, etc.). Gene targets of ERK result in a proliferative phenotype and include activation of the transcription factors Elk-1 and Myc as well as downstream regulation of G1 cyclins, E2F, and ribosomal biosynthesis(10-14).

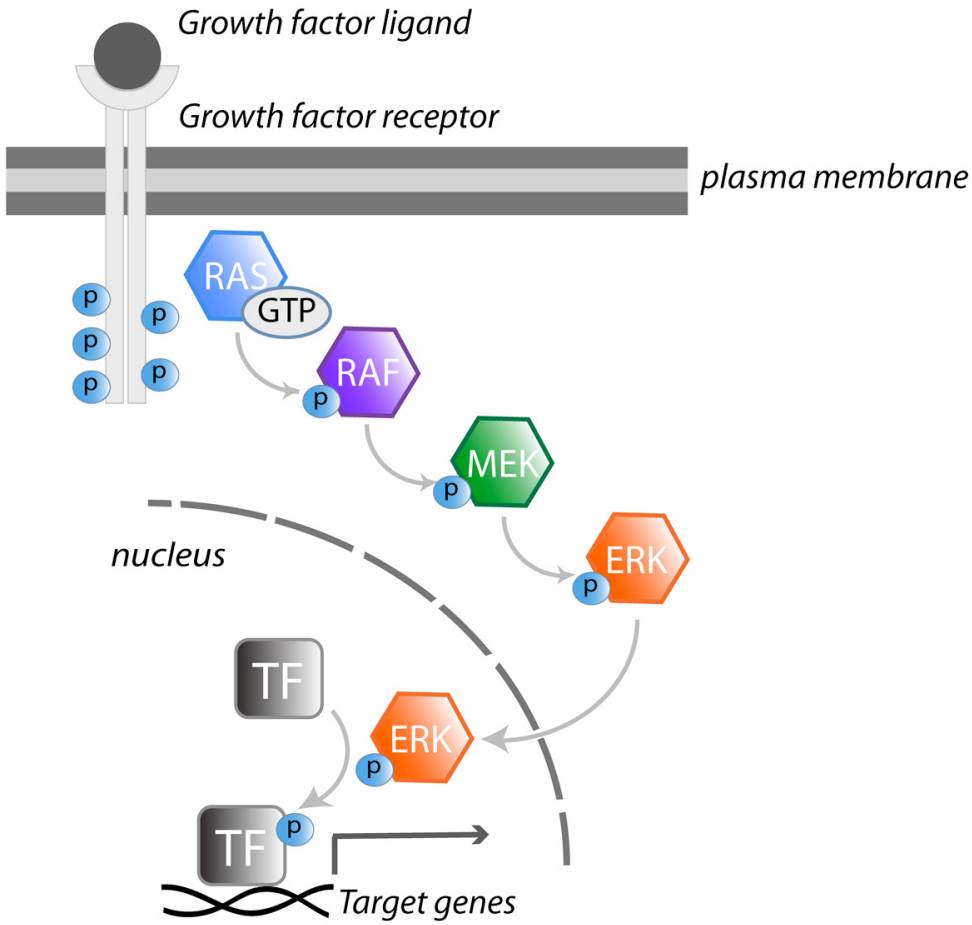

Figure 1: The Ras/MAPK signal transduction cascade

Growth factor binding initiates a cascade of events beginning with activation and autophosphorylation of receptor tyrosine kinases. Activated Ras-GTP phosphorylates and activates RAF, which phosphorylates MEK. Subsequent MEK phosphorylation of ERK results in nuclear translocation of ERK where it activates a host of transcription factors (TF) to induce target gene expression.

1.1.2 Myc Signaling

Another important regulator of cellular proliferation is Myc. Myc is a robust transcription factor whose expression is tightly regulated in the cell. Acting as a transcriptional activator or repressor, Myc heterodimerizes with Max and binds to DNA sequences through its helix-loop-helix and leucine zipper domains to promote cell growth and cell cycle progression while blocking differentiation(15-19).

Stimulatory signals for cell growth and division are distinct. Cell growth, or cell hypertrophy, is defined as an increase in protein synthesis, while cell division or proliferation is defined as an increased number of cells through mitosis. Proliferation requires cell cycle progression, which is a direct response to activation of the MAPK pathway. In somatic cell division, the cell must initiate cell proliferation in the G1 phase of the cell cycle and proceed to the S phase, where DNA is replicated, followed by further cell growth in the G2 phase until physical division is achieved in the M phase. Separating each phase are "checkpoints" that ensure completion of the preceding phase.

Tight temporal control of the cell cycle is regulated by the cyclical ubiquitination and subsequent degradation of cyclins. When present, various cyclins complex with specific cyclin-dependent kinases (Cdks). In addition to the degradation of cyclins, the cyclin-Cdk complexes themselves are also regulated by Cdk inhibitor proteins (e.g., p16, p21 and p27)(20-22). At the conclusion of each phase, the cell must have the proper complement of cyclin-Cdk complexes in order to proceed. For example, a cell with damaged DNA (via UV or X-ray

irradiation) will arrest at the G1/S checkpoint, allowing repair of the damage before DNA replication. This arrest is achieved as a result of the activity of two important proteins, p53 and one of its downstream gene targets, p21(23).

1.1.3 p53 Signaling

When DNA is damaged, p53 is stabilized and transcriptionally up-regulates p21, which inhibits the formation of G1/S-cyclin-Cdk complexes, halting cell cycle progression(23). Although this stress response role of p53 is perhaps its most well-characterized function, other evidence has linked p53 to metabolism, apoptosis, aging, autophagy and embryonic development(24-28). In each of these situations, p53 acts as a potent transcriptional enhancer or repressor of downstream genes. Recent evidence has identified a conserved p53 response element in hundreds of genes and partial response elements in thousands more(29). In its role in promoting apoptosis, stress-induced accumulation of p53 results in activation of the pro-apoptotic gene targets, Bax and IGFBP3(30,31). However, p53 represses other genes, such as Myc and cyclin D1(32,33).

Although p53 itself is regulated at the transcriptional, post-transcriptional and post-translational levels, one of the most important regulatory mechanisms is post-translational. For example, p53 protein is ubiquitinated by the E3 ubiquitin ligase, MDM2, leading to its degradation(34,35). In contrast, acetylation of p53 by the histone acetyl transferases (HATs) p300 and CBP enhances p53 activity by increasing its ability to form homo-tetramers, which have a higher affinity for DNA(36-38). Stress-activated protein MAP kinases (e.g., p38)

phosphorylate p53 on at least 7 serine/threonine residues, leading to initiation of p53 activity(39,40). The diversity of p53 responses is due, in part, to its involvement in many cell signaling cascades.

1.1.4 PI 3-kinase Signaling

The PI 3-kinase pathway is an important growth regulatory pathway downstream of or parallel to Ras. As such, many members of the MAPK pathway feed into the PI 3-kinase pathway. As described below, key oncogenic signaling pathways, including Ras, Myc and p53 converge upon PI 3-kinase itself or its downstream targets, such as Akt and the mammalian target of rapamycin (mTOR), making the PI 3-kinase pathway arguably the most important signaling cascade in tumorigenesis. I will now discuss the PI3K pathway and the interplay among Ras, Myc and p53.

The PI 3-kinase (PI3K) pathway is critical for regulating a number of vital cellular processes including growth, differentiation, survival, proliferation and migration. As with Ras, the cascade is initiated as external signals activate transmembrane receptors such as G protein-coupled receptors (GPCRs; e.g., angiotensin and adrenergic receptors) and receptor tyrosine kinases (e.g., vascular endothelial growth factor receptor (VEGFR), epithelial growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR)), leading to activation of multiple PI3K isoforms and downstream activation of effector molecules. Downstream signaling cascades are transduced, in part, through activation of distinct PI3K isoforms. PI3Kα is activated by RTKs and PI3Kγ is downstream of GPCRs. PI3Kα consists of 2 subunits: the p85 regulatory subunit

and the p110 catalytic subunit. Containing 2 SH2 domains, p85 associates with activated RTKs through phospho-tyrosine residues, thus localizing PI3K to the membrane, resulting in activation of the p110 subunit(41,42). In addition, Ras is able to activate the p110 subunit independently of p85(43). This is important given the role of Ras as described above. As seen in Figure 2, catalytically activated PI3K phosphorylates the hydroxyl residue at the D-3 position of phosphoinositides residing in the membrane, most importantly PI 4,5-bisphosphate (PIP_2)(44). The resulting PI (3,4,5)-P_3, or PIP_3, recruits downstream effector molecules, including Rac, mTOR, PKC, and Akt, among others, ultimately inducing cell behavioral changes such as migration, protein synthesis, proliferation and survival.

As effector molecules, Rac, mTOR, PKC and Akt each further regulates a host of molecules to alter protein function or gene transcription and cellular responses. As Akt is one of the primary effector molecules of PI3K, I will discuss the role of this effector in promoting cell survival.

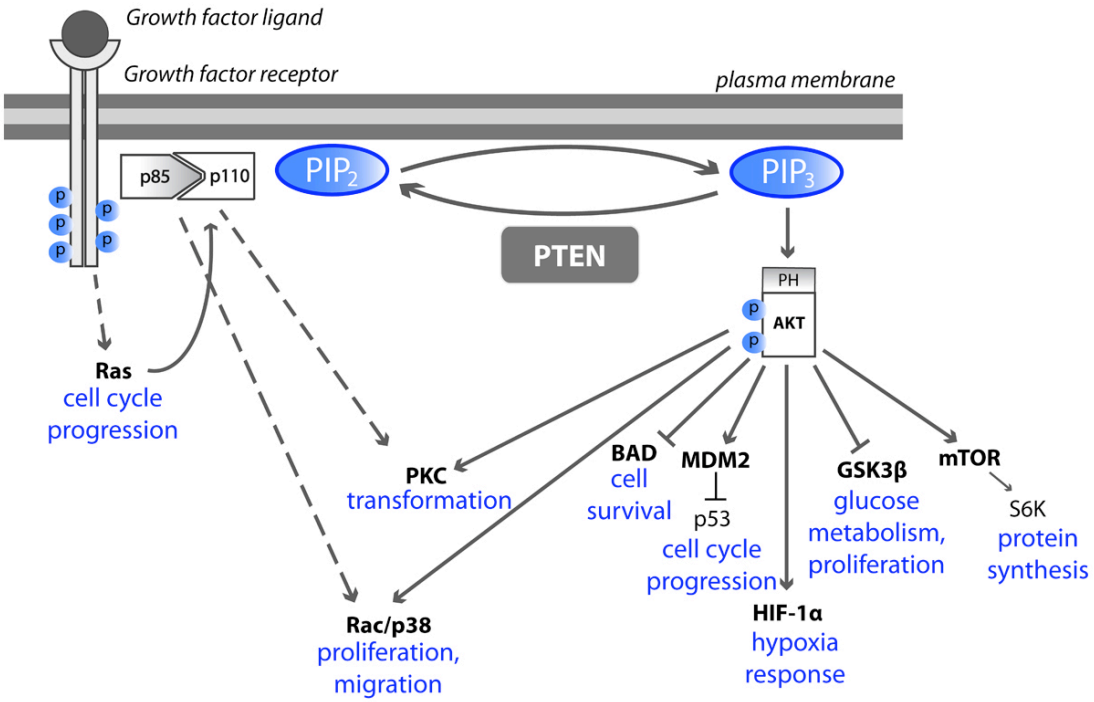

Figure 2: The PI3K/Akt signal transduction cascade

PI3Kα, which consists of the p85 and p110 subunits, is recruited to the plasma membrane by binding to phosphotyrosine residues on activated receptor tyrosine kinases, whereupon it is activated. Active PI3K phosphorylates its substrate, PIP_2, converting it to PIP_3. The lipid phosphatase PTEN, also resides at the membrane and directly antagonizes PI3K by removing the phosphate from the 3'-position of PIP_3, returning it to inactive PIP_2. PIP_3 is a potent second messenger and recruits Akt to the membrane through Akt's pleckstrin homology (PH) domain. Akt is then activated by phosphorylation on key serine and threonine residues. Akt phosphorylates many downstream targets, including PKC, Rac, BAD, MDM2, HIF-1α, GSK3β and mTOR. Through these effector molecules, Akt induces activation of a variety of cellular responses, including cell proliferation, survival and migration.

1.1.5 The Akt Effector Molecule

There are 3 isoforms of the serine-threonine kinase Akt (Akt1, Akt2, Akt3), all of which function downstream of PI3K. Akt1 is the principal isoform and will be referred to hereafter as Akt. Akt is recruited by its pleckstrin homology (PH) domain to the membrane where it interacts with phosphatidylinositides. Following activating phosphorylation events on threonine 308 and serine 473 by PDK1 and TORC2, respectively, Akt activates or inhibits many downstream molecules by phosphorylation of serine or threonine residues within the consensus motif: Arg-Xxx-Arg-Xxx-Xxx-Ser/Thr. For example, Akt phosphorylation inhibits the pro-apoptotic proteins Bad and Procaspase-9 as well as the Forkhead family of transcription factors, thereby inhibiting apoptosis and promoting cell proliferation(45). Akt also promotes survival by positively regulating IκB, which promotes stability of the survival factor NFκB(46). Thus, Akt has been shown to promote survival in virtually every cell type. Although Akt1 knockout mice are viable, they are more susceptible to genotoxic stress and have a shorter lifespan than their wild-type counterparts(47). The redundancy of Akt isoforms is made clear by the more dramatic phenotype of Akt1/Akt2 double knockout mice, which have a proliferation defect and die shortly after birth(48). Interestingly, another target of Akt is the E3 ubiquitin ligase for p53, MDM2. An activating phosphorylation of MDM2 results in a corresponding inactivation of p53. Thus, the balance between Akt and p53 activity is critical to the outcome of the cell: survival or apoptosis.

Other downstream targets of Akt affect diverse cellular processes, including cell cycle progression, metabolism and protein synthesis.

The G1/S transition of the cell cycle is also regulated by the PI3K/Akt pathway through the action of Myc and the D1 cyclins. Specifically, Akt-mediated activation of mTOR leads to the subsequent activation of p70 S6 kinase (S6K). One of the actions of S6K is to enhance Myc transcriptional activity by targeting a key antagonist of Myc, Md1, for degradation(49). Additionally, Akt has been shown to phosphorylate the Cdk-inhibitor proteins p21 and p27, rendering them unable to enter the nucleus to inhibit the formation of cyclin-Cdk complexes required to activate E2F. Consequently, active E2F promotes progression through the cell cycle. The robustness of Akt in promoting cell cycle progression is clearly seen in experiments utilizing constitutively active Akt, in which Akt is tethered to the plasma membrane by a lipid myristoyl (myr) group. Myr-Akt protects cells from undergoing apoptosis and is associated with rapid cell cycle progression and a transformed phenotype.

In addition to survival and cell cycle control, Akt modulates metabolism following alterations in glucose abundance. This is achieved by decreasing the activity of glycogen synthase kinase 3β (GSK3β) and increasing glucose transporters (GLUT-1 and -4) following Akt activation (Figure 2). Cells expressing increased levels of active Akt have been shown to have high levels of glucose consumption and lactate production, both key characteristics of tumor cells. This phenomenon is observed in cells expressing myr-Akt, although oxygen consumption is unaltered(50). Myc activity also feeds into the

metabolism of the cell, as it is a potent inducer of glycolytic genes such as LDH-A, phosphofructokinase and hexokinase 2. I will discuss tumor metabolism in more detail in later sections as it relates to the hypoxia response.

Protein synthesis is increased by Akt-mediated activation of mTOR and downstream S6K and the translational machinery component, eIF4E. As ribosomal binding is the limiting factor in protein synthesis, increasing the availability of eIF4E has dramatic effects on translational rates. If unregulated, however, expression of eIF4E can have deleterious effects, including transformation capabilities(51). Overall, the effects of Akt on inhibiting pro-apoptotic proteins, promoting cell cycle progression, adapting cellular metabolism and increasing protein synthesis combine to make Akt a dramatic promoter of cell growth and survival.

1.1.6 Dysregulation of Oncogenes and Tumor Suppressors in Cancer

As both the PI3K and MAPK signal transduction pathways are critical in maintaining proper cellular functions, dysregulation of these pathways at multiple levels results in a hyperplastic phenotype characteristic of cancer cells. Aberrant MAPK and PI3K pathways are frequently observed in cancer cells. As discussed, Ras, Myc and p53 are key regulators of each of these pathways, and as such, mutations in any of these regulatory proteins frequently leads to cancer. Thus, the functional consequences of dysregulation of these proteins in the tumor context will be addressed, and the role of an important lipid phosphatase, PTEN, as a master regulator of the PI3K pathway and its influence on each of these proteins will be discussed.

Dysregulation of the MAPK pathway classically occurs through point mutations in Ras that render it GAP-resistant. As Ras was originally discovered in the context of cancer, it is no surprise that ~30% of tumors carry a hyperactive mutant of Ras. Other mechanisms of Ras activation include inactivating or activating mutations in GAPs or GEFs, respectively. Constitutive activity of the Ras oncogene results in unrestricted signaling through the MAPK and PI3K pathways, resulting in dramatic cell proliferation and growth.

Myc is a potent transcription factor that is estimated to bind to promoter sequences in ~15% of all genes. Target genes of Myc are involved at virtually all points of transformation, including cell cycle control, protein synthesis, metabolism and adhesion. As a proto-oncogene, the Myc gene is typically overexpressed in tumor cells through chromosomal rearrangements or gene amplification. In the case of Burkitt's lymphoma, the Myc gene locus is translocated to chromosomes 2, 14 or 22 and it is juxtaposed with the promoter sequences for the immunoglobulins in ~80% of cases. These translocations result in the amplification of Myc, which may also contain an activating mutation at threonine 58, leading to a dramatic up-regulation of Myc activity.

Cell cycle regulatory proteins are also highly mutated in cancers. Because p53 has a tripartite role in apoptosis, genetic stability and the cell cycle, mutations of p53 are frequently observed in cancer. In fact, it is estimated that p53 is mutated in ~50% of tumors. Mutational analysis of p53 has uncovered "hot spots" along its 394 amino acid residue primary sequence. These highly mutated residues are amino acids 175, 248 and 273, which are within the DNA binding

domain of p53. Mutations at these sites render p53 unable to bind to the DNA of its target genes, such as p21 and PCNA. p53-null cells continue to cycle despite damaged DNA, resulting in genomic instability. Another mutation resulting in p53 inactivation includes hyperactivation of MDM2, as seen in some leukemias. The critical role of the MDM2-p53 interaction is reinforced by the observation that co-deletion of p53 rescues embryonic lethality in MDM2 knockout mice.

Likewise, aberrant or dysregulated PI3K signaling causes hyperproliferation of cells, as seen in both solid and non-solid tumors. This may be a result of activating mutations in PI3K itself or its downstream effectors, such as Akt or mTOR. In fact, Akt activity is frequently elevated in cancer and has been shown to be required for transformation of certain cell types(52). Interestingly, hyperactivation of Akt activity is generally a result of mutation of upstream proteins rather than direct mutations of Akt itself. For example, the enzyme PTEN, which directly antagonizes the action of PI3K and its downstream effectors, is frequently mutated in cancers. As PTEN is upstream of Akt, loss of its function results in a robust activation of the PI3K pathway through various effectors.

PTEN is ubiquitously expressed and is highly conserved across species, from *C. elegans* to humans. PTEN expression has been shown to correlate with decreases in the overall survival, proliferation and migration of many cell types, including endothelial cells, fibroblasts, embryonic stem cells, and others(53-56). Developmental studies have emphasized the growth inhibitory role of PTEN. In Drosophila eye development, PTEN regulates cell size, survival, and

proliferation(57). Knockdown of PTEN in zebrafish results in abnormalities in embryonic vasculogenesis and notochord formation, ostensibly due to improper cell proliferation and migration(58). In more clinical scenarios, PTEN loss and subsequent increases in Akt signaling are found in neointimal vascular smooth muscle cells(59). PTEN is clearly an important protein involved in maintaining proper PI3K/Akt pathway signaling and has dramatic implications in tumorigenesis, as will be discussed below.

Together, loss of tumor suppressor proteins such as p53 and PTEN or activation of the Myc or Ras oncogenes leads to dysregulated cell growth and division. By hydrolyzing 3-phosphoinositides, PTEN is a master regulator of the PI3K pathway and all pathways that impinge on it. As such, PTEN functions as a potent tumor suppressor protein.

1.2 PTEN as a Tumor Suppressor

Given the role of the PI3K pathway in cell growth, survival and proliferation, it is not surprising that in transformed cells the PI3K pathway is frequently dysregulated. It is estimated that the PI3K/Akt pathway is aberrantly regulated in ~40% of both colorectal and endometrial cancers. Dysregulation of the PI3K pathway can occur at various nodes, including hyperactivation of the proto-oncogenes Akt, mTOR, Ras or through PI3K itself. However, inhibition of the pathway antagonist, PTEN (also called MMAC1 and TEP1), results in a robust cell growth, survival, proliferation and migration phenotype, as it is upstream of many effector molecules.

The original identification of PTEN was made due to its notable absence in many solid tumors. In 1997, two groups independently reported that deletions or mutations within the PTEN coding region on chromosome 10(q23.3) resulted in a loss of heterozygosity of PTEN, occurring predominantly in late-stage tumors(60,61). As a direct antagonist of PI3K signaling, the dual lipid and protein phosphatase PTEN has remarkable anti-tumor properties. Although able to hydrolyze phosphates from both protein and lipid substrates, PTEN's primary target is the lipid second messenger PIP_3(62). As seen in Figure 2, PTEN removes the D-3 phosphate, converting PIP_3 to inactive PIP_2. Whereas PIP_3 levels are low in resting cells and are increased upon growth factor stimulation, PTEN activity restores them to resting levels.

Because PTEN is involved in regulation of the PI3K pathway in nearly every cell type, subtle changes in PTEN expression or activity can dramatically affect cell growth. Loss of a single PTEN allele gives rise to a haploinsufficiency phenotype, which manifests itself through an increased susceptibility to certain cancers. In fact, germ-line mutations of PTEN are observed in a number of autosomal dominant disorders, including Bannayan-Zonana and Cowden's Syndromes. It is estimated that ~80% of Cowden Syndrome patients have a germ-line mutation of PTEN. Clinically, these patients have an increased risk of developing breast, thyroid and endometrial cancers. Genetic studies investigating human subjects have determined the heritability of Cowden Syndrome and identified specific mutations within PTEN. In addition, genetically-manipulated animal models have been critical in defining the developmental and

pathological role of PTEN. PTEN knockout mice die early in embryonic development resulting from cellular hyperproliferation, while heterozygous mice are prone to spontaneous tumors, similar to Cowden Syndrome patients(63-65). However, more subtle changes in PTEN expression also have deleterious outcomes. Since complete knockout of the PTEN gene is embryonic lethal, graded PTEN expression was achieved in a mouse prostate tumor model utilizing heterozygous and/or hypomorphic alleles of PTEN(63,64). Using this approach, it was demonstrated that PTEN dose correlated with tumor progression(66).

Mutational analysis has revealed several "hotspots" along PTEN's 403 amino acid residue primary sequence. The most frequently mutated residue is Arg173, which is located in the active site of PTEN and is required for catalysis of phospholipids. However, PTEN is highly mutated throughout its sequence, resulting in many missense and nonsense forms of PTEN in various tumors.

Given that the re-expression of PTEN in tissue culture cells lacking PTEN rescues aberrant PI3K signaling, several studies have shown PTEN to be a potential therapeutic target. Direct injection of an adenovirus encoding PTEN into subcutaneous bladder tumors resulted in a reduction in tumor size through activation of apoptotic pathways(67). Another study showed some success in combining the delivery of PTEN with doxorubicin or radiation(68,69). These results are hampered by limitations of adenovirus-mediated gene therapy, such as side effects, distribution of the virus and duration of expression of the transgene. Other modes of PTEN gene therapy have not been pursued clinically.

The correlation between PTEN expression and hyperplasia has led to recent studies defining the regulation of the PTEN protein itself. To better understand the role of PTEN as a tumor suppressor, the biochemistry and stability of PTEN will be discussed.

1.3 PTEN Structure and Stability

Crystallization of PTEN in 1999 revealed a 179-residue N-terminal domain and a 166-residue C-terminal domain, as seen in Figure 3A. Within the C-tail of PTEN is located a C2 domain that interacts with the lipid membrane(70). The extreme C-terminus of PTEN was not originally crystallized with the protein, suggesting that it is not well-organized and can exist in a variety of configurations. Later two high-probability PEST domains as well as a PDZ binding motif were identified in this region, both of which are seemingly involved in protein stability(71,72). As mentioned above, further investigation into the mechanism of PTEN demonstrated that it is the lipid phosphatase activity, rather than its protein phosphatase activity, that is responsible for its ability to regulate tumor growth(73)

The active site for both lipid and protein phosphatase activities of PTEN consists of a P-loop made of residues 123-130 and a positively-charged pocket that binds the primary substrate of PTEN, the negatively charged phosphoinositides(70). Other surrounding residues (His123, Gly127 and Asp92) are also important for the catalytic activity of PTEN most notably Cys124, which mediates the phosphotransfer function of PTEN, and mutation of this residue abolishes PTEN's lipid phosphatase activity.

Although PTEN is encoded within every cell, it is not constitutively expressed or active. Moreover, regulation of PTEN is complicated and not completely understood. One of the earliest reports describing the discovery of PTEN termed the protein TEP1 (Tgf-β-regulated and epithelial cell-enriched phosphatase), as it was shown to be negatively regulated at the mRNA level following Tgf-β stimulation. PTEN transcription has since been shown to be increased by a number of transcription factors that bind directly to PTEN's upstream DNA sequences, including Egr-1 following UV exposure and PPARγ in inflammatory and transformed cells(74,75).

Several reports have presented anecdotal evidence of PTEN protein stabilization or loss in conjunction with or independent of changes in transcription. For example, BMP2 stimulation decreased PTEN degradation in breast cancer cells by altering PTEN's interaction with ubiquitin-conjugating enzymes(76). A direct interaction between MAGI-2 and the PDZ binding motif of PTEN was observed to stabilize PTEN protein and increase its lipid phosphatase activity(72,77). The earliest report of PTEN loss in a non-tumor context was in lung epithelial cells following Zn^{2+} exposure(78). This degradation of PTEN was accompanied by a loss in PTEN mRNA and was found to be proteasome-dependent. Another group demonstrated a loss of PTEN protein in vinculin-null cells, which are unable to form adherens junctions(79). As PTEN is known to scaffold directly and indirectly with structural components of adherens junctions (E-cadheren, β-catenin, MAGI-2), these data suggest that loss of this complex leads to subsequent loss of the stabilizing interaction of MAGI-2 and PTEN.

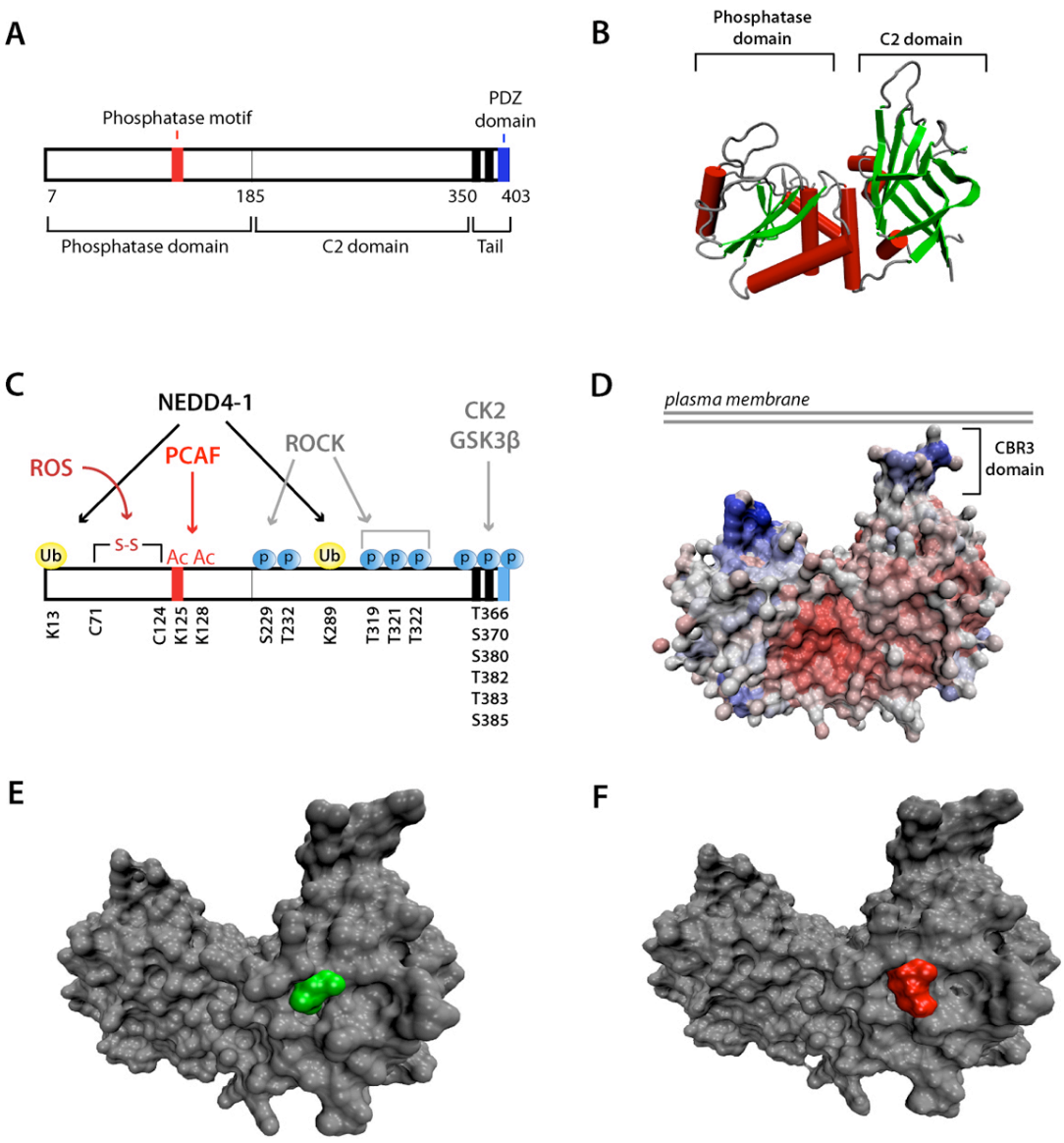

Figure 3: PTEN domain structure in linear, ribbon and 3-D representations

(A) A linear representation of the 403-amino acid residue primary structure of PTEN showing key structural domains. The catalytic site (phosphatase motif, red), PEST domains (black) and PDZ-binding motif (blue) are shown in relation to the phosphatase domain, C2 domain and C-tail of PTEN. (B) The secondary structure of PTEN consisting of alpha-helices (red) and beta-sheets (green) is shown. The protein is organized into 2 lobes: the phosphatase domain, which contains the active site, and the C2 domain, which localizes PTEN to the membrane. (C) Observed post-translational modifications within the PTEN protein are shown, including ubiquitination (yellow circles), disulfide bonds (black bracket), acetylation (Ac) and phosphorylation (blue circles). Proteins or molecules responsible for these modifications are noted above the diagram. (D) A 3-D surface rendering of PTEN depicting its orientation at the plasma membrane through its CBR3 domain. Coloring of the protein is based on the surface electrostatic potential, from positive (blue) to neutral (white) to negative (red). (E) WT PTEN showing location and accessibility of amino acid residue isoleucine 224 (green). (F) A predicted surface rendering of PTEN (performed by VMD software(1)) following mutation of Ile224 to Met (red), which is observed in some human cancers. The I224M mutation is predicted to alter the surface of the C2 domain, potentially resulting in disruption of important protein-protein interactions.

Lastly, a recent report has shown loss of PTEN protein in cardiomyocytes following myocardial ischemia(80). This loss was reversible following reperfusion and was also proteasome-dependent. The reversible nature of this degradation suggests that PTEN is a key player in the pathology of ischemia and may be important in other situations of low oxygen tension.

1.3.1 Effects of PTEN Post-translational Modification on Stability and Function

PTEN protein expression and stability are affected by a number of protein-protein interactions as well as transcription factors acting at the PTEN promoter. I will now discuss the post-translational modifications that impinge on PTEN stability and function, including phosphorylation, acetylation, ubiquitination and oxidation.

Investigation into the phosphorylation of PTEN has uncovered specific serine and threonine residues in both the C-tail and, more recently, the C2 domain that are phosphorylated by casein kinase 2 (CK2) and Rho kinase (ROCK), respectively (Figure 3C)(81,82). CK2 has been shown to specifically phosphorylate Ser370, Ser380, Thr382, Thr383 and Ser385. Although the function of these phosphorylation events is not completely understood, it is currently believed that phosphorylation of the PTEN C-tail serves to stabilize PTEN in an inactive conformation, preventing the free C2 domain from interacting with the plasma membrane. Additionally, phosphorylation of these residues may inhibit caspase-3-directed cleavage, while phosphorylation of the C2 domain may affect PTEN subcellular localization and migration of the cell(83,84). Recently, GSK3β was found to phosphorylate PTEN at Thr366, causing destabilization(85). The

phosphatase(s) that target phosphoserine/-threonine residues in PTEN remain unknown. It has been suggested that PTEN, itself, may act as the protein phosphatase, although PTEN's protein phosphatase activity is preferentially targeted toward phosphotyrosine. Phosphorylation of the C-tail of PTEN has also been shown to be elevated when PTEN is bound to PDZ domain-containing proteins (e.g., MAGI-2, hDlg, MAST205)(77). It is unclear if the stabilizing effect that these interactions seem to have on PTEN is a result of the increased phosphorylation of the C-tail of PTEN or its presence in these complexes, alone.

Several studies have demonstrated acetylation of PTEN (Figure 3C). The earliest report documented Lys125 and Lys128 acetylation of PTEN by PCAF(86). Acetylation at these active site residues decreases PTEN activity by diminishing the positive charge of lysine residues and, thus, the affinity for negatively charged PIP3. Cells underwent growth arrest when this acetylation was relieved following PCAF silencing and subsequent PTEN deacetylation. More recently, Lys402 was shown to be acetylated by CBP and deacetylated by SIRT1(87). Because this residue is located in the PDZ binding motif, scaffolding of other proteins is affected. For example, acetylation of Lys402 increases the binding affinity for other PDZ domain-containing proteins, including MAGI-2 and -3, which may alter the biologic function of PTEN(87). Thus, PTEN activity seems to be both positively and negatively regulated by acetylation.

As described previously, PTEN is degraded via the 26S proteasome. This pathway was further defined by Wang et al., who identified NEDD4-1 as the ubiquitin ligase responsible for polyubiquitinating PTEN prior to its degradation

by the 26S proteasome (Figure 3C) (88). The same group further demonstrated that monoubiquitination of PTEN stabilizes the protein and is required for nuclear import of PTEN(89). This is relevant to pathologies that show a differential localization of PTEN. For example, several mutations that inhibit PTEN from entering the nucleus have been observed in patients with Cowden Syndrome(89). Additionally, analysis of thyroid tumors, melanoma and colorectal carcinoma has shown a correlation between decreased nuclear PTEN and increased cancer progression(90-92). Thus, modifications of PTEN may have dramatic effects on tumor suppression through regulation of its compartmentalization.

PTEN has been shown to be oxidized following H_2O_2 treatment, resulting in a loss of lipid phosphatase activity(93). This oxidation was reversible, apparently as a result of thioredoxin-mediated reduction, as thioredoxin co-immunoprecipitated with PTEN. Following H_2O_2 treatment, a disulfide bond is formed between the critical active site residue Cys124 and Cys71 of PTEN, rendering PTEN unable to hydrolyze PIP3 and leading to downstream activation of PI3K pathway effector proteins (Figure 3C). Reactive oxygen species (ROS)-mediated inactivation of PTEN is significant, as H_2O_2 is a natural by-product of growth factor signaling and mitochondrial metabolism. Additionally, cells are exposed to various ROS both inside and outside the cell during times of oxidative stress, such as ischemia or hypoxia(93).

As this discussion demonstrates, loss of PTEN function occurs through a variety of mechanisms, including transcriptional repression, protein degradation,

protein-protein interactions, changes in subcellular localization and post-translational modifications, and these changes are characteristic of the development of many tumors. However, PTEN also plays a critical role in untransformed cells. In the following section I will discuss the various roles of PTEN in a non-malignant framework. Both PI3K-dependent and -independent roles of PTEN will be discussed.

1.4 Physiological Roles of PTEN

Because PI3K signaling hinges on PTEN function, PTEN activity has direct effects on PI3K-mediated cell behaviors, such as cell growth, proliferation, survival, and migration. However, increasing evidence has pointed to additional roles of PTEN in the nucleus, outside of its classical role as a negative regulator of PI3K at the plasma membrane. From crystallization and structural studies, the interaction between the CBR3 domain of PTEN and the plasma membrane has been defined (Figure 3D). It remains unclear, however, whether PTEN in the nucleus is associated with the nuclear membrane. As discussed previously, compartmentalization may be an important factor in the regulation of key protein-protein interactions with PTEN and its subsequent stability and function. Original observations of nuclear PTEN were initially thought to be artifactual due to the crude techniques used to separate cytoplasmic and nuclear proteins and the notable absence of a canonical nuclear localization signal within PTEN's coding sequence. Only after more sophisticated cell fractionation studies were performed did it become clear that PTEN was a legitimate nuclear protein. However, the question of how PTEN gained nuclear entry remained, and several

studies have proposed mechanisms to explain this phenomenon. These include diffusion or active transport by either MVP or RAN(94-96), and post-translational modifications such as phosphorylation and mono-ubiquitination have also been implicated in PTEN nuclear localization(89,95). Further analysis of PTEN's sequence has led to the identification of 4 non-canonical nuclear localization signals found throughout its coding region(96). When at least two of these sites are mutated, PTEN nuclear localization is decreased. Interestingly, a cytoplasmic localization signal was also discovered in the N-terminus of PTEN(97), and mutation of this sequence leads to an increase in nuclear PTEN, although the mechanism is not completely understood. Regardless of how PTEN enters the nucleus, it appears that PTEN has distinct functions depending on its sub-cellular locale. A role for PTEN in the nucleus could be inferred based on its effects on regulating chromosomal stability, DNA repair, cell cycle control, p53 stability and apoptosis(98-100).

The role of PTEN in maintaining chromosomal stability was first suggested from fluorescence *in situ* hybridization (FISH) analyses of PTEN-null mouse embryonic fibroblasts (MEFs), which showed a striking number of chromosomal fragments and translocations(98). A protein-protein interaction between PTEN and CENP-C, a protein found in the kinetochore required for chromosomal stability, was found to be critical for PTEN's role in regulating chromosomal stability. Thus, expression of a truncated form of PTEN inhibited this association and resulted in a loss of chromosomal stability. Interestingly, PTEN phosphatase activity was not required for its stabilizing effect on

chromosomes. This recently uncovered function of nuclear PTEN further underscores its role as a tumor suppressor and is complementary to its lipid phosphatase role in the cytoplasm. Another important protein interaction of nuclear PTEN is with p53, which will be discussed in detail later and may also contribute to PTEN's ability to protect the genome, albeit indirectly.

The presence of chromosome fragments in PTEN-null nuclei led researchers to further probe the role of PTEN in DNA repair. In addition to broken chromosomes, PTEN-null nuclei also showed an increase in DNA repair proteins associating with double-strand breaks(98). These double-strand breaks seemed to occur spontaneously in PTEN-deficient cells. The mechanism was uncovered when a reciprocal relationship was found between PTEN expression and transcription of Rad51, a key player in protecting the cell against double-strand breaks. Notably, PTEN was localized to the Rad51 promoter, where it apparently inhibited Rad51 transcription. However, it is unclear if this function of PTEN is dependent on its catalytic activity.

Early evidence linking nuclear PTEN to mitotic index in the progression of various tumors was validated with the observation of a cyclical expression pattern of nuclear PTEN. PTEN was shown to be localized to the nucleus in G0-G1 and more in the cytoplasm during S phase. This finding, together with early evidence that PTEN was excluded from the nuclei of transformed cells, is consistent with a constantly cycling cell and one of the hallmarks of cancer: "insensitivity to anti-growth signals"(101,102). PTEN has long been known to induce cell cycle arrest at the G1 checkpoint, ostensibly through its inhibition of

the PI3K pathway. This role has been further defined in PTEN-null U87 tumor cells, in which re-expression of PTEN led to cell cycle arrest by limiting the nuclear accumulation of cyclin D1(99). The lipid phosphatase activity of PTEN was required for this effect. A separate effect of PTEN on the G2 checkpoint protein, CHK1, also links PTEN to cell cycle control, DNA damage repair and apoptosis. Like p53, CHK1 is stabilized following DNA damage, where it degrades multiple cyclin-Cdk complexes and halts cell cycle progression. CHK1 activity is negatively regulated by Akt, thus depletion of functional PTEN results in loss of CHK1-dependent regulation of the cell cycle via Akt-mediated phosphorylation. Therefore, PTEN functions in multiple capacities and subcellular compartments to regulate the cell cycle.

p53 is often referred to as the "guardian of the genome." This designation is in large part due to its role in cell cycle checkpoint control. The interactions between PTEN and p53 are complex, each appearing to positively regulate the other's stability or expression. In addition to PTEN's indirect role in p53 stability through Akt-mediated phosphorylation of MDM2, PTEN has been shown to directly associate with p53 in the nucleus(100). This interaction increased p53 protein expression and transcriptional activity, and was not dependent on PTEN phosphatase activity, although it did require the C2 domain of PTEN. A follow-up study extended these findings by implicating the histone acetyl transferase (HAT) p300 in PTEN's modulation of p53 activity(103). As p300 has been shown to interact with both PTEN and p53, it is not surprising that all three proteins may complex together. PTEN overexpression resulted in an increase in p53

acetylation and stabilization via p300. Interestingly, activation of p53 was independent of PTEN's phosphatase activity. The formation of the PTEN-p300 complex occurred in the nucleus and was required for p53 stabilization. Based on these findings, it was suggested p53 acetylation and tetramerization facilitates PTEN binding to p53, which promotes continued p53 acetylation via p300, ultimately increasing p53 stabilization. These findings provide strong evidence for non-canonical, nuclear-specific roles for PTEN.

In addition to PTEN's role in promoting apoptosis through Akt blockade, PTEN seems to also have direct effects on cell survival. Although results have been conflicting, it is unclear whether predominantly nuclear or cytoplasmic PTEN is a promoter of apoptosis. Cell type, stress, and presence of other proteins may account for these discrepancies. Despite the controversy, the role of PTEN in apoptosis is another example of the importance of subtle changes in PTEN localization. A study showing that nuclear PTEN induced apoptosis in U87 glioma cells found this phenomenon to be dependent on its catalytic activity, suggesting some lipid phosphatase activity in the nucleus. In fact, Akt as well as PI3K and its lipid substrates have all been observed in the nucleus, insinuating that canonical PI3K pathway activity occurs within the nucleus (reviewed in Neri, et al.)(104). The mechanism by which the nuclear PI3K pathway is activated given the absence of RTKs in the nuclear membrane remains unknown. It is becoming clear, though, that many components of PI3K signaling, including PTEN, have nuclear-specific roles in maintaining normal cell function. Although these functions of PTEN occur within individual cells, the summation of these

discrete events results in global changes in tissue growth or survival. This is most evident in tumors where PTEN expression or function is frequently altered. One of the hallmarks of tumors that is intimately tied to several of PTEN's known functions is the ability of tumors to maintain sustained angiogenesis(102). Initially a vascularly quiescent tissue, it is hypothesized that the tumor acquires an angiogenic phenotype in a step-wise fashion, by shifting the balance of expression from angiogenic inhibitors (e.g. Thrombospondin-1) to activators (e.g. VEGF, FGF). Although the kinetics are unknown and likely tumor-specific, temporal regulation of the angiogenic switch is expected to be affected by selection pressure from hypoxic microenvironments. Thus, the role of PTEN in hypoxia is critical to its global role in tumor biology.

1.5 Tissue Responses to Hypoxia

Adult tissues require oxygen for aerobic respiration. The delivery of oxygen to body tissues is influenced by a host of variables, an important one of which is the partial pressure of O_2 (pO_2). pO_2 is calculated as the percentage of inspired O_2 multiplied by the barometric pressure (mmHg) (pO_2^{elevY}=%O_2 x barometric pressure^{elevY}). Thus, the pO_2 of ambient air (21% O_2) at atmospheric pressure at sea level (760 mmHg) is 159 mmHg (0.21 x 760 mmHg=159 mmHg).

Remarkably, eukaryotic systems are able to survive across environmental extremes of inspired pO_2. Humans, for example, can survive at altitudes extending from sea level to 4000 m in the Himalayan Mountains where Tibetans have lived for centuries. Animals are also highly adaptive to hypoxic niches, whether at high-altitude or below sea level. A notable example is the

subterranean mole rat (*Spalax ehrenbergi*), which survives underground in a hypoxic environment ~5% O_2 (pO_2 ~38 mmHg). The molecular modifications underlying these adaptations will be discussed in detail below.

In addition to pO_2, another important determinant of oxygen delivery is the diffusion distance of O_2 from the capillary network. Although each tissue has distinct oxygen and metabolic needs, this distance remains essentially constant (~100 μm). It is estimated that most tissues are subjected to pO_2 of at least 20 mmHg, as it has been observed that pO_2 lower than 10 mmHg results in induction of hypoxia inducible factor (HIF)-1α binding to target DNA sequences(105,106). In this discussion, hypoxia will be defined as ~1-2% O_2 (pO_2 ~7 mmHg), and normoxia will be defined as ~21% O_2 (>3% O_2 = pO_2 of >20 mmHg).

Generally, normal adult tissues function under normoxic conditions, although there are a few notable exceptions. Specific regions of the thymus and bone marrow are very hypoxic (5-10 mmHg), which may facilitate maintenance of a less differentiated phenotype of immature T cells and hematopoietic stem cells, respectively(107-109). These cells have developed a glycolytic metabolism in order to survive such conditions, thus potentially minimizing the production of ROS, which are byproducts of oxidative phosphorylation. Studies using 2-nitroimidazole drugs, which form adducts with proteins and DNA at <2% O_2, have led to an increase in the understanding of the role of hypoxia in developmental, physiological and pathophysiological conditions(110-113). For example, it is now estimated that the human embryo develops in an

environment of 1-5% O_2 (pO_2 0.5-30 mmHg)(114). The subsequent expression of HIF-1α in the embryo is required for the formation of many tissues and organs, including the heart, the placenta and the vasculature, among others. HIF-1α is also required for differentiation and migration of key cellular compartments, including trophoblasts and neural crest cells (reviewed by Dunwoodie(115)). Although the developing embryo is considered to be chronically hypoxic before complete development of the vasculature, intermittently or acutely hypoxic microenvironments may be more physiologically relevant to adult tissues and pathologies.

More precise methods for measuring tissue oxygenation have revealed the cellular microenvironment to be quite heterogeneous. Complex measurements using a combination of oxygen probes, dyes and microscopy in both normal and tumor tissues have demonstrated cyclical hypoxia of variable duration, from minutes to several hours. As reviewed by Dewhirst et al., tissue oxygenation is simply a balance between oxygen availability and tissue need. Factors such as diffusion distance, the shape and geometry of the blood vessels and flux of red blood cells all determine how well that need is met(116). These cycles of intermittent hypoxia seem to correlate with red cell flux, as tissue pO_2 itself positively correlates with red cell flux(117). Inconsistent red cell flux may be due to ongoing vascular remodeling, intermittent claudication or changes in arterial pressure or oxygen-carrying capacity of the blood (hypoxemia).

Tissue responsiveness to hypoxia must be robust and rapid given the dynamic nature of oxygen availability. Although the response to hypoxia may

be tissue-type specific, there are several general outcomes following a decrease in oxygen tension. In hypoxemic hypoxia, all tissues are affected due to the global decrease in oxygen in the blood. At high altitudes, for example, the decreased availability of O_2 is offset by a reduction in the affinity of hemoglobin for oxygen, thereby increasing the delivery efficiency of available oxygen. Hypoxemia results in a series of physiological/molecular changes that result in increased production of red blood cells and hemoglobin to increase the hematocrit, and sprouting of capillaries from existing blood vessels to extend the blood supply. Heart rate and breathing also increase to improve O_2 delivery. Athletes commonly train at high altitudes to increase their performance by manipulating this natural process of increasing their oxygen delivery. Cellular energy production mechanisms quickly adapt to hypoxia by switching from oxidative to glycolytic metabolism and conserving ATP where possible, often by halting protein, DNA and mRNA synthesis. If the hypoxic stress is too severe, cellular necrosis and apoptosis occur, eventually resulting in organ failure. The brain is particularly susceptible to hypoxia given its large energy demands.

Hypoxia may also be limited to specific tissues as in ischemic diseases caused by atherosclerotic arterial obstruction, such as coronary artery disease and peripheral artery disease. These conditions affect a variety of cell types, including endothelial and vascular smooth muscle cells, skeletal myocytes, and cardiac myocytes. Data from our lab have shown that endothelial cells are more resistant to oxygen deprivation than skeletal myocytes, which may maintain tissue perfusion to preserve surrounding, more susceptible tissues during

hypoxia. Endothelial cells (ECs) subjected to hypoxia rapidly release growth factors and inflammatory cytokines that not only protect the endothelial cell but also recruit inflammatory cells. Intercellular adhesion molecule (ICAM)-1 and other molecules specific to neutrophil attachment are increased on the endothelial cell surface(118-120). Pulmonary blood vessels constrict in hypoxic conditions, resulting in increased blood flow velocity, while systemically, vessels vasodilate to improve perfusion. Additionally, vascular smooth muscle cells (VSMC) may respond to hypoxia through increased proliferation and migration, resulting in intimal hyperplasia, or thickening of the muscular layer of the arterial wall (121). However, this VSMC response is thought to be mediated by ECs through secretion of various soluble growth factors(122). Survival of VSMCs is also prolonged in hypoxia following activation of the hypoxia-responsive gene telomerase reverse transcriptase (TERT)(123).

Studies in skeletal muscle have shown substantial decreases in basal expression of the primary modulator of the hypoxia response, HIF-1α, suggesting a more global role for HIF in regulating skeletal myocyte homeostasis. HIF-1α is still stabilized in hypoxic skeletal muscle, although to a lower extent compared to other cell types. However, a recent study in skeletal muscle has shown that peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α is able to induce a number of hypoxia-responsive genes through HIF-1α stabilization, even in the setting of normoxia(124). Thus, PGC-1α may be relevant to hypoxia-dependent gene regulation in skeletal muscle. As exercise results in a transient hypoxic microenvironment, some researchers have

studied hypoxia in skeletal muscle through exercise studies. Although this hypoxic stimulus resulting from exercise is quite different from that occurring in ischemic vascular diseases, exercise induces hypoxia in the skeletal muscle sufficient to induce capillary growth and mitochondrial biogenesis. Evidence for metabolic products involved in HIF stabilization (succinate, fumarate) suggests that although HIF is activated in exercise, it may be through a non-hypoxic mechanism. However, HIF is required for capillary and metabolic changes in skeletal muscle, as deletion of HIF-1α specifically in the skeletal muscle of mice resulted in an inability to shift to a glycolytic metabolism during exercise, leading to increased muscle damage and decreased endurance capacity in these animals(125). The role of HIF in maintaining metabolism seems to be a key function of HIF in the skeletal muscle, one of the largest tissues for glucose storage in the body.

Other pathologies resulting in hypoxic microenvironments include stroke, myocardial infarction and tumors. Regardless of whether the source of hypoxia is environmental or pathological, inadequate oxygen delivery to the tissues elicits a cascade of events in order to relieve the effects of hypoxia. The degree of hypoxia required to initiate these events may be cell-type specific, although the hypoxia program itself is ubiquitous and is responsible for hypoxia-responsive signal transduction pathways and target gene activation. In the next section, I will discuss the molecular mechanisms underlying hypoxia-adaptive responses.

1.6 Hypoxic Signal Transduction, Co-activators and Target-gene Expression

The survival of cells in hypoxic microenvironments requires induction of specific genes that adapt the cellular metabolism while simultaneously acting to improve oxygen supply by increasing blood flow and oxygen-carrying capacity of the existing capillary network. As small hydrophobic molecules, O_2 and CO_2 freely diffuse across the lipid bilayer. Thus, changes in extracellular tissue oxygen tension are immediately reflected intracellularly. The mechanisms by which cells are able to sense changes in O_2 tension are complex and may not be completely defined, although our understanding of this process has greatly improved over the last 20 years with the discovery of the hypoxia inducible factor pathway and elucidation of its regulatory mechanisms. The HIF pathway represents the chief mechanism of cellular oxygen sensing, and an understanding of this pathway is critical to any investigation of cellular responses to hypoxia.

For many years it was known that exposure to low oxygen levels at high altitudes led to an increase in the hematocrit or percentage of red blood cells in the serum, although it was not until the discovery of HIF-1α that a molecular link was made between oxygen concentration and induction of erythropoiesis. Discovered by Wang and Semenza in 1992, HIF-1α is the primary oxygen sensor in the cell, and is found in every cell type in the body, and is required for normal vascular development(106). HIF-1α-null embryos are hypoxic and die by embryonic day (E) 10.5 due to vascular malformations, although morphological differences are seen as early as E8(111,126). HIF-1α is part of a heterodimeric transcription factor complex that potently regulates expression of a large

number of hypoxia-responsive genes. HIF-1α's oxygen sensing ability lies in an exquisite oxygen-dependent proteolytic mechanism that results in its degradation within minutes in the presence of oxygen tensions above 5%. HIF-1α is constitutively expressed at both the mRNA and protein levels, and in the absence of oxygen HIF-1α can be stabilized and thus regulate the cellular hypoxia response.

The HIF-α proteins consist of HIF-1α and 2 other known isoforms, HIF-2α and HIF-3α. Unlike HIF-1α and -2α, HIF-3α has no known role as a transcription factor, but has exhibited some dominant negative behavior to suppress HIF signaling(127). A closer look at the domain structure of the HIF-α proteins shown in Figure 4 reveals several domains. Two oxygen-dependent degradation domains (ODDD) at the N- (N-ODDD) and C-terminus (C-ODDD) of HIF-α regulate its stability as described further below(128,129). In HIF-1α these domains contain 2 key proline residues (Pro402 and Pro564) that are hydroxylated in the presence of oxygen. This hydroxylation is performed by prolyl hydroxylases (PHD) 1-3. As diagrammed in Figure 5, prolyl hydroxylation and acetylation of Lys582 allows binding of the E3 ubiquitin ligase von Hippel-Lindau (VHL) protein to HIF-α leading to polyubiquitination and subsequent degradation by the 26S proteasome(130,131). The association between VHL and HIF-α occurs at each of the ODDDs (N-ODDD and C-ODDD), independent of one another(132). VHL acts as a tumor suppressor protein and is crucial to proper HIF signaling. Germ-line mutations in VHL are the cause of von Hippel-Lindau syndrome, in which unregulated HIF signaling results in a predisposition

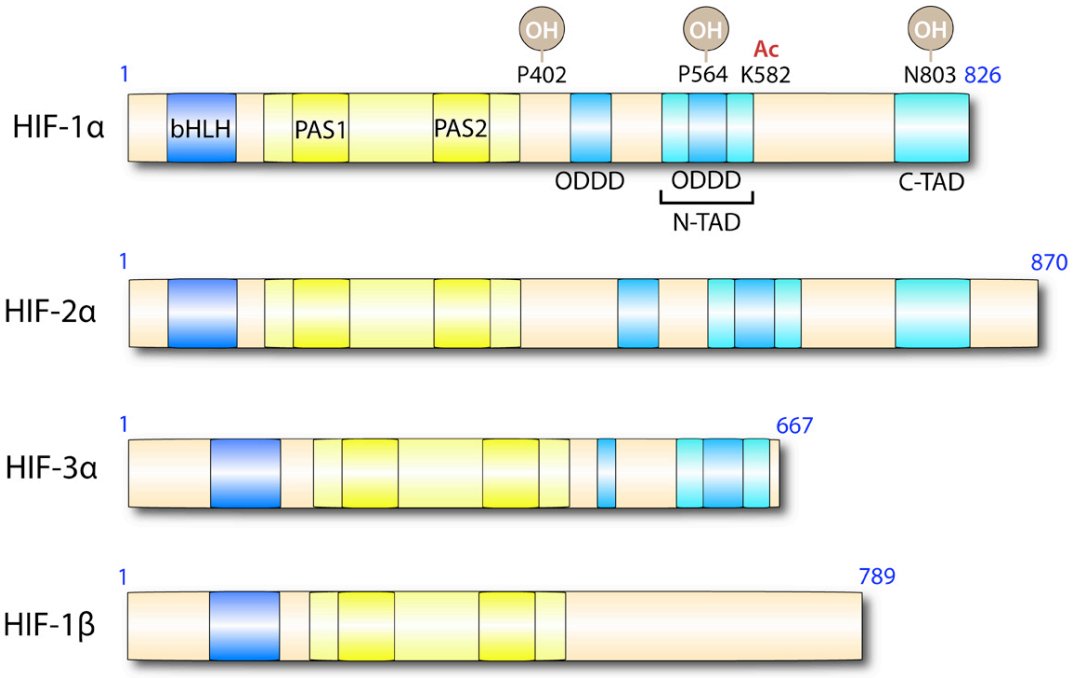

Figure 4: Domain structures and key amino acid residues of HIF proteins

The HIF proteins each contain a basic helix-loop-helix motif (bHLH) and PAS domains, which facilitate DNA-binding and heterodimerization, respectively. Oxygen regulation of HIF-α stability is mediated by hydroxylation (OH) of key proline residues within and outside of the oxygen-dependent degradation domains (ODDDs) as well as acetylation (Ac) of Lys582. HIF-1β is constitutively expressed and the protein is stabilized due to its lack of ODDDs. Transactivation domains (TAD) are important in regulating protein stability and serve as a binding site for co-activators, including p300.

to many tumors, particularly renal carcinomas, which are classically associated with impaired VHL function(133).

Two transactivation domains (TAD) are found at the N- (N-TAD) and C-terminus (C-TAD) of HIF-1α and -2α. While the ODDDs function to regulate HIF-α stability, the C-TAD binds the transcriptional co-activator p300, which is important for regulating HIF-α activity(134). However, hydroxylation of Asn803 in the C-TAD by asparagine hydroxylases, such as factor inhibiting HIF-1 (FIH)-1, inhibits p300 binding in the presence of oxygen(135-137). Interestingly, HIF-3α is truncated and does not contain the C-TAD domain, hence its characterization as a potential inhibitor of HIF-α activity.

Stabilized, active HIF-α translocates to the nucleus by virtue of two nuclear localization sequences, one of which is in the C-TAD, whereupon it heterodimerizes with the constitutively expressed HIF-1β protein. As seen in Figure 4, HIF-α and HIF-1β both contain basic helix-loop-helix (bHLH) domains as well as two PER-ARNT-SIM (PAS1 and PAS2) domains, both of which are critical for heterodimerization with HIF-1β. These proteins, along with other co-activators, activate gene transcription by complexing at hypoxia response elements (HRE) containing the core consensus site ACGTG. As hundreds of genes are known to contain HREs upstream of their promoters, HIF stabilization and target gene activation affects many diverse cellular processes. Interestingly, the components of the heterodimer may lend some specificity to which genes are activated, such that HIF-1α/HIF-1β and HIF-2α/HIF-1β heterodimers may activate overlapping genes (EPO, VEGF), but they may also have exclusive

targets. HIF-mediated gene transcription has been shown to be dependent on the N-TAD domain as well as a region of the C-terminus that regulates transcriptional activation following DNA binding(138,139). HIF-2α/HIF-1β has been shown to activate Tgf-α, lysyl oxidase (LOX), Octamer-4 (Oct4), and cyclin D1, while activation of PGK-1, LDH-A and GLUT-1 have been suggested to be HIF-1α/HIF-1β-dependent. This is an important distinction, as various tissues may differ in the expression of the individual components of the hypoxia regulatory machinery, including PHDs, coactivators or the HIF transcription factors themselves. HIF-2α is nonuniformly expressed and was originally identified as endothelial PAS-1 (EPAS1) in 1997 due to its high expression in the endothelium of embryos(140). Since that time, however, HIF-2α expression has been found in many other cell types and tissues, including kidney, heart, brain, pancreas and intestine, among others(141). Although HIF-1α and HIF-2α are 48% homologous overall and their bHLH, PAS and TAD domains are highly homologous (> 80%), it is currently unclear whether their functions are complementary or redundant.

The recruitment of transcriptional repressors (DEC1, DEC2, ZEB2) or activators (NEMO, p300/CREB binding protein (CBP), ZEB1, Ets-1, Smad3, ELK-1, TIF2, STAT3) to the HIF complex also dictates the activity and specificity of the HIF transcription factors, although repressors and activators may be gene-specific. Investigations into the inhibiting effects of FIH-1 on HIF transcriptional activity have determined that binding of the co-activator p300 and FIH-1 to HIF-1α are mutually exclusive(142). Thus, p300/CBP recruitment to the HIF complex

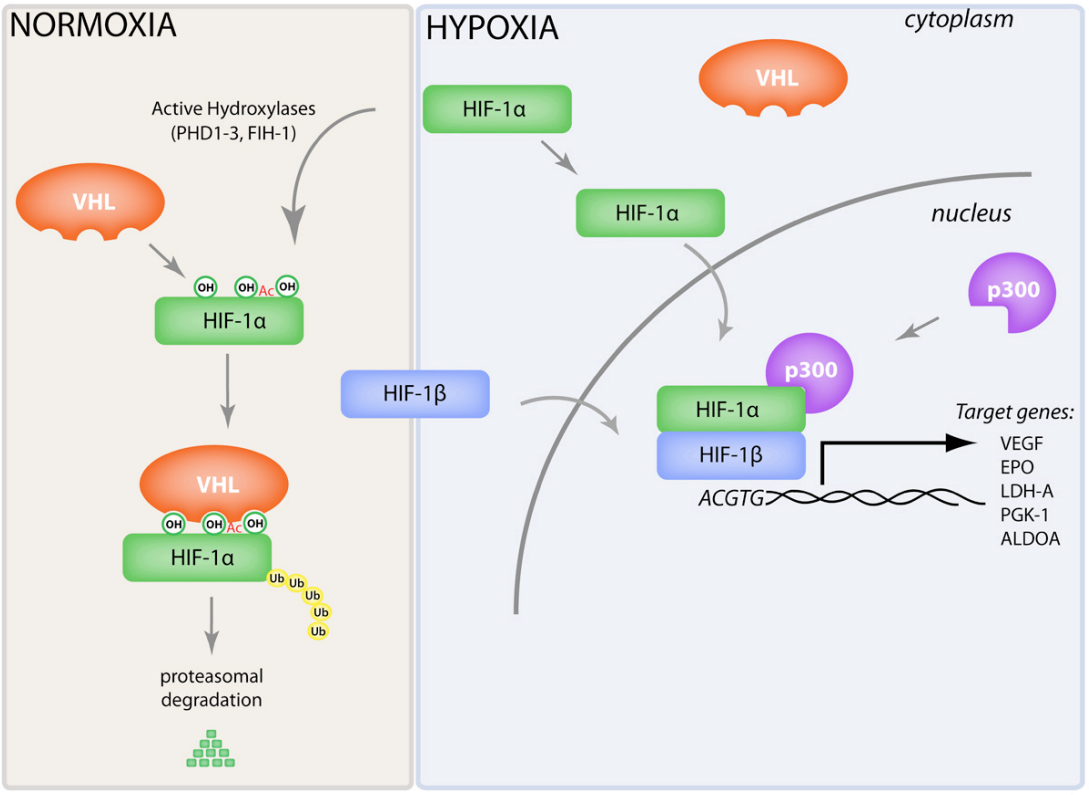

Figure 5: Oxygen-dependent HIF degradation and signaling

In normoxia (~21% O_2), HIF-1α is rapidly degraded following proline and asparagine hydroxylation, as well as acetylation of its C-tail. An E3 ubiquitin ligase, the von Hippel-Lindau (VHL) protein, binds to hydroxylated HIF-1α and polyubiquitinates it, leading to HIF-1α degradation by the 26S proteasome. In hypoxia (~1-2% O_2), however, hydroxylases are inactive, thus leaving HIF-1α unmodified and unable to bind to VHL. HIF-1α protein is stabilized and translocates to the nucleus where it heterodimerizes with the constitutively expressed HIF-1β. The co-activator p300 binds to HIF-1α in the absence of Asn803 hydroxylation and activates transcription of genes containing the HRE consensus sequence (ACGTG) in their promoter regions.

is required for hypoxic gene expression, suggesting that it is a global co-activator of HIF signaling.

Although p300 and CBP are paralogs with high sequence homology and many overlapping functions, they are not completely redundant. For the sake of this discussion, I will focus primarily on p300. The p300 histone acetyltransferase interacts with hundreds of transcription factors in addition to HIF-α, including p53 and CITED2. With many of these transcription factors competing for p300 binding, p300 itself may be a limiting factor in the activity of HIF and other transcription factors. For example, increases in both p53 and CITED2 transcriptional activity due to p300 binding have shown corresponding decreases in HIF transcriptional activity(143,144). p300 may elicit activation of transcription factors through different mechanisms, including direct acetylation of chromatin or transcription factors (by p300 itself or other associated HATs) or scaffolding of other transcription factors at the promoter. Scaffolding by definition increases the local concentration of proteins (in this case, transcription factors) on the DNA increasing the likelihood of interactions. Regardless of its mechanism, p300 is clearly required for many important cellular processes, including cell cycle progression and differentiation, as p300-null embryos die at E10.5 due to incomplete closure of the neural tube and heart(145).

The structure of the p300 gene contains 3 cysteine/histidine-rich (CH1, CH2, CH3) domains, a HAT domain, a bromodomain, a KIX domain and a glutamine-rich domain. The combination of these domains results in the remarkable ability of p300 to scaffold with various proteins in a cell-type or

context-specific manner. The C-TAD and N-TAD of HIF-1α bind to the CH1 and CH3 domains of p300, respectively, while p53 binds to several domains within p300, including the KIX and glutamine-rich domains(146-148). Interestingly, the interacting domains between p300 and another binding partner described above, PTEN, are unknown.

Normally, p300 is expressed in the nucleus where it seems to be regulated by a number of kinases. The cyclin-dependent kinases CDK2 and CDC2 have been suggested to phosphorylate p300, although the functional consequences of this phosphorylation are unclear(149). More recently, p38-mediated phosphorylation of p300 has been shown to result in p300 degradation(150). p300 is also regulated by other post-translational modifications including sumolation, methylation and acetylation(151-153). Thus, p300 activity is modulated by the quantity (expression) as well as the quality (post-translational modifications) of p300 in the cell. As reviewed by Ghosh and Varga, several proteins and chemicals may affect p300 expression and activity(154). Assuming that p300 abundance is limited, the regulation of p300 stability is critical for transcription of hypoxia-regulated genes, since HIF transcriptional activity relies on p300 co-activation.

Once a functional HIF-α/HIF-1β heterodimer is formed in the nucleus and combines with appropriate co-activators, transcription of HRE-containing target genes is initiated. Currently, 1-5% of genes in the human genome are estimated to contain HREs in their promoters. As might be expected based on the physiological response of cells to hypoxia, HIF-regulated genes modulate a

number of important cellular behaviors, including metabolism, angiogenesis, erythropoiesis, vasodilation, proliferation, survival, migration, and adhesion, among others.

1.7 The Hypoxia Response in Tumors

Generally speaking, tumors show an increase in HIF-α transcriptional activity compared to non-transformed cells. Stabilization of HIF proteins may occur through hyperactivation of cell growth pathways (PI3K/Akt) or loss-of-function mutations in regulatory proteins, such as VHL or PHDs, or mutation of the HIF ODDDs. An increased hypoxia response typical of tumors results in increased target gene expression and has been correlated with a poor prognosis, as reviewed previously(155). Hypoxic tumors, or those with stabilized HIF expression even in normoxia, are considered to have an aggressive phenotype, as they are more migratory, less likely to undergo apoptosis, more glycolytic and highly angiogenic due to the induction of LOX, BNIP3, LDH-A and VEGF genes, respectively, among others. Because the diffusion distance of oxygen is limited, tumors cannot grow beyond several millimeters in size without recruiting new blood vessels. Thus, VEGF-mediated angiogenesis is critical to tumor growth. Interestingly, tumor metabolism is characteristically glycolytic despite the fact that tumors display a highly angiogenic phenotype.

In the 1920s, the German scientist Otto Warburg first published the observation that cancer cells utilize anaerobic metabolism even in the presence of oxygen. This effect, termed the "Warburg effect" or "aerobic glycolysis" was hypothesized by Warburg to be the cause of cellular transformation. Although

this hypothesis has not yet been proven, it is now well recognized that the Warburg effect is present in a number and variety of tumors, and this is the basis for positron emission tomography (PET) scanning, which visualizes tumors based on uptake and metabolism of ^{18}fluorodeoxyglucose. In addition to increased glucose uptake, some other characteristics of a tissue that is aerobically glycolytic are increased production of lactate and a lower pH. Tumors with increased lactate secretion are correlated with poor prognosis(155). For example, HIF induced overexpression of LOX has been correlated with a poor prognosis in breast and head and neck cancer(156). Likewise, overexpression of LDH-A in tumors has been linked to poor prognosis in colorectal and non-small cell lung cancer(157,158). Despite being a less efficient means of producing ATP, compensation occurs and cellular ATP levels are sufficient. Cellular metabolism is most efficient when abundant glucose is completely reduced to H_2O and CO_2 through oxidative respiration in the mitochondria. In the absence of oxygen, however, the cell undergoes fermentation and instead converts pyruvate (the end product of glycolysis) to lactate through LDH-A (Figure 6). This anaerobic metabolism is 18 times less efficient than aerobic metabolism, yielding only 2 molecules of ATP compared to the 36 ATPs that are generated when pyruvate is completely reduced and converted to acetyl-CoA. However, the process of anaerobic metabolism is faster than oxidative phosphorylation.

Despite this apparent drawback, some hypotheses for the predominance of the Warburg effect in tumors have been postulated and reviewed in detail elsewhere ((159,160)). First, byproducts of the pentose phosphate shunt include

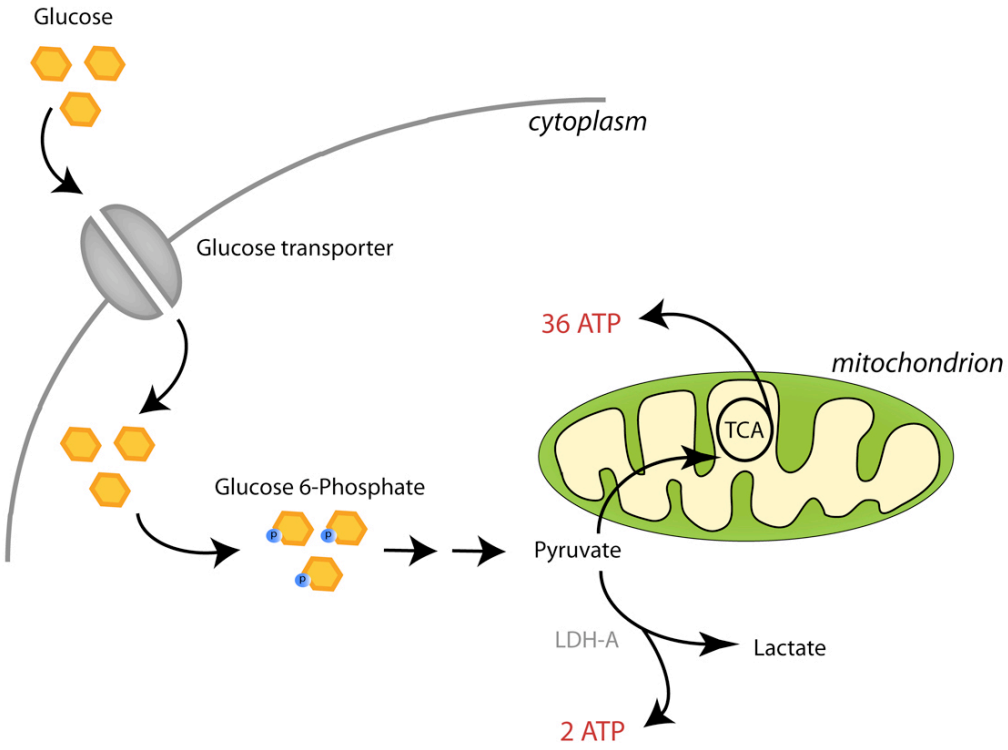

Figure 6: Glycolysis, fermentation and oxidative phosphorylation

Cellular glucose uptake is mediated by the glucose transporters (GLUT). The increase in cytoplasmic glucose stimulates glycolysis, a step-wise breakdown of glucose to two pyruvate molecules (see Fig. 7). Following glycolysis and production of pyruvate, the cell may undergo the energetically inefficient process of fermentation, yielding 2 ATP molecules plus lactate; or, the cell may undergo the energetically favorable complete reduction of pyruvate to 36 molecules of ATP through oxidative phosphorylation in the mitochondria.

nucleotide and ribose building blocks required during cellular replication. Second, the low pH may act as a selective pressure for transformation and a protection from immune cells(159,161). Third, the increased rate of fermentation compared to respiration (~100 times faster) may give a selective advantage if resources are scarce. And lastly, preferential aerobic glycolysis may decrease oxidative stress by limiting production of reactive oxygen species produced as a result of mitochondrial respiration, or by increasing endogenous ROS scavengers (pyruvate, NADPH)(162,163).

The impetus for the switch to the glycolytic phenotype has been shown to be initial exposure to hypoxia or activation of distinct oncogenes. Exposure to hypoxia would select for cells relying on glycolytic metabolism, killing off tumor cells unable to adapt their metabolic requirements. This theory, however, does not explain the existence of many cancers (leukemias and lymphomas) that are characteristically glycolytic despite never being exposed to hypoxia.

The degree of hypoxia in a given tumor has direct implications for the treatment of that tumor. As radiation-induced cell death requires oxygen to produce genotoxic free radicals, hypoxic tumors are characteristically radiation-resistant(164). Moeller et al. demonstrated apparently opposing roles for HIF-1α in both enhancement of tumor cell killing and the protection of endothelial cells from ionizing radiation treatment, the latter of which would be expected to maintain perfusion and tumor survival(165). Because these responses vary among different cells and tissues, the overall effect of HIF-1α on tumor survival following radiation therapy is tumor-type specific. As HIF is regulated both

transcriptionally and translationally by PI3K signaling, I will next discuss the classical role of PI3K in hypoxia and the role of PTEN in regulating global metabolism and hypoxia in normal and pathological conditions.

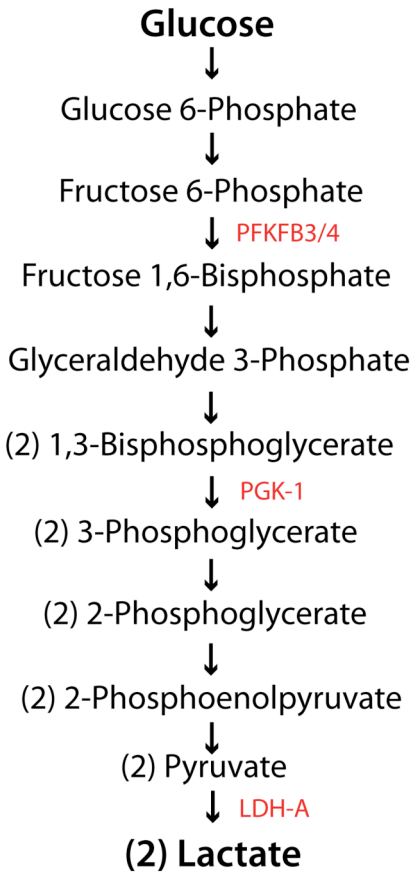

Figure 7: The steps of glycolysis

The intermediates of glycolysis are shown, with PI3K-regulated enzymes denoted in red.

1.8 The Canonical Role of the PI3K Pathway in Hypoxia and Metabolism

The strongest and most well defined link between PI3K signal transduction and hypoxia stems from the transcriptional and translational activation of HIF-1α downstream of Akt and mTOR. Various growth factors (IGF, EGF, FGF) bind to RTKs and activate PI3K and downstream effector molecules to promote HIF-1α gene transcription in either the presence or absence of oxygen(166). Akt has also been shown to stabilize HIF-1α protein in normoxia independent of prolyl hydroxylation(167). Thus, activation of the PI3K pathway typically correlates with induction of the hypoxia response. One of the preeminent physiological responses to hypoxia is production and secretion of vascular endothelial growth factor (VEGF), which results in angiogenesis to restore blood flow to ischemic tissues. Therefore, angiogenesis is a frequent surrogate for a tissue's ability to respond to hypoxia.

The earliest observation of PI3K/Akt signaling promoting angiogenesis was made in 2000 following overexpression of activated forms of PI3K and Akt in an *in vivo* angiogenesis model(168). Constitutively active PI3K (myr-PI3K) or Akt (myr-Akt) resulted in increased sprouting of vessels compared to basal PI3K signaling. This effect was abolished following inhibition of PI3K, suggesting that the PI3K pathway is critical in the angiogenic response. Several years later, new evidence demonstrated that PI3K's ability to induce VEGF expression and angiogenesis was mediated through HIF(169,170). These studies further delineated the pathway by defining HDM2 and p70 S6K as the downstream targets of Akt that are required for HIF-1α transcription and VEGF secretion

prior to vascular sprouting. Studies in prostate carcinoma cells showed that HIF-1α expression levels correspond with PI3K activity, as treatment with a pharmacological inhibitor of PI3K decreased growth factor-induced expression of HIF-1α(171). Expansion of these biochemical studies to clinical scenarios demonstrated that pharmacological inhibition of PI3K signaling led to a decrease in angiogenesis and prostate tumor size. Reconstitution with a dominant-negative form of Akt in prostate cancer cells also led to a reduction in tumor angiogenesis, tumor size and expression of HIF-1α and VEGF(172). There have been many similar studies in various tumor models suggesting that PI3K activity correlates positively with tumor growth by enhancing the angiogenic response.

PI3K signaling is also intricately tied to metabolism and energy usage of cells, complementary to its role in hypoxia. PI3K signaling regulates metabolism by modulating transcription of glycogen synthase, hexokinase and phosphofructokinase as well as glucose transporter expression and localization. PI3K also regulates many other genes involved in glycolytic metabolism downstream of HIF-1α, including lactate dehydrogenase A (LDH-A), 6-phosphofructo-2-kinase/fructose-2,6-biphosphate (PFKFB)-3 and -4 and phosphoglycerate kinase-1 (PGK-1)(Figure 7) (111,173-176). All of these genes are important in adapting the cell to oxygen-independent metabolism.

In normal cells, energy availability and usage dictates cell growth. As discussed with respect to hypoxia in tumors, one characteristic of the tumor phenotype is its unique metabolism, termed "aerobic glycolysis," in which the

tumor preferentially metabolizes glucose, even in the presence of oxygen, resulting in the production of high levels of lactate.

The importance of PI3K signaling in determining energy usage is observed by the shift in metabolism following dysregulation of PI3K signaling. Akt hyperactivation has been linked to an increase in glucose transporter translocation to the plasma membrane and subsequent glucose uptake, as well as an increase in lactate production. Similar observations have been made in tumor cells using artificial hyperactivation of Akt signaling through expression of membrane-targeted myr-Akt(50). Akt activity was shown to correlate in a dose-dependent manner with glycolytic output of cells. Interestingly, these cells did not proliferate any faster than control cells, and no effect was observed on respiration or oxygen uptake, suggesting that these cells were aerobically glycolytic. These results suggested that Akt activation is sufficient to induce aerobic glycolysis, although this idea is still somewhat controversial. This effect may or may not be through Akt activation of hexokinase, the pathway-determining enzyme that phosphorylates glucose to glucose-6-phosphate, thereby committing the cell to glycolysis(177). Regardless of whether Akt is sufficient to induce an aerobically glycolytic phenotype, the data indicate that hyperactive PI3K signaling "addicts" cells to glucose, such that they undergo apoptosis when glucose is withdrawn(50). Thus, manipulation of PI3K signaling or glucose availability may be effective anti-cancer modalities.

1.9 The Role of PTEN in Hypoxia and Metabolism

Given the positive correlation between PI3K pathway activity and HIF-1α activation, together with PTEN's role as a direct antagonist of PI3K signaling, catalytically active PTEN would be expected to be a negative regulator of hypoxia-mediated gene transcription, and this finding has been made in a number of published reports. However, findings presented in this thesis will demonstrate that PTEN, in fact, paradoxically *promotes* hypoxic gene transcription, and this phenomenon is independent of its lipid phosphatase activity. Before presenting these data, it is important first to understand the canonical role of PTEN as a negative regulator of HIF-1α and its gene targets in order to provide a framework on which to understand PTEN's apparently conflicting functions.

An initial study linking PTEN and hypoxia was performed in a PTEN-null glioblastoma cell line, where HIF-1α and its target genes were observed to be highly expressed. However, HIF-mediated gene expression was lost when PTEN expression was reconstituted, an observation that is consistent with PTEN's role in the PI3K pathway(178). Experiments in prostate carcinoma cells lacking PTEN have shown high basal levels of HIF-1α expression, which is ablated following re-expression of PTEN(171). These studies suggest an inverse relationship between PTEN and HIF-1α expression.

Another group coupled PTEN with more functional effects of HIF signaling by selectively knocking out PTEN in the endothelium(179). When melanoma or lung cancer cells were implanted subcutaneously in these animals,

they displayed larger, more vascular tumors compared to those in mice expressing PTEN in the endothelium. These effects were attributed to an increase in proliferation and migration of endothelial cells, resulting in a robust angiogenic response and faster tumor growth. Another study using an ectopic prostate tumor model clearly demonstrated that tumor size and angiogenesis were decreased in the presence of PTEN(172). The outcomes of all of these studies hinge on PTEN's role as a negative regulator of PI3K signaling.

However, one study arguing a role for PTEN that is counter to its canonical role in the PI3K/Akt pathway concluded that in renal cell carcinoma cells WT PTEN *activated* HIF-2α gene targets through suppression of yin yang 1 (YY1), a repressor or HIF-2α(180). It is not clear if PTEN catalytic activity was required for this effect. Renal cell carcinoma (RCC) is characterized by a loss of von Hippel-Lindau (VHL) protein, resulting in hyperactivation of HIF pathways (as described in detail above). HIF-2α stabilization has been implicated particularly in RCC progression and in that context is considered to be an oncogene. Although my work demonstrates a similar action of PTEN in promoting HIF-mediated gene expression, as will be described, it is through an independent mechanism.

Hypoxia-induced angiogenesis has immediate effects on the growth rates and metabolism of surrounding cells. The ability of cells to appropriately adapt to oxidative and glycolytic metabolism in the presence or absence of oxygen, respectively, is critical for cell survival. As described above, PI3K signaling through Akt regulates metabolism through various effector molecules. In

addition to dysfunction of the PI3K pathway resulting from dysregulated Akt activity, mutation or inactivation of PTEN has drastic effects on cellular metabolism, complementing its role in hypoxia. A study in glioblastoma cells noted that glucose uptake was twice as high in PTEN-null cells compared to cells expressing PTEN(181). In addition to loss of PTEN protein, loss of PTEN activity is also sufficient to affect cellular metabolism. From previous discussions it is clear that PTEN expression can be modulated by oxidation. A dramatic example of this has been demonstrated in thioredoxin-interacting protein (Txnip) knockout mice(182). These animals had high levels of Akt activity and signaling due to PTEN inactivation through oxidation. Not surprisingly, normally oxidative tissues in these animals (skeletal muscle and the heart) also displayed a dependence on anaerobic glycolysis.

It is unclear whether the observed effects of PTEN on the hypoxia response and subsequent metabolism changes are due exclusively to PTEN's antagonism of PI3K or to other, catalytically-independent roles of PTEN. There is a precedent for distinct functions of PTEN independent of its catalytic activity. For example, PTEN's ability to stabilize chromosomes as well as the p53 protein was independent of its phosphatase activity, suggesting alternative non-canonical roles for PTEN. This dissertation project was initiated in order to parse out the contribution of PTEN to the hypoxia response. As discussed in subsequent chapters, the results of these studies revealed a paradoxical role of PTEN as a *positive* regulator of the hypoxia response that was independent of its catalytic activity and canonical role as an antagonist to PI3K signaling.

2. Materials and Methods

2.1 Materials and Methods

Antibodies

Rabbit polyclonal antibodies against Akt (9272), BNIP3 (3769), E-cadherin (4065) and cleaved caspase-3 (D175), as well as monoclonal antibodies against phospho-Akt (S473) and caspase-3 (clone 8G10), were purchased from Cell Signaling Technology. Rabbit polyclonal antibodies specific for p300 (C-20) and p53 (FL-393), as well as goat polyclonal PTEN (N-19) and mouse monoclonal PTEN (clone A2B1) antibodies were obtained from Santa Cruz Biotechnology. A PGK-1 (ab71257) rabbit polyclonal antibody was obtained from Abcam; HIF-1α (NB100-449) and -2α (NB100-122) rabbit polyclonal antibodies as well as monoclonal antibodies against ALDOA (clone 3C12-6D11) and GAPDH (clone 1D4) were purchased from Novus Biologicals; a rat monoclonal tubulin (clone YL1/2) antibody and rat anti-mouse CD31 (MCA-1364) antibody from Serotec. All HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology.

Reagents

Firefly D-Luciferin potassium salt (4,5-Dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid potassium salt) was obtained from Caliper Life Sciences. Deferoxamine mesylate salt, cycloheximide, puromycin, doxycycline, mitomycin C and polybrene (hexadimethrine bromide) were

purchased from Sigma. Hoechst 33342 was purchased from Invitrogen. The hypoxia marker EF5 (2-8-*N*-[2,2,3,3,3-pentafluoropropyl]acetamide) and Cy-3-labeled EF5 antibody (ELK3-51) were obtained from Dr. Cameron Koch (University of Pennsylvania, Philadelphia, PA).

Cell lines

Human umbilical vein endothelial cells (HUVECs) were isolated as described previously by Marin et al.(183). The immortalized HUVEC line, EC-RF24 (ECRF), was provided by Dr. Ruud Fontijn (VU University Medical Center, Amsterdam). Both HUVECs and ECRFs were maintained in Endothelial Basal Medium (EBM)-2 (Clonetics) supplemented with growth factors and 5% fetal bovine serum (FBS; EGM-2-MV, Clonetics). HUVECs were used through passage 6. All other cells (NIH 3T3 murine fibroblasts, human embryonic kidney [HEK]-293, COS-7, PC-3 human prostate adenocarcinoma, and DU-145 human prostate carcinoma) were obtained from the Cell Culture Facility of the Duke Comprehensive Cancer Center or the American Type Culture Collection and were maintained in Dulbecco's Modified Eagle's Media (DMEM, Gibco) supplemented with 10% FBS (HyClone) and 1% penicillin/streptomycin (Gibco). All cells were routinely passaged and housed in a humidified 37°C incubator in 5% CO_2.

HEK-293 cells were engineered to stably express either a scrambled (scr) or PTEN-targeted shRNA. Cells were infected with retrovirus containing scr or

PTEN shRNA and positive clones were selected with puromycin (5 μg/mL). PTEN knockdown was verified by western blotting.

A Tet-inducible PTEN U87 glioblastoma cell line ($U87^{Tet\text{-}On:PTEN}$) was provided by Dr. Maria-Magdalena Georgescu (MD Anderson, Houston, TX). Cells were exposed to 1 μg/mL doxycycline for a minimum of 12 hours to induce PTEN expression, which was validated by western blotting.

Constructs, silencing and transgene delivery

Full-length wild-type (WT) murine PTEN (PTEN-WT) was isolated from a PY-4-1 murine endothelial cell cDNA library and sub-cloned into the pcDNA3 mammalian expression vector (Invitrogen) downstream of and in-frame with an HA-tag and verified by sequencing. The catalytically inactive C124S mutant of PTEN (C/S) was also generated using site-directed mutagenesis (QuikChange; Stratagene). This method was similarly used to mutate leucine 182, isoleucine 224, arginine 234 and lysine 341 to generate PTEN-L182V (L/V), PTEN-I224M (I/M), PTEN-R234W (R/W) and PTEN-F341V (F/V) in both the WT and C/S backgrounds. PTEN C-tail truncation mutants (1-396 PTEN, 1-400 PTEN) were generated by PCR with the reverse PTEN primer containing a stop codon after the desired amino acid. PTEN expression constructs containing either a nuclear-exclusion signal (NES) or nuclear-localization signal (NLS) were generously provided by Dr. Wiliam Sellers (Dana-Farber Cancer Institute). Adenoviruses encoding either PTEN-WT or PTEN-C/S were generated as described previously(53). Truncation mutants of the PTEN C2 domain (1-274 PTEN, 1-373

PTEN) were provided by Anita Hjelmeland (Cleveland Clinic, Cleveland, OH). Lentivirus constructs containing shRNAs targeting HIF-1α or HIF-2α were generously provided by Dr. Zhizhong Li (Duke University, Durham, NC).

A PTEN shRNA (GATCTTGACCAATGGCTAAGT) targeting base pairs 319-339 of the coding regions of both human and mouse PTEN was generated and cloned into the pSuper-Retro retroviral vector (Clontech; OligoEngine) as described previously(184). A non-targeted rescue construct containing five silent mutations (underlined) within the shRNA-targeting region of PTEN (GA<u>C</u>CT<u>C</u>GACCA<u>G</u>TGGCT<u>G</u>AG<u>C</u>) was impervious to PTEN shRNA silencing and was generated using QuikChange mutagenesis. This rescue PTEN construct was used as the template for all previously mentioned constructs. Another PTEN shRNA (CGACTTAGACTTGACCTATAT) obtained from Open Biosystems (Lafayette, CO) targeted base pairs 63-83 of the coding region of both human and mouse PTEN. This PTEN shRNA was able to silence expression of both endogenous PTEN and rescue PTEN constructs.

A p53-luciferase reporter construct (generously provided by Dr. Joe Nevins, Duke University Medical Center) was used to assess p53 transcriptional activity. The hypoxia response element (HRE) was cloned from sequences upstream of the VEGF promoter, and consisted of five tandem repeats of the core HRE sequence (TACGTG). These tandem repeats were cloned upstream of the luciferase cDNA in the pGL3-Basic vector (Promega), as described previously(185). An adenovirus containing the HRE-luciferase reporter described

previously was generously provided by Dr. Dawn Bowles (Duke University, Durham, NC)(186).

Transient transfections were performed with FuGene 6 (Roche), according to manufacturer's instructions. Viral transductions were performed overnight in whole media containing polybrene (8 μg/mL).

Immunoprecipitation and western blotting

Whole cell lysates were obtained in RIPA buffer (Sigma) supplemented with protease inhibitors (Complete, Roche), sodium fluoride (1 mM), sodium orthovanadate (1 mM), and deferoxamine (200 μM). For immunoprecipitations (IPs), an antibody specific for p300 was incubated with cell lysates and allowed to bind overnight at 4°C. Antibody-protein complexes were immunoprecipitated with Protein A/G agarose (Santa Cruz) for 1 hour at 4°C, washed and then eluted by boiling in 4× Laemmli sample buffer containing 5% β-mercaptoethanol. Immunoprecipitated proteins and whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes prior to probing with the indicated antibodies.

RNA isolation and quantitative real-time PCR

RNA was isolated using TRIzol reagent (Invitrogen) and cDNA was synthesized using the Superscript III First-Strand cDNA synthesis kit (Invitrogen) from 3-5 μg total RNA template using oligo(dT) primers, according to manufacturers' instructions. For real-time qPCR, cDNA (1 μg) was added to a 25

μl reaction mix containing human primer and probe sets for specific genes and amplified in an ABI Prism 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Cycle thresholds (Ct) were calculated for all samples and the ΔΔCt method was then used to compare mRNA quantity between samples ($2^{-\Delta\Delta Ct}$), using β-glucuronidase, a housekeeping gene involved in carbohydrate metabolism, as a reference.

A custom real-time oligo-array was designed to contain primer and probe sets specific for 46 hypoxia-responsive genes in duplicate in a 96-well plate format (Applied Biosystems). Equal amounts of cDNA were added to each well, each treatment was repeated two times, and relative mRNA expression was calculated as described above. Each sample was normalized to untreated cells (293^{scr}) and the average of two experiments was taken.

2.2 In vitro Studies

Hypoxia and luciferase reporter assays

Hypoxia was induced by incubating cells in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed with a clinical blood gas mixture of 5% CO_2, 95% N_2. The chamber was placed inside an incubator at 37°C for the duration of the hypoxic treatment, and hypoxia induction was verified by HIF-1α protein stabilization.

Reporter assays measuring the activity of hypoxic gene transcription and p53 target gene transcription were performed using HRE-luciferase and p53-luciferase reporter constructs, respectively. For HRE-luciferase assays, cells were

transiently transfected or adenovirally-transduced with the reporter construct and subjected to 12 hours of hypoxia. For p53-luciferase assays, cells were transiently transfected with the reporter construct and subjected to genotoxic damage by treatment with Mitomycin C (2 μg/mL) for 4 hours. Luciferase activity was measured using a bioluminescence imaging system (IVIS, Xenogen Corporation) and accompanying software (Living Image, Xenogen Corporation) following exposure to a standard solution of luciferin (5.7 mg/mL) for 5 minutes.

DNA fragmentation assay

Genomic DNA was isolated using an apoptosis and DNA-laddering kit (Roche) and assessed for fragmentation following separation by agarose gel electrophoresis, according to manufacturer's directions. Densitometry was performed on laddering gels using ImageJ software (NIH).

VEGF and lactate quantification and HAT activity assays

A VEGF ELISA (R&D Systems, Minneapolis, MN) was performed according to manufacturer's instructions on hypoxic cell supernatants. Values were quantified according to a standard curve and replicates were averaged.

A colorimetric lactate assay (Abcam, Cambridge, MA) was performed according to manufacturer's instructions on hypoxic cell supernatants. Values were quantified according to a standard curve and replicates were averaged.

In vitro p300 acetyltransferase activity was measured using a HAT assay kit (Active Motif, Carlsbad, CA) following immunoprecipitation of endogenous

p300 from cell lysates. p300 protein was immunoprecipitated on Protein A/G agarose (Santa Cruz), and immune complexes were washed, incubated with assay mix containing acetyl-CoA and histone H4 peptide for 30 minutes at room temperature, and read on a fluorescent plate reader (FLUOstar Omega, BMG Labtech).

p300 stability assay

p300 stabilization in hypoxia was determined by subjecting treated cells to cycloheximide (25 μg/mL) simultaneously with hypoxia exposure. Whole cell lysates were obtained at baseline (0 hours) and after 6 and 12 hours of hypoxia and separated by SDS-PAGE. Protein expression of p300 was quantified and normalized to a loading control (tubulin).

2.3 In vivo Studies

Tumor implantation, immunofluorescence and hypoxia quantitation

All animal studies were approved by the Duke University Institutional Animal Care and Use Committee. Male, nude athymic (nu/nu) mice (4-6 weeks old) were purchased from Jackson Labs (Bar Harbor, ME) and housed in a temperature-controlled room on a 12-hr light/dark cycle. Animals were given water and food *ad libitum*. 1 x 10^6 293-PTEN^{low} or 293^{scr} cells were suspended in 100 μl PBS and injected subcutaneously into the flanks of mice (n=3 per group) and tumor growth was monitored. When the 293-PTEN^{low} tumors reached ~1.5 cm in diameter, all tumors in both groups were resected. Three hours prior to

tumor removal, animals were injected intraperitoneally with the hypoxia marker EF5 (100 μM), as described previously (187). Perfused vessels were labeled by intravenous injection of Hoechst 33342 (1 mg/kg), which was allowed to circulate for 5 minutes prior to sacrifice. Excised tumors were snap-frozen over liquid Nitrogen vapor, embedded in optimal cutting temperature (OCT) medium and serially sectioned on a Leica CM3050S cryostat. Seven-micron thick sections were immunostained for CD31 as well as to verify PTEN status. Complete tumor section images were obtained with a Retiga 1300R camera (QImaging, Surrey, BC, Canada) mounted on a Zeiss Axioskop 2 fluorescence microscope (Thornwood, NY) equipped with a motorized stage (Marzhauser-Wetzlar, Germany) at 5× magnification and stitched together using MetaMorph software (Molecular Devices). Images were overlaid using Adobe Photoshop CS3 software.

EF5 staining was performed as described previously(187). EF5 images captured at the same exposure for all tumor sections were inverted and the relative density of EF5 was quantified relative to total tumor area using ImageJ software (NIH). Tumors were grouped according to PTEN expression, and EF5 density/tumor area ratios were averaged. CD31 staining was quantified similar to EF5.

2.4 Statistical Analysis

Results are expressed as the mean ± standard error of the mean (SEM). Analysis of variance was used to compare means among different groups and a

Student's *t* test was used to determine inter-group differences. For all comparisons, $P < 0.05$ was considered statistically significant.

3. PTEN Positively Regulates Hypoxia-responsive Genes by Modulating p300 Activity

3.1 Introduction

PTEN, a well-studied tumor suppressor protein, is highly mutated in a variety of solid tumors, including breast, brain and colon carcinomas. The lipid phosphatase activity of PTEN as a negative regulator of the PI3K/Akt pathway underscores PTEN's tumor suppressive function. However, emerging research has shown an increasing number of roles for nuclear PTEN as well as lipid phosphatase-independent functions. This work sought to better define the role of PTEN in cellular hypoxia.

Previous studies have shown PI3K and PTEN to be negative regulators of the hypoxia response, as hyperactivation of the PI3K pathway, by expressing either a constitutively active Akt (myr-Akt) or PTEN siRNA, results in HIF stabilization in normoxia(168,171,178). Downstream effects of HIF stabilization include a glycolytic phenotype and greater induction of angiogenesis in tumors(177). Thus, it was hypothesized that PTEN would act as a negative regulator of the hypoxia response.

In these studies, the role of PTEN in hypoxia was investigated using an *in vitro* HRE reporter assay, validated by real-time quantitative PCR and western blotting for specific hypoxia-responsive genes, as well as in a tumor xenograft model. Interestingly, PTEN loss led to a striking decrease in HRE activity, an inability to induce specific hypoxia-responsive genes and a greater degree of tumor hypoxia. These effects were independent of PTEN's lipid phosphatase

activity and seemed to be augmented by forced nuclear expression of PTEN. Furthermore, mutation of a single residue (Ile224) in the C2 domain of PTEN inhibited the positive effect of PTEN on hypoxia-mediated gene transcription, thus providing a separation-of-function mutation. The ability of PTEN to *positively* regulate the hypoxia response was shown to be mediated through its modulation of p300 activity. These findings and represent a novel, nuclear-specific role for PTEN independent of its canonical lipid phosphatase activity.

3.2 Results

PTEN silencing by shRNA and successful rescue

To investigate the role of PTEN in the hypoxia response, PTEN expression levels were modulated in a variety of cell types using either PTEN-targeted shRNA knockdown or PTEN overexpression with a recombinant adenovirus or transient plasmid transfection. PTEN expression was validated by western blotting cell lysates. PTEN-targeted shRNA was effective at knocking down human and mouse PTEN. A non-targeted rescue PTEN cDNA (PTEN-WT-R) was impervious to silencing and was expressed even in the presence of stably expressed PTEN shRNA (Figure 8).

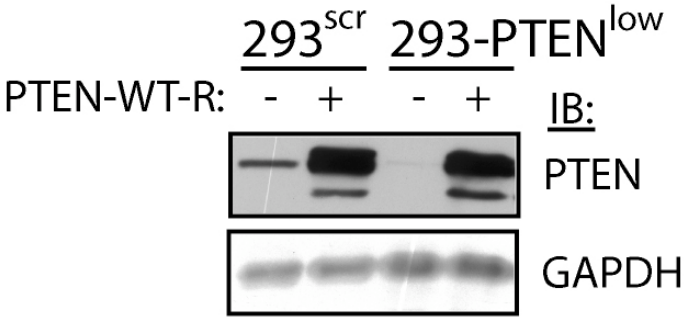

Figure 8: PTEN-WT-R rescues PTEN expression in the presence of a PTEN shRNA

HEK-293 cells stably expressing a PTEN shRNA (293-PTEN^{low}) have diminished endogenous PTEN expression compared to HEK-293 cells, which express a scrambled shRNA (293^{scr}). Transient transfection of a wild-type PTEN rescue construct (PTEN-WT-R) results in successful rescue of PTEN expression in 293-PTEN^{low} cells and overexpression of PTEN in 293^{scr} cells.

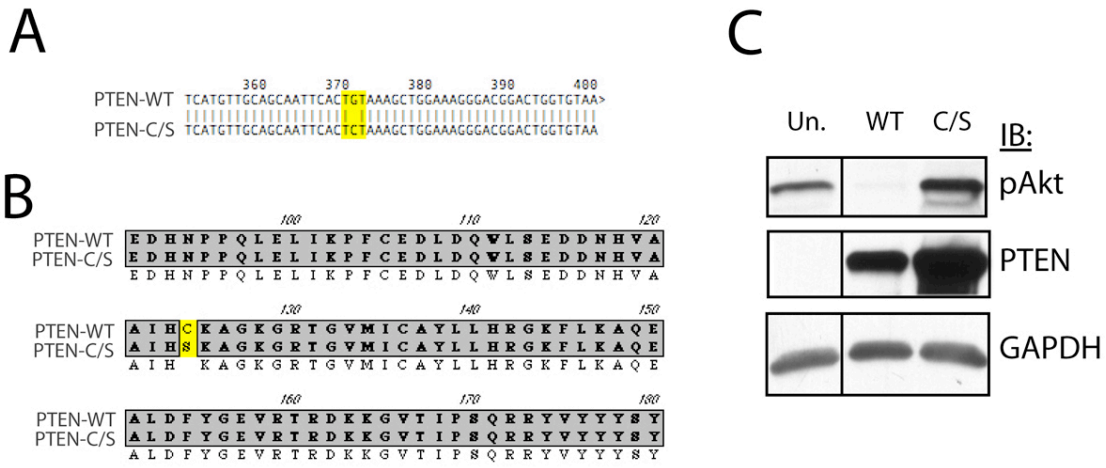

Figure 9: Characterization of PTEN-C124S

(A) Nucleotide sequence alignment of PTEN-WT and PTEN-C124S (C/S) demonstrates a single base mutation of nucleotide 371, from G to C. (B) Partial amino acid alignment of PTEN-WT and PTEN-C/S confirms C124S mutation. (C) Western blot analysis verifying catalytic inactivity of the PTEN-C/S construct was performed following overexpression of either PTEN-WT or PTEN-C/S in a PTEN-null cell line and immunoblotting for phospho-Akt (pAkt). GAPDH is used as a loading control.

Characterization of PTEN-C/S construct

A catalytically inactive PTEN construct was generated by mutating the catalytic cysteine in the active site of PTEN to serine (TGT to TCT). Mutation of the catalytic activity was verified by sequencing and loss of catalytic activity was indirectly verified by evaluating the phosphorylation status of Akt by western blotting. Specifically, cells treated with PTEN-C/S showed a marked increase in phospho-Akt levels compared to cells overexpressing PTEN-WT (Figure 9).

PTEN expression positively modulates hypoxia-mediated gene expression in transformed and non-transformed cells

A hypoxia response element (HRE)-luciferase reporter construct containing 5 tandem HRE repeats from the VEGF promoter has been previously characterized(185), and was used to assess global cellular hypoxia response following PTEN modulation.

The HRE-luciferase reporter construct was co-expressed with different PTEN constructs in a variety of cell types, and luciferase activity was measured after 12 hours of hypoxia. Contrary to PTEN's role as a negative regulator of PI3K signaling(55) and its reported role as a negative regulator of the hypoxia response(178), loss of PTEN expression led to a dramatic and significant decrease in HRE activity, whereas overexpression of PTEN led to an increase in hypoxic gene transcription. The modulation of HRE-luciferase activity in the setting of hypoxia was observed in a wide range of cells, including primary human umbilical vein endothelial cells (HUVEC), immortalized EC-RF24 cells derived from HUVECs (ECRF), several commonly used immortalized cell lines (HEK-

293, NIH 3T3, COS-7), and a number of tumor-derived cell lines (DU-145, PC-3, U87). Results from HUVECs, 293, and 3T3 cells are shown (Figure 10A-C). The ability of PTEN to inhibit or augment HRE activity was PTEN dose-dependent following either PTEN silencing or overexpression, respectively (Figure 10D,E).

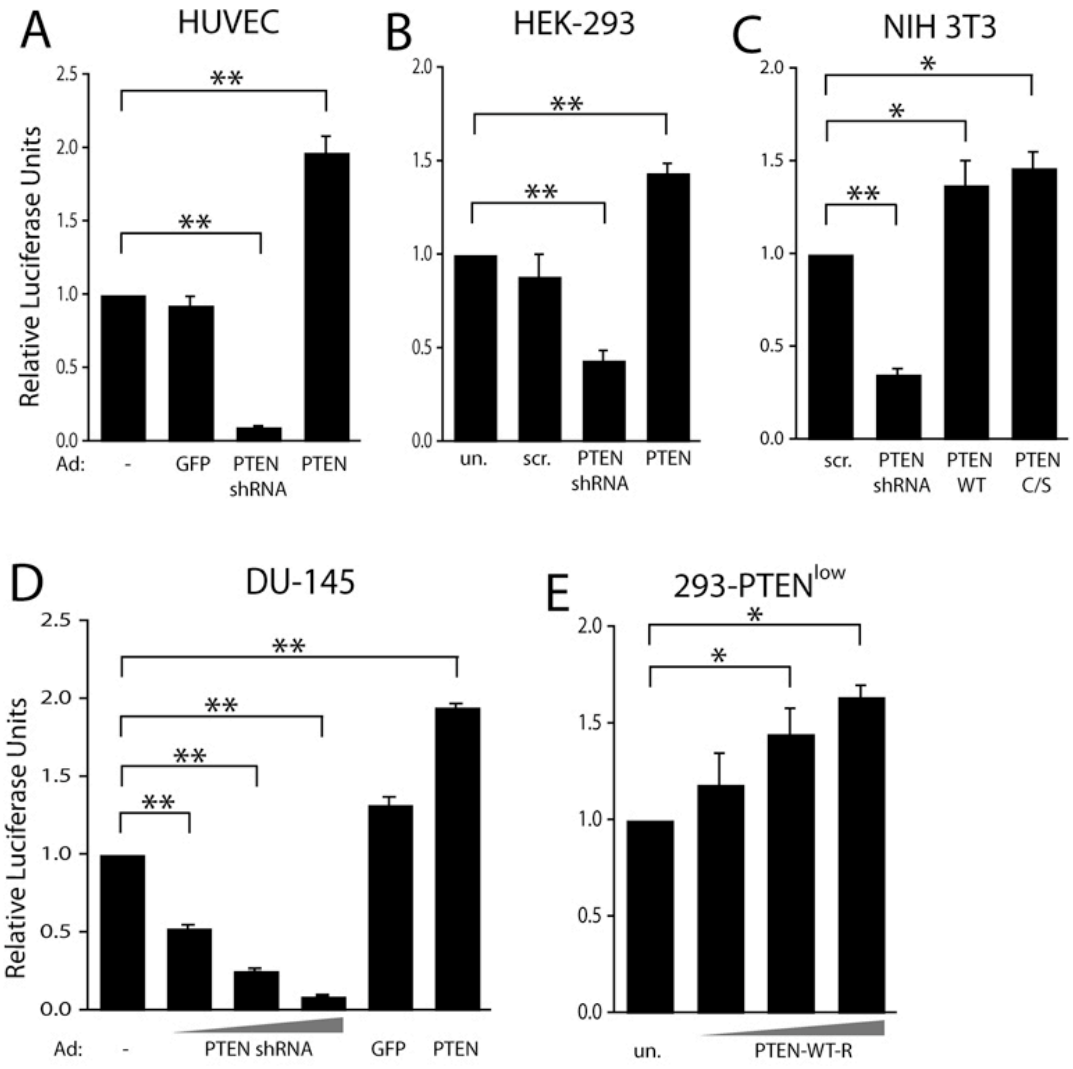

Figure 10: PTEN positively regulates HRE activity in a dose-dependent manner

PTEN levels were modulated by recombinant adenovirus (Ad) transduction or transient transfection in a variety of cell lines in the presence of an HRE-luciferase reporter construct. Ad-GFP (green fluorescent protein) and scrambled shRNA were used as controls. As seen in the primary endothelial line HUVEC (A), HEK-293 (B), and 3T3 fibroblast line, PTEN expression correlated positively with HRE activity following hypoxia. PTEN's effect on HRE activity was dose-dependent following either PTEN silencing, as shown in the prostate tumor line DU-145 (D), or delivery of a PTEN rescue construct (PTEN-WT-R) as shown in 293 cells stably expressing a PTEN shRNA (293-PTEN^{low}) (E). Gray triangles denote increasing dosage. (*, $P < 0.05$; **, $P < 0.005$).

PTEN's C2 domain but not catalytic activity are required to modulate hypoxia-mediated gene expression

Surprisingly, overexpression of the catalytically inactive C124S (C/S) mutant of PTEN had the same effect on HRE activity as wild-type PTEN (Figure 11A,B). PTEN expression and catalytic activity were evaluated by assessing phospho-Akt by western blot (Figure 11C). As PTEN-C/S is sometimes able to act as a dominant negative, treatment with PTEN-C/S in ECRF cells actually increased phospho-Akt levels compared to untreated cells (Figure 11C).

As the C2 domain of PTEN has previously been implicated in catalytic-independent functions of PTEN, we determined the requirement of the C2 domain of PTEN in modulating the hypoxia response by overexpressing two PTEN truncation mutants in comparison to the full-length PTEN (403 amino acids). Interestingly, a PTEN mutant truncated at amino acid residue 274 was unable to promote HRE activity, while expression of a mutant containing residues 1-373 of PTEN somewhat rescued this effect although still decreased compared to WT (Figure 11D). Clearly, the presence of the C2 domain is critical to PTEN's ability to modulate the hypoxia response.

The C-tail of PTEN contains two high-probability PEST domains as well as a PDZ- binding domain from residues 400 to 403. Unlike the C2 domain, overexpression of PTEN truncation mutants lacking the second PEST domain and PDZ domain (PTEN 1-396) or PDZ domain alone (PTEN 1-400) behaved as full-length PTEN (Figure 11E). These results suggest that PTEN's role in the hypoxia response is independent of interactions between PTEN and PDZ domain-containing proteins.

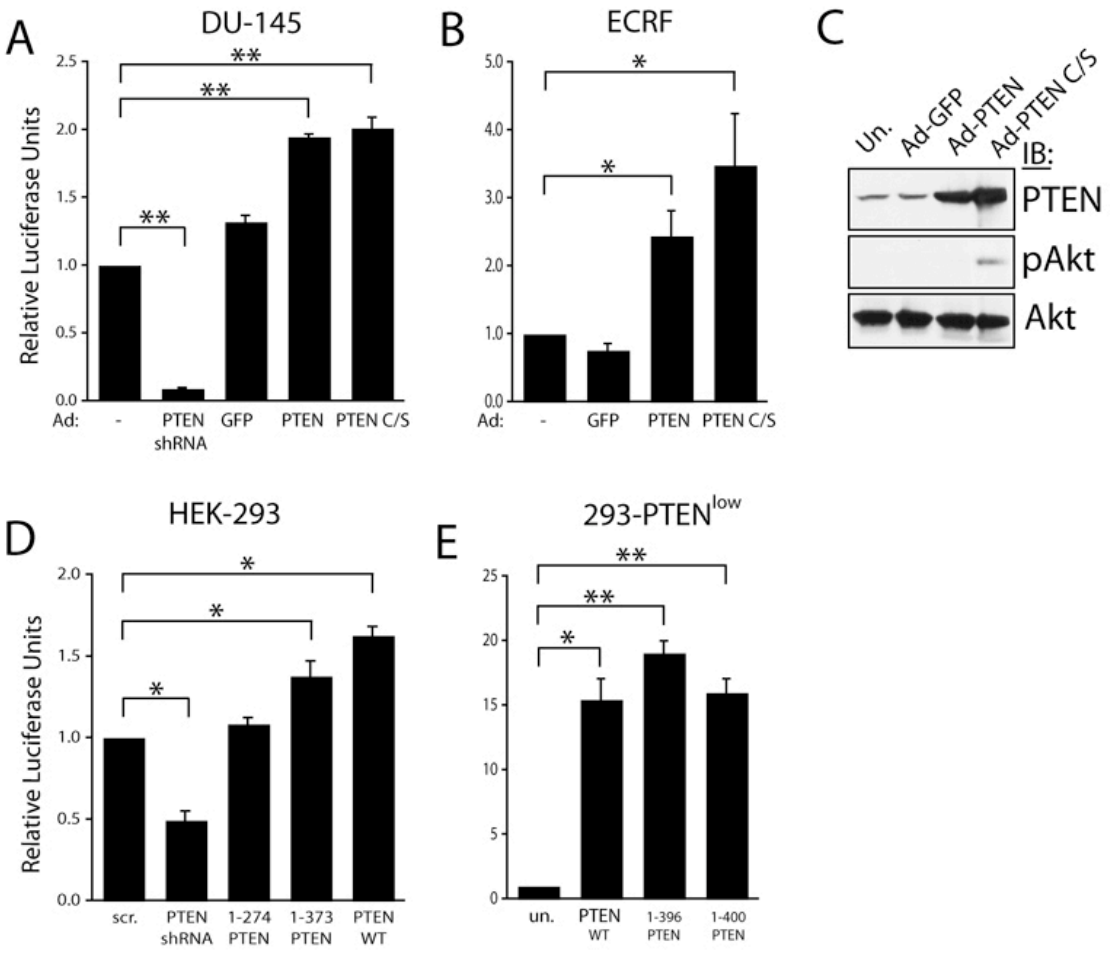

Figure 11: PTEN's C2 domain, but not catalytic activity, are required to regulate the hypoxia response

PTEN levels were modulated by recombinant adenovirus (Ad) transduction or transient transfection in a variety of cell lines in the presence of an HRE-luciferase reporter construct. Ad-GFP (green fluorescent protein) and scrambled shRNA were used as controls. PTEN catalytic activity is not required for PTEN's ability to positively regulate HRE activity, which was observed following overexpression of PTEN-C124S (C/S) in DU-145 (A) and ECRF (B) cells. (C) A representative western blot taken from ECRF cell lysates assayed in (B) verifies loss of catalytic activity. (D) The PTEN C2 domain is required, as truncation mutants (1-274 PTEN, 1-373 PTEN) had a lesser effect on HRE activity compared to full-length PTEN. (E) Loss of a PEST domain and PDZ-binding domain (1-396 PTEN) or PDZ-binding domain alone (1-400 PTEN) in the C-tail of PTEN had no effect on PTEN's ability to positively regulate the hypoxia response. (*, $P < 0.05$; **, $P < 0.005$).

Modulation of hypoxic gene expression by PTEN is HIF-1α- and -2α-dependent

To investigate whether PTEN's positive effect on hypoxic gene transcription was dependent on hypoxia-inducible factors (HIF), we evaluated the effects of silencing HIF-1α, HIF-2α, or both on HRE-luciferase activity. Because HIF-2α expression is somewhat restricted, we tested these effects in endothelial cells, which are known to express both HIF-1α and -2α. Silencing either HIF-1α or HIF-2α individually significantly blunted PTEN's effect on HRE activity by more than 50% (Figure 12A-B). Although silencing both HIF-1α and -2α further reduced PTEN's effect, it was not completely abolished. This may be due to incomplete silencing of the HIFs or contribution of a HIF-independent pathway. However, these findings demonstrate that PTEN's ability to enhance the hypoxia response is dependent upon the HIF-α proteins.

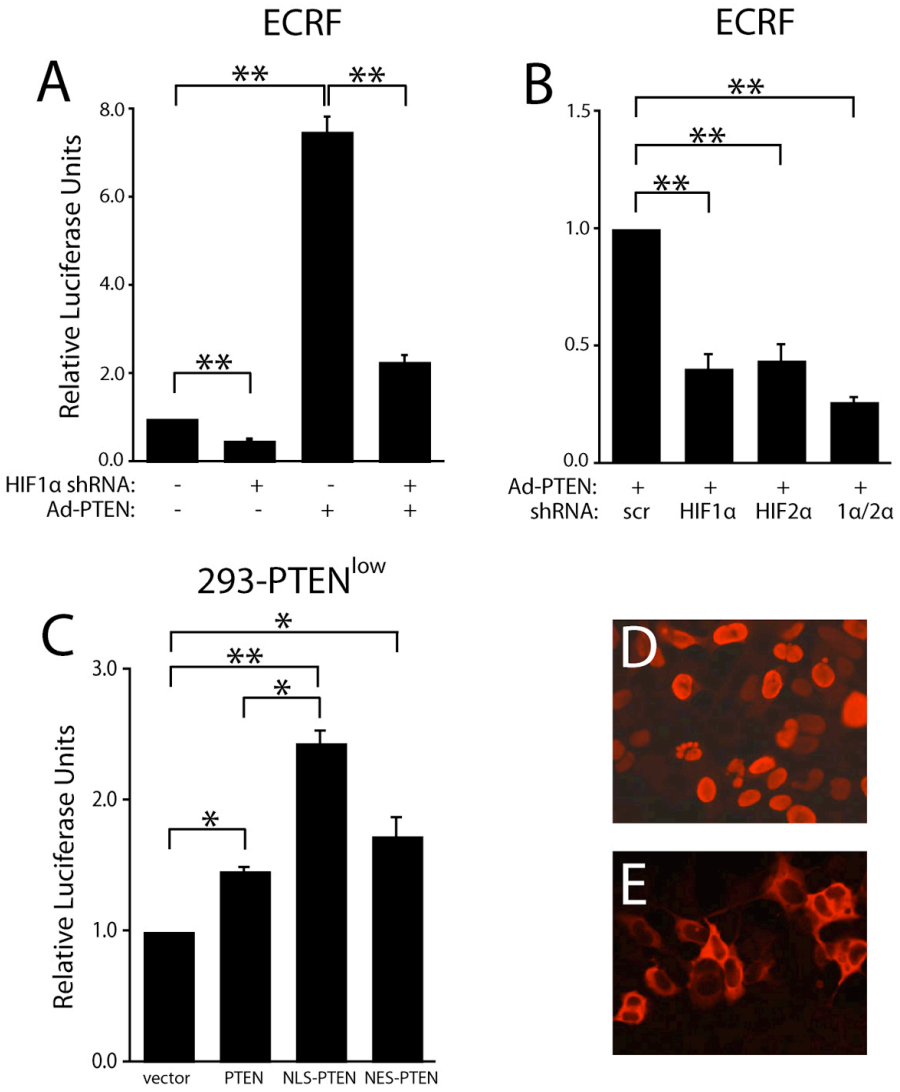

Figure 12: PTEN's effects on the hypoxia response are mediated by HIF and are more pronounced following PTEN nuclear localization

HIF-1α (A) and -2α (B) knockdown in hypoxic ECRF cells blunted PTEN's ability to increase HRE-luciferase activity in hypoxia. The relative contribution of HIF-1α and -2α appears to be similar, although knockdown of both HIF proteins was not additive. (C) Nuclear localized PTEN (NLS-PTEN) rescue was more effective than nuclear-excluded PTEN (NES-PTEN) rescue in positively regulating HRE activity in hypoxic 293-PTEN^{low} cells. Compartmentalization of NLS- (D) and NES-PTEN (E) was validated by immunofluorescence microscopy with anti-PTEN (*, $P < 0.05$; **, $P < 0.005$).

Nuclear PTEN enhances the hypoxic response more efficiently than cytosolic PTEN

PTEN is known to localize and function in both the cytoplasm and the nucleus. Because PTEN was found to alter hypoxia-mediated gene expression, which is initiated in the nucleus, we hypothesized that a nuclear-localized PTEN construct would enhance this effect. To test this, PTEN that was stably silenced in HEK-293 cells with a retrovirally-expressed shRNA to generate 293-PTEN^{low} cells and was rescued with PTEN constructs fused to either a nuclear localization signal (NLS) or a nuclear exclusion signal (NES). Compartment specificity of these constructs was achieved, as demonstrated by immunofluorescence microscopy (Figure 12D,E). When we examined effects of these constructs on HRE-luciferase reporter activity, NLS-PTEN induced significantly greater HRE activity than wild-type, non-localized PTEN (Figure 12C). Interestingly, HRE activity induced by NES-PTEN was similar to that of non-localized PTEN. This result may be due to incomplete silencing of endogenous PTEN by our shRNA, incomplete nuclear exclusion by the NES-PTEN constructs or some threshold effect of PTEN or the ability of PTEN in the cytoplasm to signal. Taken together, however, our results demonstrate that PTEN positively modulates the hypoxia response, likely through a nuclear scaffolding function, as it is independent of PTEN's lipid phosphatase activity and enhanced by nuclear localization of PTEN.

PTEN enhances expression of multiple hypoxia-responsive genes

Our results with the HRE-luciferase reporter demonstrated that PTEN alters the global hypoxia response. To determine PTEN's effects on expression of

specific genes, we generated a custom real-time qPCR array containing 46 genes known to be hypoxia responsive and containing HREs in their promoters. Four conditions were tested: 1) 293 cells stably expressing a scrambled shRNA (293^{scr}); 2) 293 cells stably expressing a PTEN-specific shRNA (293-$PTEN^{low}$); 3) 293-$PTEN^{low}$ cells transiently overexpressing a non-targeted wild-type PTEN rescue construct (293-$PTEN^{WT-R}$); and 4) 293-$PTEN^{low}$ cells transiently overexpressing a non-targeted (impenetrable to shRNA degradation) catalytically inactive PTEN rescue construct (293-$PTEN^{C/S-R}$). Cells were then subjected to hypoxia and cDNA was applied to the array. Each condition was performed in duplicate, normalized to unmodulated cells (293^{scr}) (Table 1) and results were averaged and graphed (Figure 13A). Responsive or non-responsive genes were grouped accordingly, and candidate genes were validated independently by quantitative real-time PCR analysis. Genes validated to be positively regulated by PTEN in the setting of hypoxia included those involved in angiogenesis (VEGF, EPO), metabolism (ALDOA, PFKFB3, PFKFB4, PGK-1), cell cycle and apoptosis (Tgf-α, BNIP3, TERT, cyclin D1), cell motility (E-cadherin), and transcription (DEC2) (Figure 13B). In agreement with our HIF knockdown studies, genes regulated by PTEN include those regulated either exclusively by HIF-1α (ALDOA, PGK-1) or HIF-2α (Tgf-α) as well as those regulated by both HIF-α proteins (VEGF).

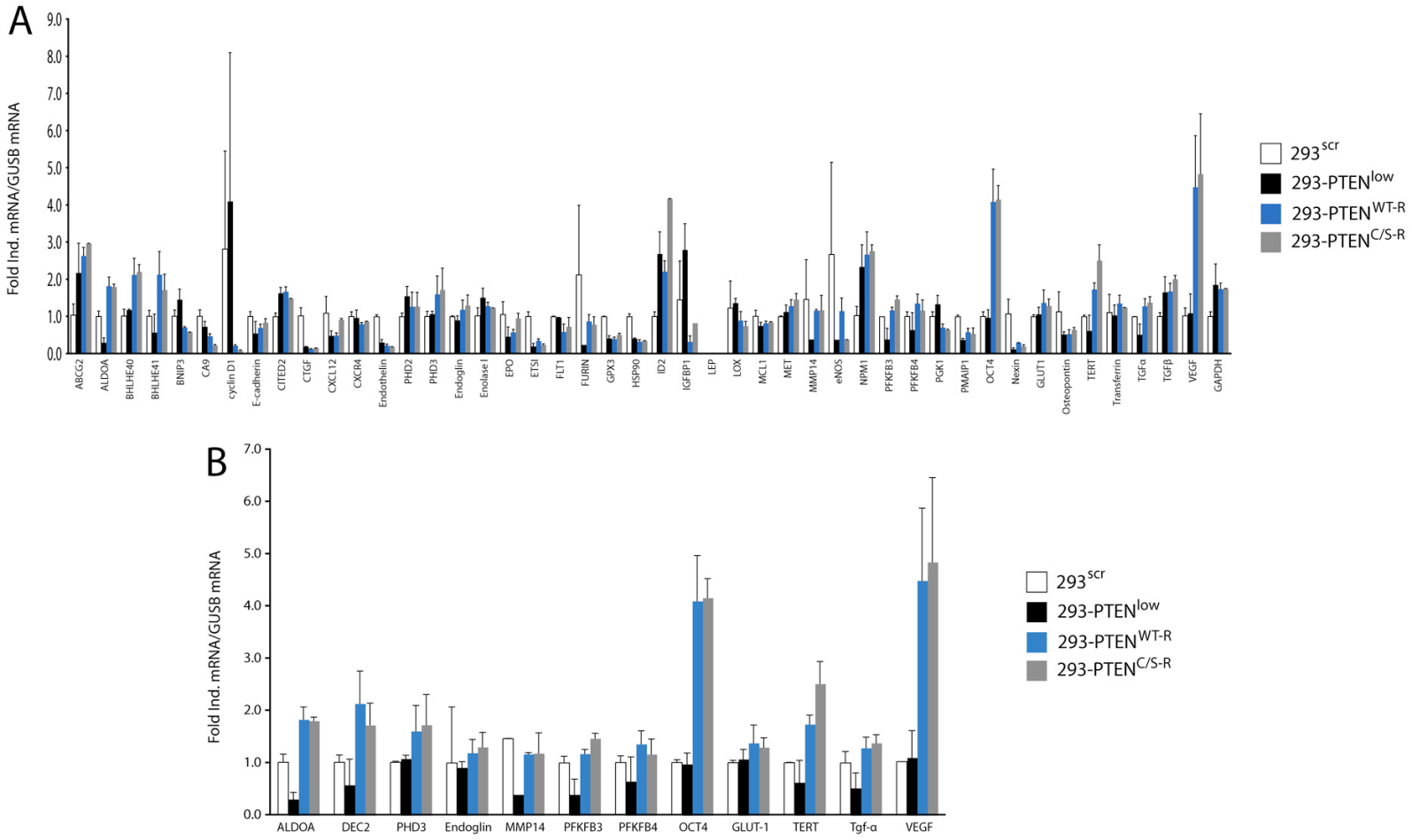

Figure 13: Real-time quantitative PCR of HRE-containing gene induction following PTEN modulation in hypoxia

(A) Cumulative real-time results for 46 HRE-containing genes following PTEN silencing or induction and hypoxia treatment illustrates the presence of 4 classes of HRE genes: 1) genes negatively regulated by PTEN; 2) genes positively regulated by PTEN; 3) genes upregulated with PTEN silencing or loss; and 4) genes downregulated with PTEN silencing or loss. (B) Candidate genes that appear to be regulated by PTEN in hypoxia.

Table 1: Relative expression values of hypoxia-responsive genes

ABI Gene ID	Gene Name	293^{scr} rel. exp.	$293\text{-}PTEN^{low}$ rel. exp.	SEM	$293\text{-}PTEN^{WT\text{-}R}$ rel. exp.	SEM	$293\text{-}PTEN^{C/S\text{-}R}$ rel. exp.	SEM
Hs01053787_m1	ABCG2	1	2.184898127	0.79435354	2.64284	0.22796	2.97925	0.00000
Hs00765620_m1	ALDOA	1	0.306308506	0.12798732	1.82991	0.24098	1.80724	0.07005
Hs00969291_m1	BNIP3	1	1.463502557	0.28140427	0.717035097	0.02094305	0.591049145	0
Hs00154208_m1	CAIX	1	0.734601282	0.14075971	0.480735974	0.06242435	0.231275519	0.02615411
Hs00366696_m1	CITED2	1	1.641119939	0.14787586	1.682756128	0.12631609	1.493244078	0
Hs00170014_m1	CTGF	1	0.195350041	0.00643624	0.132036846	0.01059816	0.159509077	0.00356507
Hs00237052_m1	CXCR4	1	0.971850221	0.21078078	0.806939072	0.03951283	0.882152509	0
Hs99999004_m1	Cyclin D1	1	4.110011547	3.99772944	0.227448636	0.02089333	0.101282932	0.00725716
Hs00186419_m1	DEC1	1	1.188112821	0.01544045	2.133543377	0.43793852	2.212045524	0.19506102
Hs00229146_m1	DEC2	1	0.576672414	0.49632753	2.136012393	0.6243353	1.724957522	0.42104236
Hs01013959_m1	E-Cadherin	1	0.556838721	0.31967586	0.709569878	0.0921388	0.843571185	0.10646212
Hs00923996_m1	Endoglin	1	0.910140992	0.1135285	1.19174517	0.25544047	1.304756276	0.28108678
Hs00174961_m1	Endothelin	1	0.312233436	0.07829513	0.231983082	0.03381452	0.195264285	0
Hs00361415_m1	Enolase 1	1	1.518737797	0.24691295	1.291488921	0.09881472	1.247552043	0
Hs01574659_m1	eNOS	1	0.38780411	0	1.157775969	0.35102238	0.38780411	0
Hs00171267_m1	EPO	1	0.470469548	0.25359991	0.5861601	0.07611384	0.963184477	0.13035098
Hs00428287_m1	ETSI	1	0.210016551	0.07157969	0.362044548	0.04138817	0.247046295	0.02650817
Hs00176573_m1	FLT1	1	0.985074861	0.00266292	0.594897976	0.21118486	0.736808955	0.24560298
Hs00159829_m1	FURIN	1	0.250060658	0	0.882031225	0.17744411	0.792305501	0.20793713
Hs99999905_m1	GAPDH	1	1.863583579	0.56161016	1.752928343	0.15300809	1.764305018	0
Hs00892681_m1	GLUT-1	1	1.07105347	0.18513245	1.382047848	0.34026831	1.298590875	0.18033526
Hs00173566_m1	GPX3	1	0.424221083	0.06684871	0.407996882	0.0411627	0.523331336	0.03226154
Hs00427665_g1	HSP90	1	0.418900578	0.01135029	0.337615237	0.04282718	0.35912301	0
Hs00747379_m1	ID2	1	2.697360929	0.59027376	2.2214731	0.2884619	4.17608719	0.00665767
Hs00426285_m1	IGFBP1	1	2.802271901	0.70269301	0.328114202	0.15939454	0.833873242	0
Hs00174877_m1	LEP	1	undetermined	0	undetermined	0	undetermined	0
Hs00184700_m1	LOX	1	1.373998739	0.12380646	0.905106805	0.23838815	0.750532048	0.12543591
Hs01050896_m1	MCL1	1	0.758004655	0.0918865	0.828670957	0.05131353	0.859602795	0.00971164
Hs01565581_m1	MET	1	1.134773205	0.17889415	1.291785625	0.16774046	1.465302465	0.16359975
Hs00237119_m1	MMP14	1	0.394213749	0	1.172095001	0.02542521	1.182641248	0.39421375
Hs00167155_m1	Nexin	1	0.135268418	0.03391968	0.309583407	8.5835E-05	0.209941104	0.040753
Hs02339479_g1	Nucleophosmi	1	2.349570318	0.58917432	2.676646669	0.60654733	2.772248513	0.16649612
Hs01895061_u1	OCT4	1	0.977103615	0.21020913	4.099340962	0.87187921	4.160459779	0.36887758
Hs00960942_m1	Osteopontin	1	0.525710899	0.07050221	0.538134869	0.11815269	0.653551986	0.05733888
Hs00190079_m1	PFKFB3	1	0.395015067	0.29211256	1.17440057	0.08256924	1.470217613	0.09596235
Hs00190096_m1	PFKFB4	1	0.646370184	0.46805518	1.360229172	0.25350171	1.169373857	0.28722135
Hs99999906_m1	PGK-1	1	1.34711916	0.2291348	0.71413398	0.09273146	0.649448294	0.01770953
Hs00254392_m1	PHD2	1	1.560394121	0.25968353	1.27283702	0.39190659	1.283190073	0.37315125
Hs00222966_m1	PHD3	1	1.079552829	0.06819046	1.610683425	0.48889547	1.728157063	0.58041377
Hs00382168_m1	PMAIP1	1	0.384960061	0.03468747	0.592092205	0.09861683	0.545706131	0.15483905
Hs00930455_m1	SDF-1	1	0.494810131	0.12407776	0.501011864	0.06505721	0.925277058	0.02997277
Hs99999022_m1	TERT	1	0.626439811	0.4192464	1.739803834	0.17308098	2.516026419	0.42465399
Hs00608187_m1	Tgf-α	1	0.519123274	0.28858152	1.28822825	0.20382601	1.38281941	0.15661457
Hs01086000_m1	Tgf-β	1	1.662721964	0.41694137	1.68356179	0.21861324	2.019268453	0.09601353
Hs01067777_m1	Transferrin	1	1.043481463	0.27392451	1.361183941	0.22010389	1.250933651	0.00338161
Hs99999070_m1	VEGF	1	1.10186254	0.51371501	4.49013947	1.38673874	4.846732638	1.61557755

Lactate and VEGF concentrations correlate with PTEN expression in hypoxia

As several glycolytic genes seemed to be positively regulated by PTEN in hypoxia, a lactate production assay was performed to determine differences in cell metabolism following PTEN modulation. Interestingly, PTEN loss led to a corresponding decrease in lactate production in hypoxia compared to WT cells, ostensibly due to the diminished expression of glycolytic genes (Figure 14A). As the glycolytic pathway is a step-wise process, enzymatic deficiencies upstream of pyruvate production, such as PFKFB3, PFKFB4 and PGK-1, may account for the observed difference in lactate production.

Similarly, a VEGF ELISA was performed to quantify VEGF secreted protein into the cell supernatant over a timecourse of hypoxia. As expected from the HRE-luciferase reporter assay, PTEN loss resulted in a decrease in VEGF expression and secretion compared to cells expressing PTEN (Figure 14B).

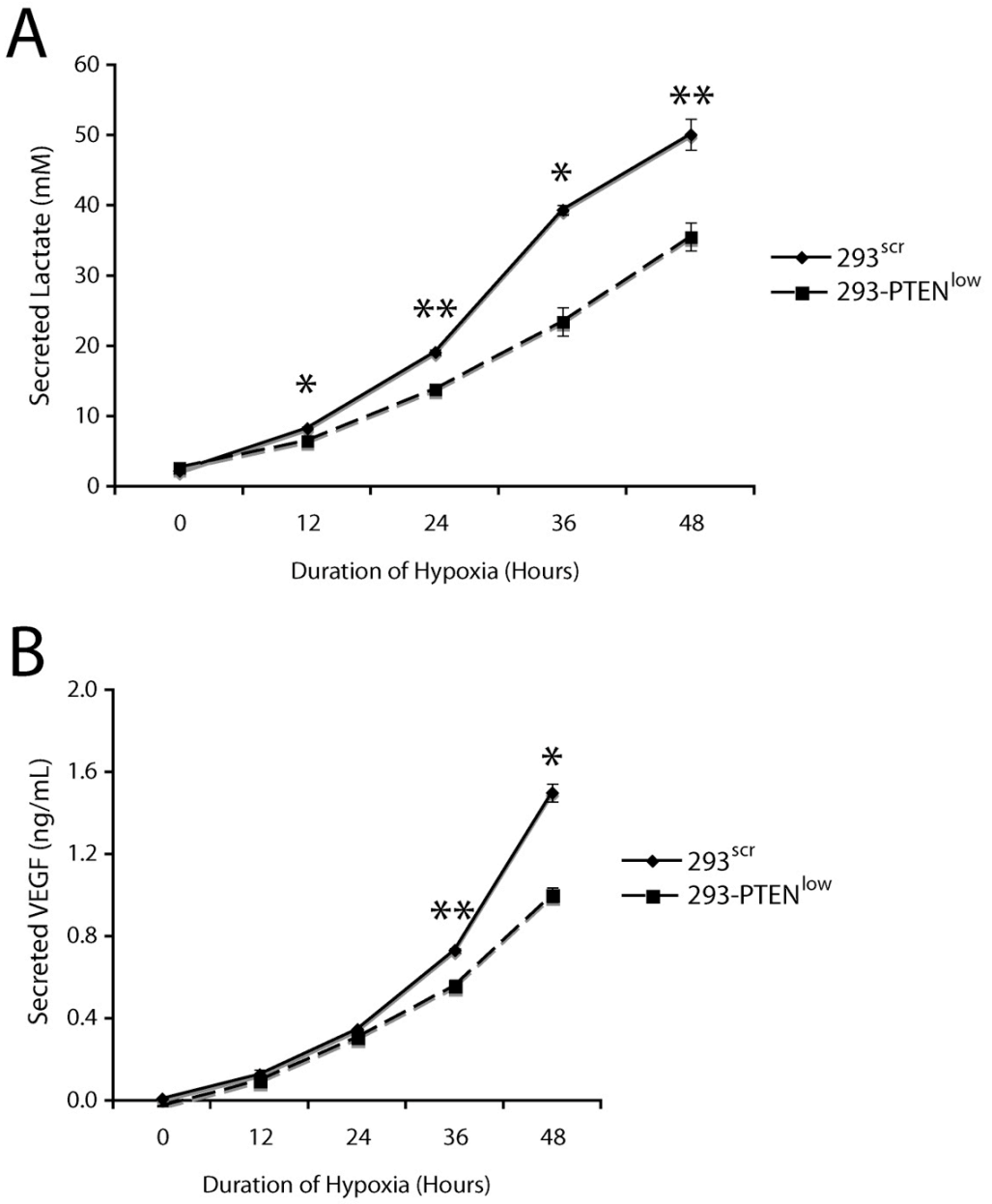

Figure 14: Lactate and VEGF production in hypoxic cells correlate with PTEN expression

A timecourse from 0 to 48 hours of hypoxia comparing supernatants from 293^{scr} and $293\text{-}PTEN^{low}$ cells demonstrates diminished lactate (A) and VEGF (B) production in cells lacking PTEN compared to those expressing endogenous PTEN levels (*, $P < 0.05$; **, $P < 0.005$).

Identification of PTEN mutations in cancer cell lines

During the screening of a variety of both normal and transformed cell lines for PTEN's effect on the hypoxia response, we observed a paradoxical response in the HRE-luciferase assay in an inducible-PTEN U87 cell line ($U87^{Tet\text{-}On:PTEN}$). Despite expressing catalytically active PTEN, these cells had minimal HRE-luciferase activity in the setting of hypoxia following induction of PTEN by doxycycline (Figure 15A). However, uninduced U87 cells transduced with a PTEN adenovirus demonstrated the previously observed augmentation of HRE activity, which was significantly blunted when PTEN was simultaneously induced with doxycycline (Figure 15A), suggesting that the PTEN expressed in these cells might act in a dominant-negative manner relative to exogenously expressed PTEN. Sequencing revealed 11 amino acid mutations throughout the PTEN cDNA induced in these cells (Figure 15B).

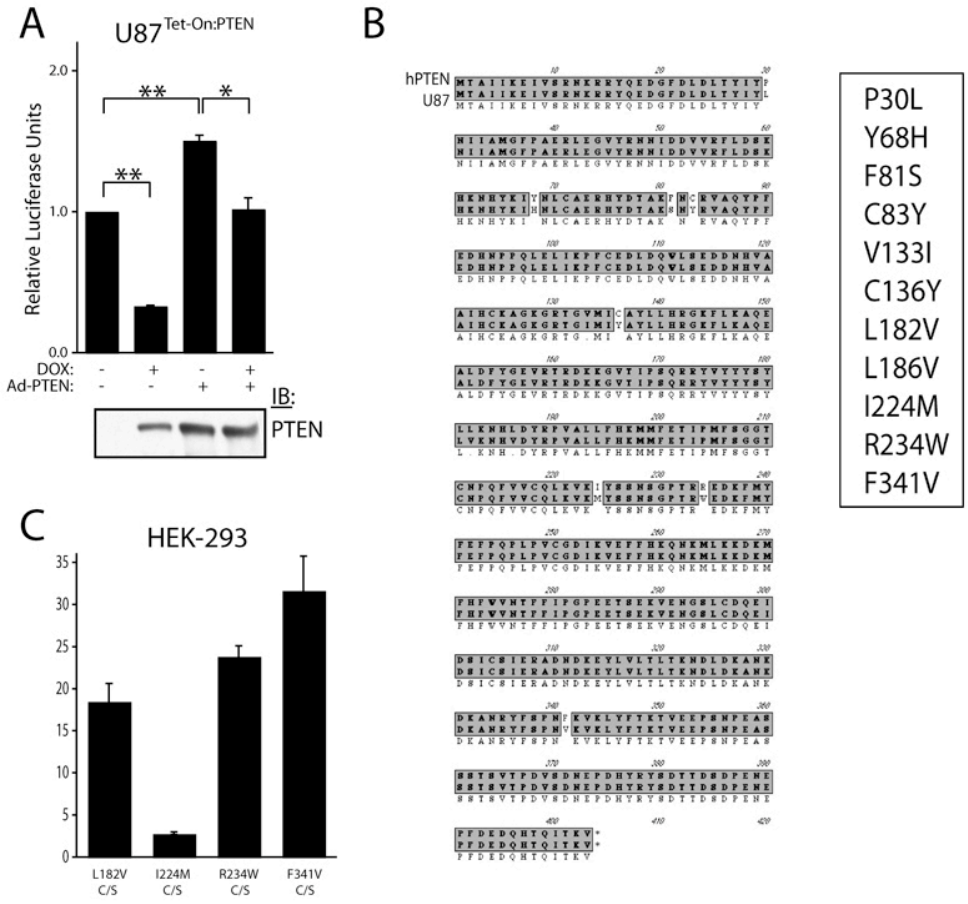

Figure 15: I224M recapitulates the dominant-negative phenotype of induced PTEN in U87 cells

(A) Doxycycline (DOX) induction of PTEN in the PTEN-null U87$^{\text{Tet-On:PTEN}}$ cell line resulted in a decrease in baseline HRE activity, in opposition to results obtained following adenoviral transduction of PTEN-WT despite similar levels of PTEN expression (bottom). Co-expression of induced PTEN and (Ad) PTEN-WT resulted in an intermediate level of HRE activity, suggesting a dominant-negative effect of the induced PTEN. (B) Alignment of the Dox-induced PTEN (U87) with the published PTEN cDNA sequence (hPTEN) (*left*) revealed 11 missense mutations (*right*). (C) Overexpression of a PTEN cDNA containing a single Ile224Met mutation in hypoxic HEK-293 cells recapitulated the dominant-negative phenotype observed with induced PTEN in U87$^{\text{Tet-On:PTEN}}$ cells, while L182V, R234W, and F341V did not show this dominant-negative phenotype (*, $P < 0.05$; **, $P < 0.005$).

Isoleucine 224 is required for PTEN's effect on hypoxia-mediated gene expression

From previous results demonstrating that PTEN's effect on HRE activity was independent of PTEN's catalytic activity and seemed to be augmented by forced nuclear PTEN expression, we hypothesized that PTEN was acting as a nuclear scaffold. This, in combination with reports of an interaction between PTEN and p300 that required the C2 domain of PTEN, led us to focus on mutations within the C2 domain of $U87^{Tet\text{-}On:PTEN}$ PTEN. Mutagenesis of PTEN-WT was performed to generate single amino acid mutations representing four of the identified mutations.

cDNA constructs containing each of 4 different amino acid substitutions observed in the U87 mutant PTEN were generated individually by site-directed mutagenesis, transiently transfected into HEK-293 cells, and HRE-luciferase activity was assessed. Interestingly, a single amino acid substitution at residue 224 (I224M, I/M) recapitulated the dominant negative phenotype observed in $U87^{Tet\text{-}On:PTEN}$ cells when this cDNA was overexpressed in HEK-293 cells (Figure 15C). PTEN-I/M was also unable to induce expression of specific genes previously shown to be positively regulated by wild-type PTEN in hypoxia when it was overexpressed in HEK-293 cells (Figure 16A). Importantly, PTEN-I224M overexpression also inhibited the hypoxia-induced up-regulation of protein expression for these targets after 12 hours of hypoxia (Figure 16B). Expression of PTEN-I224M had no untoward effects on cell viability after 12 hours of hypoxia as assessed by DNA fragmentation and caspase-3 cleavage (Figure 17A,B).

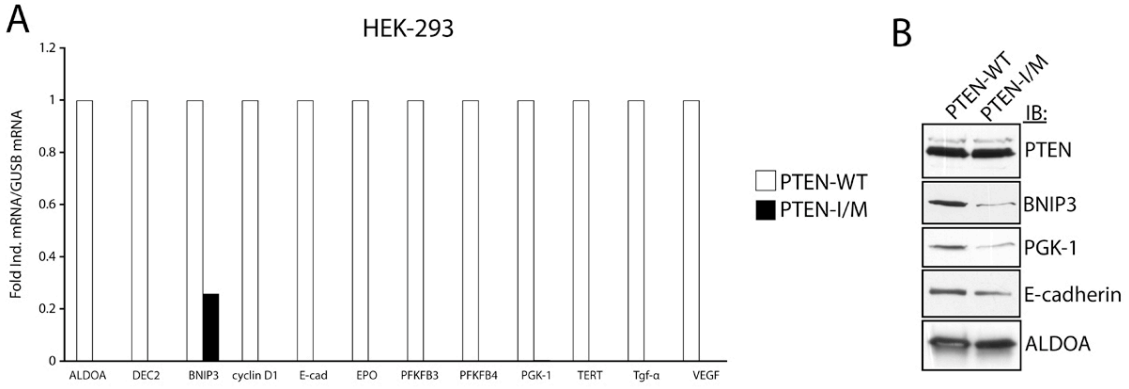

Figure 16: PTEN-I224M is unable to induce hypoxia-responsive gene transcription or protein expression after 12 hours of hypoxia

(A) Overexpression of PTEN-I224M in HEK-293 cells dramatically inhibits hypoxic gene transcription of 12 candidate genes independently validated by real-time PCR. (B) Immunoblotting lysates from cells overexpressing PTEN-I224M demonstrates inhibition of hypoxic upregulation of several hypoxia-responsive proteins after 12 hours of hypoxia compared to cells overexpressing PTEN-WT.

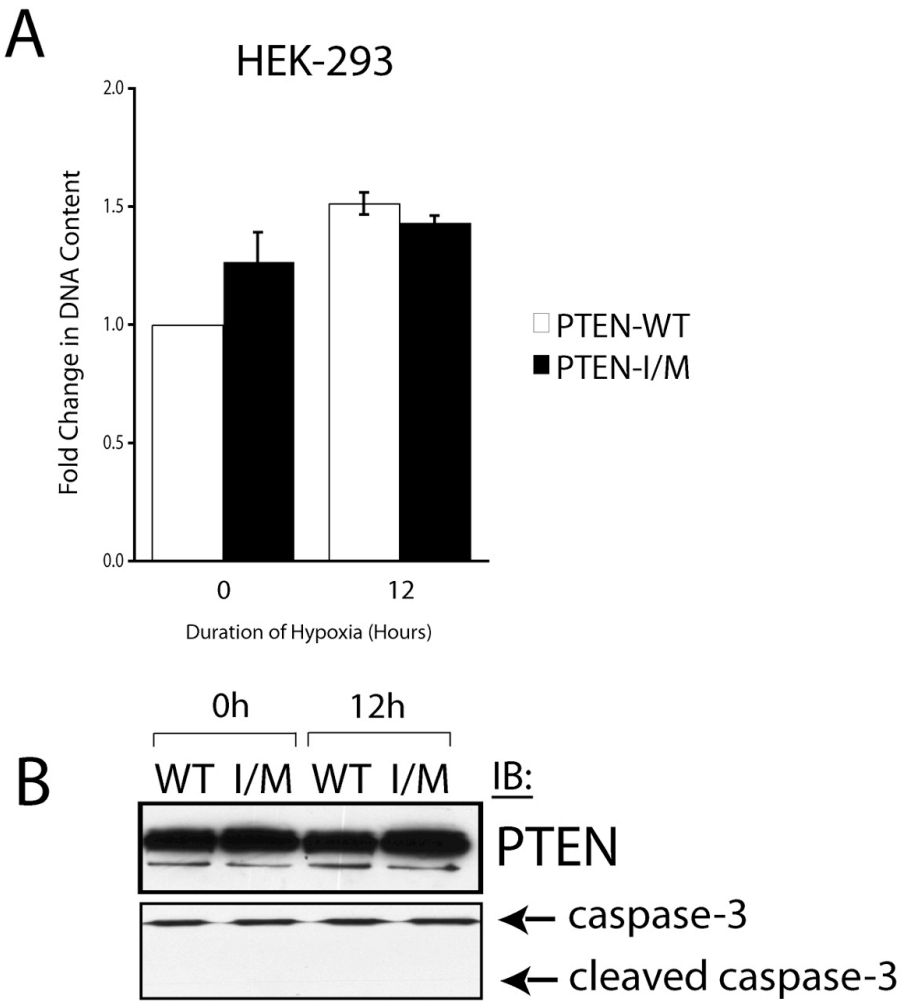

Figure 17: Expression of PTEN-WT or PTEN-I224M has no cytopathic effects on cells after 12 hours of hypoxia

(A) Genomic DNA harvested from cells overexpressing PTEN-WT or PTEN-I224M at normoxia and after 12 hours of hypoxia shows no loss in total DNA content, a hallmark of DNA fragmentation. (B) Caspase-3 cleavage is not observed in lysates from cells overexpressing PTEN-WT or PTEN-I224M in normoxia or after 12 hours of hypoxia.

PTEN promotes stabilization and activation of p300

Because the histone acetyltransferase (HAT) p300 is an important co-activator for HIF, we investigated whether the effects of PTEN on hypoxia-mediated gene expression were regulated through p300. HEK-293 cells were transiently transfected with constructs expressing PTEN-WT or PTEN-I224M or were infected with a PTEN shRNA, and effects on p300 stability and HAT activity were analyzed. Overexpression of PTEN-WT and PTEN-I/M resulted in an increase in p300 protein at baseline and prolonged stability of p300 after 6 hours of hypoxia compared to cells expressing endogenous levels of PTEN (Figure 18A). However, the quality of p300 appears to be different between PTEN-WT and PTEN-I/M treated cells (Figure 18B). PTEN silencing led to destabilization of p300 at baseline and greater loss of the protein after 6 hours of hypoxia (Figure 18A,B). To test the functional effects of PTEN on p300, immunoprecipitated p300 from these lysates was subjected to an *in vitro* HAT assay. In cells in which PTEN had been silenced or in those overexpressing PTEN-I224M, p300 HAT activity decreased, whereas overexpression of PTEN-WT increased p300 HAT activity compared to control vector-transfected cells (Figure 19). Taken together, these data demonstrate that the positive effect of PTEN on the hypoxia response is mediated in part through effects on the HIF co-activator p300.

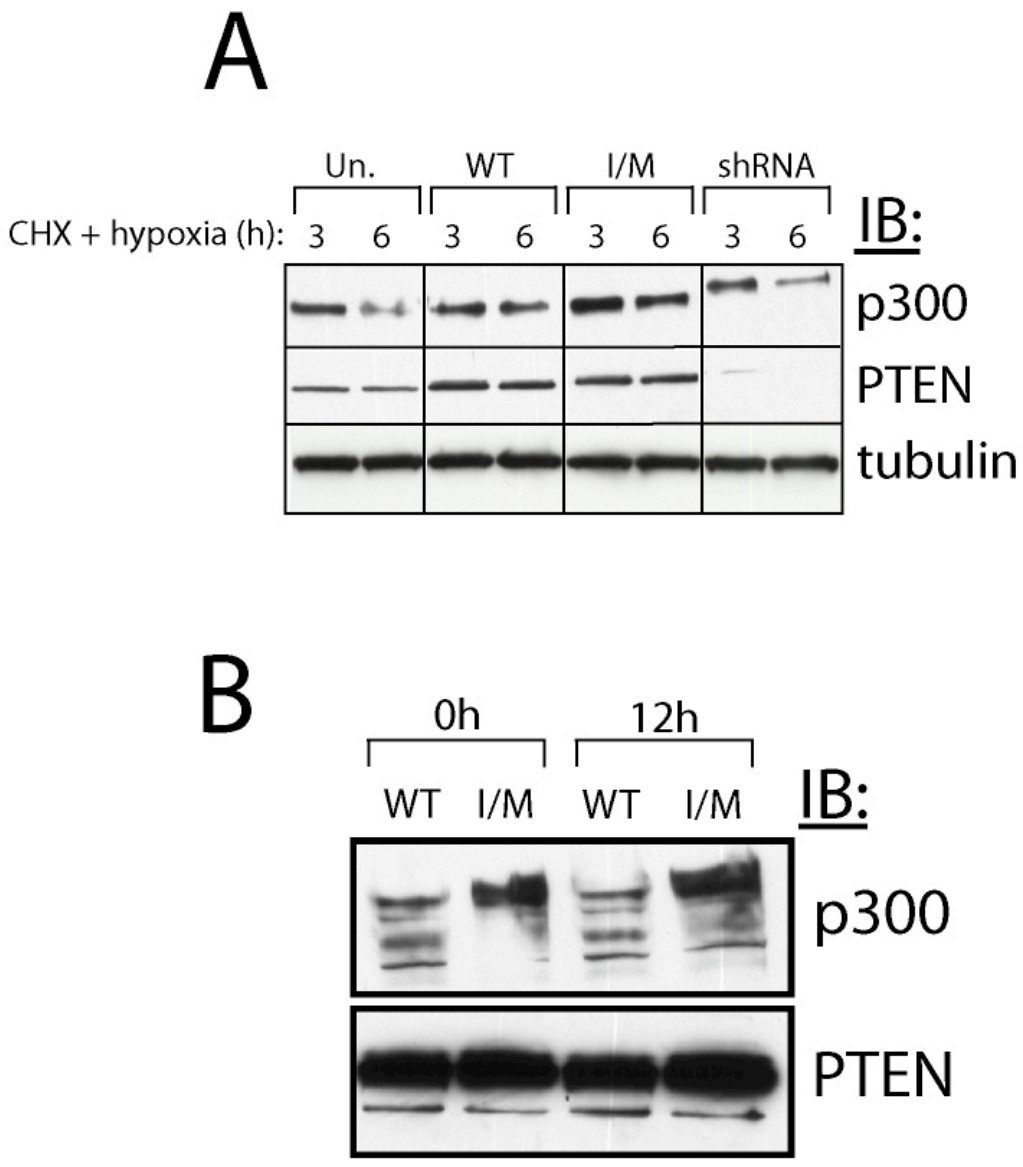

Figure 18: PTEN modulation affects p300 stabilization in hypoxia

(A) Quantitative and qualitative differences in p300 are observed between cells overexpressing PTEN-WT and PTEN-I224M (I/M) in normoxia and after 12 hours of hypoxia. (B) PTEN overexpression stabilizes p300 in hypoxic cells compared to untreated (Un.) or PTEN silenced (shRNA) cells. Cells were simultaneously treated with cycloheximide (CHX; 25 μg/mL) and hypoxia, and lysed after 3 and 6 hours.

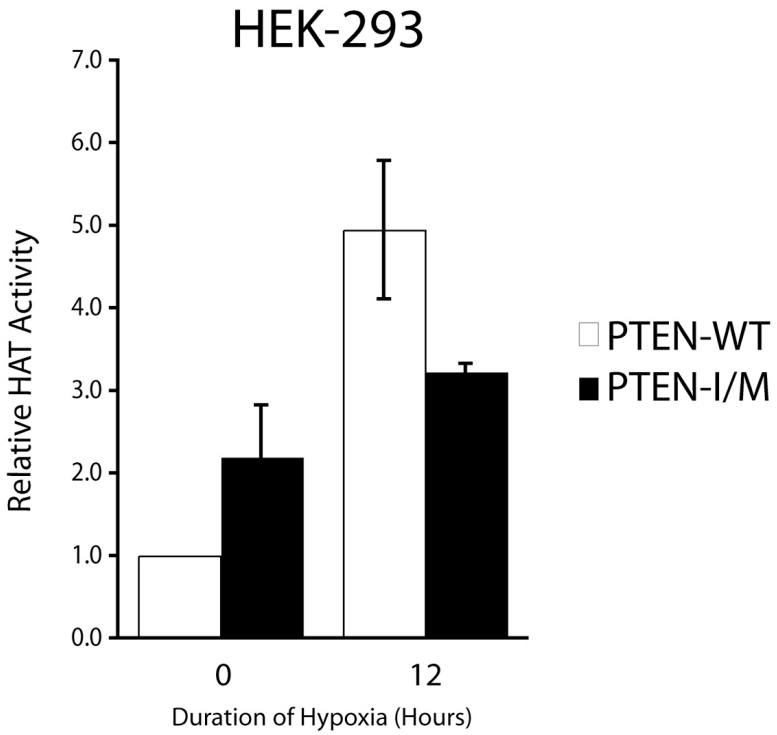

Figure 19: PTEN overexpression enhances p300 HAT activity

HEK-293 cells transiently overexpressing PTEN-WT or PTEN-I224M were subjected to hypoxia or left untreated, lysed and p300 was immunoprecipitated. An *in vitro* HAT assay was performed on the immobilized p300 and HAT activity was compared relative to PTEN-WT treated cells in normoxia.

PTEN regulation of p300 modulates p53 target gene transcription

p300 is involved in many cellular processes, including acetylation and stabilization of p53. Therefore, we next sought to determine whether modulation of PTEN expression would affect p300-dependent p53 stabilization. PTEN expression was either silenced in 293 cells or overexpressed with constructs encoding PTEN-WT or -I224M. Cells were subjected to genotoxic stress with mitomycin C, and cell lysates were used in a p53-luciferase reporter assay. Consistent with previous reports, PTEN-WT was able to increase p53 transcriptional activity, whereas overexpression of PTEN-I224M had no effect. Results for p53 transcriptional activity at baseline and following DNA damage resulting from mitomycin C treatment are shown (Figure 20).

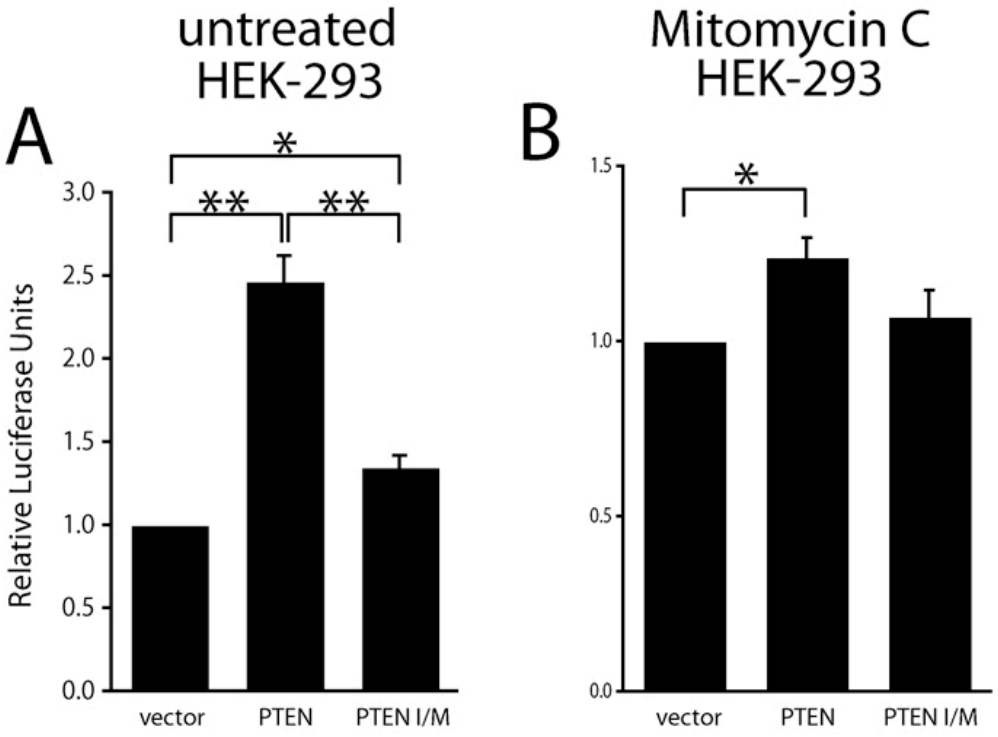

Figure 20: PTEN-WT but not –I224M enhances p53 activity at baseline and following mitomycin C treatment

HEK-293 cells were co-transfected with a p53-luciferase reporter and either empty vector, PTEN-WT or PTEN-I224M and left untreated (A) or exposed to mitomycin C (2 μg/mL) for 4 hours (B), and luciferase activity was quantified relative to vector-treated cells. (*, $P < 0.05$; **, $P < 0.005$).

PTEN expression correlates with the degree of hypoxia in tumors

PTEN expression in tumors has been negatively correlated with tumor growth and angiogenesis(188,189). Based on the unexpected finding that PTEN is both necessary and sufficient for hypoxia-mediated gene transcription, we investigated the effect of PTEN loss on tumor hypoxia *in vivo*. HEK-293 cells stably expressing either a scrambled shRNA (293^{scr}) or a PTEN-targeted shRNA ($293\text{-}PTEN^{low}$) were injected into the flanks of nude athymic (nu/nu) mice. Prior to sacrifice, mice were injected intravenously with Hoechst 33342 as a marker of perfusion and with the EF5 hypoxia marker. EF5 is a 2-nitroimidazole drug that is reduced in live cells and forms adducts with protein thiols in < 1% O_2. Bound EF5 is detected by a monoclonal antibody conjugated to a fluorophore. This detection allows quantitation of EF5 binding, one of the primary advantages to using EF5 preferential to other hypoxia markers. Quantitation of hypoxic regions on resultant tumor sections demonstrated that $PTEN^{low}$ tumors had a significantly greater hypoxic area compared to control, PTEN-expressing tumors (Figure 21A). Although $PTEN^{low}$ tumors were significantly larger (Figure 21B), this size difference did not appear to be responsible for the difference in hypoxia, as 293^{scr} tumors had grown well beyond the diffusion distance for oxygen. Capillary density was not significantly different between tumor groups (Figure 21C). Representative tumor images show the dramatic difference of EF5 staining between the groups (Figure 21D,E). Immunofluorescence microscopy of serial sections validated PTEN status (Figure 21H,I) and captured capillary density (Figure 21F,G). These findings suggest that the accumulation of larger hypoxic

regions resulted from a failure to mount an appropriate hypoxia response in the absence of PTEN expression.

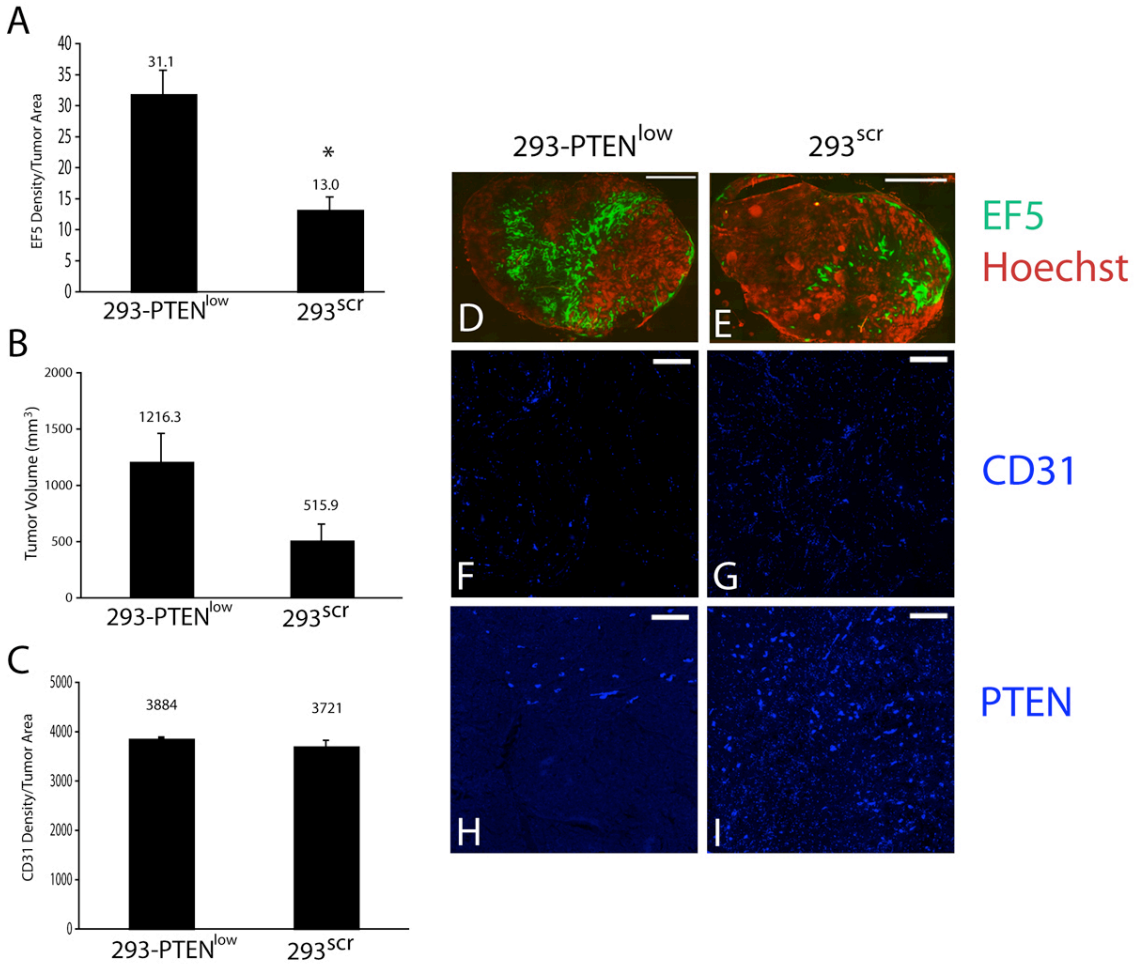

Figure 21: PTEN expression negatively correlates with the degree of hypoxia in tumors

Subcutaneous tumors were generated by injecting 1 x 10^6 293-PTEN^{low} or 293^{scr} cells into the flanks of nude mice. Prior to sacrifice, mice were injected with EF5 to quantify hypoxic regions and Hoechst 33342 to quantify perfusion. EF5 was stained with a monoclonal antibody. (A) Hypoxic area was quantified as the area of EF5 staining per total tumor area. (B) Tumor volume was estimated from caliper measurements using the formula: $V=(\pi LW^2)/6$, where V is tumor volume, L is tumor length and W is tumor width, and averaged within groups. (C) Average ratios of CD31 density between 293-PTEN^{low} and 293^{scr} tumors. (D-E) Representative images of EF5 (hypoxia) and Hoechst (perfusion) staining are shown for similarly sized tumors from each group (scale bar=3 mm). (F-G) The endothelial stain CD31 was performed on serial sections to assess capillary density (scale bar=500 μm). (H-I) PTEN staining verified decreased PTEN expression in 293-PTEN^{low} tumors compared to 293^{scr} tumors (scale bar=500 μm) (*, $P < 0.05$).

3.3 Discussion

These data, together with previous reports, further define an important new role for PTEN in the nucleus apart from its canonical role as a lipid phosphatase in the PI3K/Akt pathway. As shown in Figure 10, PTEN is able to modulate hypoxia-mediated gene expression in a variety of cell types, although some tumor lines did not respond to PTEN overexpression. This failure to respond to PTEN modulation may be due to stabilization of HIF at baseline in these cells or hyperactivation or mutation of HIF activators or repressors.

Early observations that PTEN-C/S was as effective as PTEN-WT in positively regulating HRE activity suggested that the physical presence of PTEN was sufficient to elicit this effect. Together with localization studies, these findings suggest that PTEN scaffolding with other proteins is integral to the mechanism of this process. Although we did not identify proteins that differentially associate with PTEN in hypoxia compared to normoxia, identification of Ile224 as a key residue that is able to modulate PTEN's hypoxic function may facilitate these types of studies in the future.

We have shown that PTEN modulates both p300 stability and activity in hypoxia. Although modest, even small differences in p300 function may have dramatic effects, as p300 is often the limiting factor in a number of cellular processes. As seen in Figure 18A, overexpression of either PTEN-WT or PTEN-I/M is sufficient to stabilize p300 in hypoxia, although the quality of p300 may vary depending on the species (WT or I/M) of PTEN present (Figure 18B).

Together with modeling data (Figure 3E,F), we hypothesize based on these results that PTEN-WT is able to stabilize p300 in hypoxia either through a direct or indirect interaction. This stabilization effect on p300 seems to be abolished following either loss of, or mutation of PTEN (I/M). Based on modeling observations, it is likely that the Ile224Met mutation primarily effects PTEN's tertiary structure, which may account for its dramatic phenotype in hypoxia.

Furthermore, this work has validated the proposed role of PTEN in hypoxia with an animal tumor model. *In vivo*, PTEN seems to have dramatic effects on the ability of cells to overcome tissue hypoxia and mount a hypoxic response. This has clear implications in radiation and chemotherapy, which are more effective in perfused and oxygenated tissues. As PTEN continues to be implicated in novel processes, including those not requiring its catalytic activity, a better classification of PTEN mutations arising in tumors may be warranted in order to better understand and characterize their respective functional effects, whether they are catalytically-dependent or –independent.

4. Conclusions and Future Directions

The role that hypoxia plays in tumors is complex, as hypoxia is known to regulate tumor growth, metabolism and angiogenesis, and to influence the efficacy of many therapeutics. A better understanding of the cellular responses and signaling pathways resulting from hypoxia in non-transformed cells may shed light on tumorigenesis, a process in which these pathways are often usurped and hyperactivated. A more comprehensive evaluation of the hypoxia response will require identification of previously unrecognized modulators of this process. To date, there have been very few studies that have investigated the role of PTEN in tumor hypoxia outside of its role in the PI3K/Akt pathway, despite its known association with the HIF co-activator p300. The published studies demonstrate a negative regulatory role for PTEN consistent with its role in the PI3K/Akt pathway. However, emerging research from this body of work and others suggests a broader function for PTEN in the cell apart from its lipid phosphatase activity. Numerous reports have documented disparate nuclear functions of PTEN including the regulation of chromosomal stability and the cell cycle. Here, we have defined yet another function for PTEN independent of its lipid phosphatase activity.

PTEN's tumor suppressor action lies in its negative regulation of the PI3K/Akt pathway by dephosphorylating PIP_3 to inactive PIP_2, directly antagonizing the action of the PI 3-kinase. Thus, the presence of PTEN regulates PI3K's ability to propagate growth stimulatory signals transduced by growth factors and their receptors. As the PI3K/Akt pathway is a primary signal

transduction cascade able to robustly affect cell behavioral changes such as proliferation, growth, and migration, PTEN is crucial in maintaining cellular homeostasis. Thus, the understanding of how PTEN is regulated at the molecular level and identification of additional functions of PTEN outside of its canonical role as a negative regulator of the PI3K pathway, are of critical importance.

As described in this thesis, we have identified a novel role for PTEN in positively regulating the cellular response to hypoxia. Given the ubiquitous presence of PTEN and its frequent loss during cellular transformation, together with the regular oscillations in tissue oxygen tension, the study of PTEN in hypoxia may reveal key insights into cellular transformation and tumor metabolism, and these insights may lead to more effective therapeutic strategies for tumor cell killing.

This dissertation work demonstrates a role for PTEN independent of its lipid phosphatase activity and apart from its role in the cytosol as a positive modulator of HIF-mediated gene transcription in hypoxia. To our knowledge, only one other report has shown a role for PTEN in promoting HRE activity, and that study was restricted to HIF-2α gene targets in VHL-null renal cell carcinoma cells(180). My findings expand on this observation, as the presence of PTEN increased the transcription of both HIF-1α and -2α gene targets. Also, my studies have been performed in a wide range of cell types, including primary, immortalized, and transformed cells. These observations naturally led to an investigation of the mechanisms responsible for PTEN's positive effect on the hypoxia response.

PTEN interacts with a number of proteins, primarily through its C-tail or PDZ-binding motif. Some of these interactors include MAGI-2 and β-catenin, which associate with PTEN at adherens junctions, a host of PDZ-domain containing proteins, as well as p53 and p300. Although the association of PTEN and p300 was initially reported to increase p53 activity, this interaction may have other functions as well. p300, a known HAT, is a co-activator for the HIF-α proteins. Thus, p300 activity and stability were tested and found to be modulated by PTEN in hypoxia. Although the precise residues required for PTEN's interaction with p300 are unknown, the C2 domain is known to be required(103). Likewise, the C2 domain of PTEN was required to augment the hypoxia response, which we predict to be through interaction with the p300 co-activator. The PDZ-like binding domain of PTEN was not required, suggesting that PTEN scaffolding with PDZ-domain containing proteins is likely not responsible for PTEN's effect (Figure 11D,E).

A loss-of-function mutation of PTEN was serendipitously discovered during the screening of cancer cell lines for PTEN's effects of the hypoxia response. Despite having functional lipid phosphatase activity, PTEN-I224M was unable to induce hypoxic gene transcription. This single amino acid mutation separates PTEN's role in the PI3K pathway from its role in the hypoxic nucleus. Although it is not entirely clear how this residue changes PTEN's function, protein modeling suggested that the Ile224 residue is on the surface of PTEN, within a groove, and that mutation of this residue to a larger Met may alter the conformation of the face of the C2 domain, which is predicted to interact with

p300 (Figure 3E,F). However, it is unclear from our results whether this mutation affects PTEN's ability to bind p300.

We further delineated specific gene targets affected by PTEN's modulation of the hypoxic response. A custom array consisting of 46 hypoxia-responsive genes demonstrated that ~25% (11) of genes were positively regulated following PTEN overexpression. These candidate genes and others were validated independently through real-time and protein analysis. Although p300 is a global co-activator of HIF proteins, the inability of PTEN to modulate 100% of the genes tested may be due to gene specific co-activators or repressors. Given the range of target genes PTEN expression affects, the next step was to determine the functional consequences of this effect.

In vivo studies clearly showed a dramatic difference in the degree of hypoxia observed in tumors derived from $PTEN^{scr}$ and $PTEN^{low}$ cells. Although $PTEN^{low}$ tumors were larger than $PTEN^{scr}$ tumors, both groups of tumors were well beyond the diffusion limit for O_2. A comparison of similar-sized tumors between the two groups demonstrates the difference in hypoxic regions. This experiment served as proof of principle that the effects of PTEN on cellular responses observed *in vitro* translate to physiological effects *in vivo*.

4.1 Implications for Tumor Biology

The dramatic difference in the degree of hypoxia between PTEN-null and PTEN-positive tumors suggests a potential role for PTEN mutation status, in conjunction with p53 status, in predicting the response to radiation therapy. As

discussed below, the opposing functions of PTEN in the PI3K pathway and as a positive regulator of HIF and p53 make prediction of the benefits of radiation therapy difficult. Since radiation therapy requires HIF and oxygen to kill tumor cells, one would expect a poor response in PTEN-null cells, in which the hypoxic response is blunted, compared to PTEN-positive tumors. Consistent with this notion, re-expression of PTEN in combination with radiation therapy has been shown to induce a greater degree of cell death compared to radiation or PTEN alone(69). However, an important implication of this thesis work is the recognition that mutations in PTEN that disrupt its catalytic activity while preserving PTEN expression may lead to a "double hit." With such mutations, tumors would be expected to have enhanced growth and metastatic potential due to increased PI3K/Akt activity, but they would also have a preserved hypoxic response through PTEN's nuclear scaffolding activity, which could confer a growth advantage. Moreover, other roles of PTEN that are independent of its lipid phosphatase activity would be preserved, such as its roles in promoting p53 activity and chromosomal stability(98,100). Improved screening for PTEN mutations may shed light on additional functions of PTEN and key residues involved in these functions.

Another approach to studying the effects of PTEN on tumor hypoxia is to investigate the breast, thyroid and endometrial cancers that frequently arise following germ-line mutations of PTEN, as found in Cowden Syndrome and Bannayan-Zonana patients. It would be interesting to correlate the hypoxic area in these tumors with the nature of the PTEN mutation. Again, more

sophisticated characterization of the type of PTEN mutation in these patients may lead to more successful treatment of these tumors and a better understanding of the key residues involved in various PTEN functions.

4.2 Implications for Normal Physiology

PTEN's role in modulating hypoxic gene expression has biological implications that extend beyond tumor pathology and physiology. The majority of human tissues are exposed to normoxic conditions ($pO_2 \geq 20$ mmHg), however brief periods of intermittent or transient hypoxia are common in the cellular microenvironment. This intermittent hypoxia may arise from normal vascular remodeling, a decrease in red blood cell flux, thrombosis or an increase in the cellular oxygen demand. These events result in a drop in cellular pO_2 and induction of hypoxia-responsive genes, which elicit various physiological responses that simultaneously decrease cellular oxygen demand and increase oxygen delivery. As studies on primary endothelial cells (HUVECs) demonstrate, PTEN is required for hypoxic gene transcription in normal, nontransformed cells. This finding, together with the ubiquitous expression of PTEN, places PTEN at the heart of the oxygen sensing pathway inherent to each cell.

PTEN may also play an important role in chronically hypoxic tissues, such as the thymus and bone marrow. Although the role of chronic hypoxia in these tissues is not completely understood, it is thought that the decreased O_2 tension characteristic of the bone marrow and thymus is required to maintain immature hematopoietic and immune cells found therein. It would be interesting to

determine the requirement for PTEN in the maintenance of these cells. The study of patients with diminished PTEN expression, such as Cowden Syndrome and Bannayan-Zonana patients, could potentially provide insight into the role of PTEN in chronically hypoxic niches and the maintenance of immature cells within these hypoxic tissues.

Another example of chronic hypoxia in normal physiology is in the developing embryo prior to complete vascular development. PTEN-null embryos are embryonic lethal, ostensibly due to hyperproliferation, although this thesis provides data for the requirement of PTEN in hypoxic gene transcription. Genetic studies with the separation of function mutant identified in this work, PTEN-I224M, would delineate the relative contributions during embryogenesis of PTEN's lipid phosphatase activity and its role in regulating hypoxia-mediated gene transcription. The definitive experiment would be to determine the viability of embryos homozygous for the PTEN-I224M mutant, which is catalytically active. Other genetic manipulations in which only one allele contains the I224M mutation would be useful in parsing out the role of PTEN in the developing hypoxic embryo.

4.3 Future Directions

This dissertation has defined an important phenotype of the PTEN-I224M mutant, such that it acts as a dominant negative inhibitor in hypoxia but retains its lipid phosphatase activity. This mutant is intriguing and should be tested both *in vitro* and *in vivo* to further define the functional outcomes following its

expression in hypoxia. For example, would I224M cells, which are unable to induce HIF target genes such as E-cadherin, PGK-1 and ALDOA, or cyclin D1 in hypoxia, be more migratory, less glycolytic or more quiescent, compared to cells that can induce these genes? Furthermore, it would be important to understand the functional outcomes of cells overexpressing PTEN-WT, which have seemingly opposing functional effects on the PI3K pathway and the hypoxia response. These questions have important implications for understanding the mechanisms of PTEN's role in tumor progression, as both the timing and nature of PTEN mutation or loss likely have distinct effects on the ultimate outcome.

An important outstanding question is what effects the I224M mutant will have on the hypoxia response *in vivo*. To eliminate growth effects of PTEN loss observed with the *in vivo* study in this dissertation, all implanted tumors should stably express a catalytically inactive PTEN mutant containing either Ile224 or Met224. Tumors expressing PTEN with both of these mutations (C124S and I224M) would expect to have different characteristics compared to a tumor expressing WT PTEN, no PTEN, or a tumor expressing only one of these mutations. Table 2 projects tumor characteristics for tumors with various degrees of PTEN expression, and the cumulative effect on radiation response. Although PTEN and PTEN-I/M have opposing effects in the context of tumor angiogenic response (Table 2, shaded in gray), we hypothesize that the loss of the hypoxic function of PTEN (I/M) will overcome the ability of PTEN to promote tumor angiogenesis.

PC-3 and U87 cells stably expressing either I224 PTEN and M224 PTEN constructs in the C/S backbone have recently been generated in our lab. By eliminating the PI3K component of PTEN's function, effects on growth will be controlled, addressing a more reliable comparison of tumor hypoxia independent of tumor size. This would also enable radiation therapy outcomes to be tested without the confounding factor of size discrepancies. Differences in expression of hypoxia-responsive proteins in hypoxic regions of the tumors would also provide an important link to our biochemical data and would provide some insight into the effect of PTEN's scaffolding function on cancer cell phenotype.

This body of work also established a role for PTEN in stabilizing and activating p300 in hypoxia, likely through a direct interaction. We hypothesize that endogenous PTEN scaffolds with p300 and HIF-α in the hypoxic nucleus through PTEN's C2 domain and increases hypoxic gene transcription by increasing p300 protein and activity and recruiting additional co-activators to the HIF complex. This proposed mechanism also explains the effects of PTEN-WT overexpression, which robustly activates HRE activity. Loss of PTEN, however, fails to stabilize p300, which is still able to promote HIF-mediated gene transcription, although to a lesser degree. The presence of gene-specific co-activators and co-repressors at the HRE may lessen or enhance, respectively, the effects of loss of PTEN. The presence of the PTEN-I224M mutant exerts a dominant negative behavior on hypoxic gene expression by recruiting

transcriptional co-repressors and/or inhibiting co-activator association due to PTEN's altered tertiary structure. This proposed model is illustrated in Figure 22.

A better understanding of the mechanism by which PTEN-WT promotes and PTEN-I224M inhibits p300 is needed. As PTEN and p300 are both known to have many protein interactors, a mass spectrometry approach may be the most efficient means to uncover potential differences in co-activators or co-repressors interacting with PTEN-WT and PTEN-I224M.

Table 2: Projected characteristics of tumors with varying PTEN expression states

	$PTEN^{low}$	$PTEN^{scr}$	$PTEN^{WT\text{-}R}$	$PTEN^{C/S\text{-}R}$	$PTEN^{WT\text{-}I224M\text{-}R}$	$PTEN^{C/S\text{-}I224M\text{-}R}$
PTEN expression relative to $PTEN^{scr}$:	↓	—	↑	↑	↑	↑
PTEN catalytic activity relative to $PTEN^{scr}$:	↓	—	↑↑	↓↓	↑↑	↓↓
PTEN hypoxic activity relative to $PTEN^{scr}$:	↓	—	↑↑	↑↑	↓↓	↓↓
tumor size relative to $PTEN^{scr}$:	↑	—	↓	↑	↓	↑
tumor angiogenesis relative to $PTEN^{scr}$:	↑	—	↓	↑	↓	↓
hypoxia response relative to $PTEN^{scr}$:	↓	—	↑	↑↑	↓↓	↓
tumor hypoxic area relative to $PTEN^{scr}$:	↑	—	↓	↓↓	↑↑	↑
response to radiation relative to $PTEN^{scr}$:	↓	—	↑	↑↑	↓	↓↓

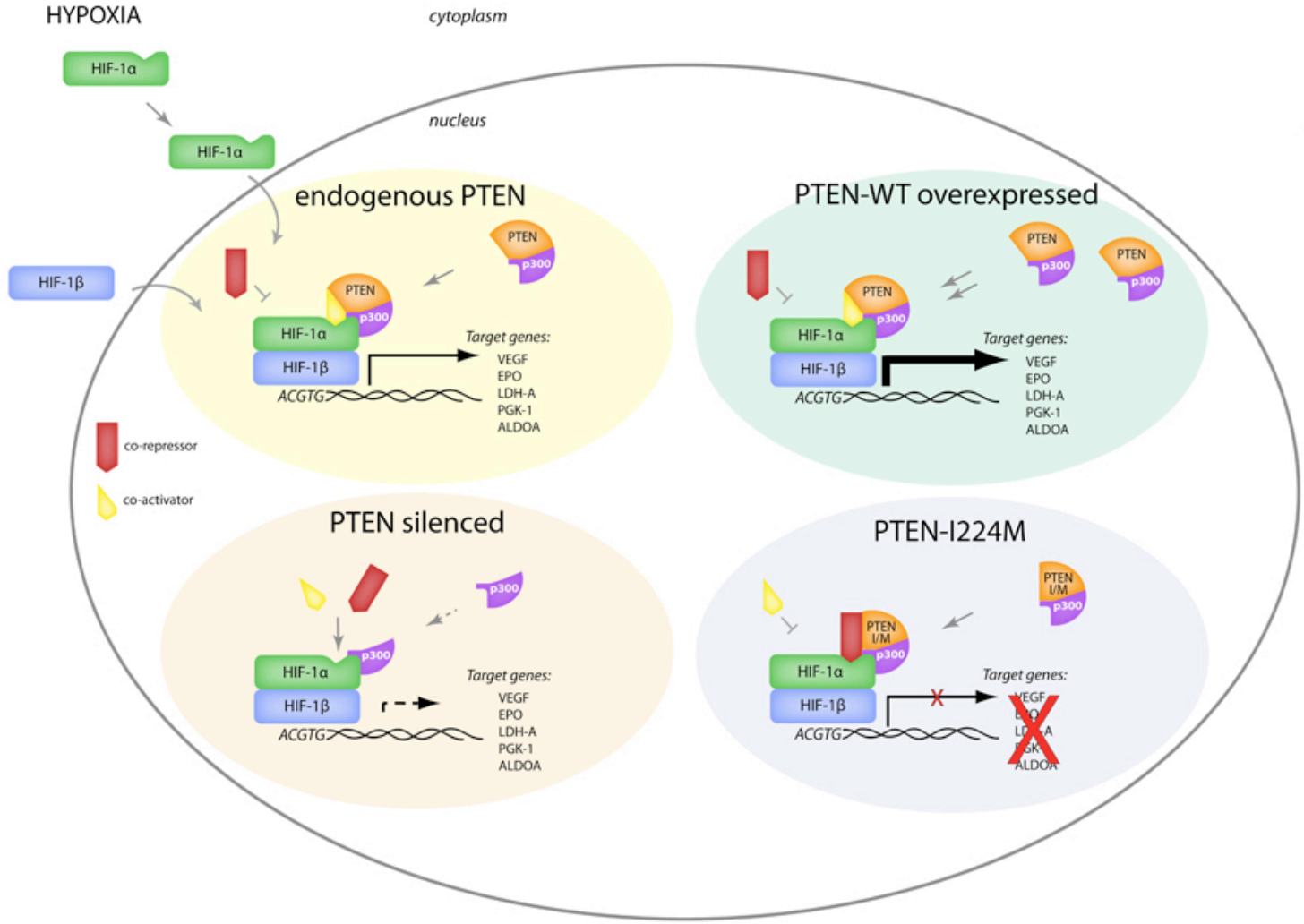

Figure 22: Proposed model of PTEN's regulation of the cellular response to hypoxia

In a cell with endogenous PTEN, PTEN promotes the hypoxic response by stabilizing p300 and increasing p300 HAT activity in the hypoxic nucleus. PTEN's association with the HIF co-activator p300 promotes target gene expression following binding of p300 with the HIF complex and recruitment of additional co-activators. Overexpression of WT PTEN functions in a similar manner, although to a higher degree. Silencing of PTEN diminishes HIF transcriptional activity as p300 is not stabilized in hypoxia by PTEN. The absence of PTEN may affect genes individually based on binding of gene-specific co-activators or co-repressors. Finally, the presence of PTEN-I224M (I/M) elicits its dominant-negative effect on the hypoxia response through an inability to scaffold with co-activators or gained ability to scaffold with co-repressors, likely through its altered C2 tertiary structure.

Consistent with previous reports, PTEN overexpression resulted in activation of p53 transcriptional activity and downstream gene expression, similar to its effects on hypoxia-mediated transcription. Ostensibly, both of these effects are mediated through p300 activation. Unique to this thesis work, however, was the finding that PTEN-I224M was unable to activate p53. Future experiments should investigate the functional consequences of this effect. In particular, this observation suggests that PTEN-I224M cells would be more susceptible to DNA damage compared to cells expressing wild-type PTEN. As a result, radiation-induced cell killing would be predicted to be more effective in PTEN-I224M cells. However, this possibility seems at odds with the hypothesis that wild-type PTEN would be required to enhance radiation-induced cell death by promoting the hypoxia response and increasing oxygen availability for the production of oxygen radicals. It would be interesting to see which of the following of PTEN's functions would predominate following radiation therapy in a PTEN-I224M tumor: the inability to up-regulate hypoxic gene expression (decreased killing), the ability to inhibit PI3K/Akt activity (increased cytotoxicity), or the inability to inhibit p53 activation (increased or decreased killing depending on the stress). Clearly, the interactions among PTEN, p53 and p300 are complex in nontransformed cells, and they likely become more so in a transformed cell in which mutations in these proteins and others are not uncommon.

It is also possible that the PTEN-I224M mutant could act as a dominant-negative inhibitor of other nuclear-defined roles for PTEN, such as chromosomal

stability or cell cycle regulation. In our analysis, expression of PTEN-I224M did not have cytopathic effects, but expression was only transient and the maximum duration of hypoxia was 24 hours.

The observation that PTEN's modulation of the hypoxia response is augmented when PTEN is localized to the nucleus provides another nuclear function for PTEN. Despite the number of nuclear functions previously described for PTEN, it is unclear where in the nucleus PTEN is acting. Structural representations of PTEN have identified key residues in the C2 domain responsible for PTEN's intimate association with membrane lipids. For example, the CBR3 domain interacts with the plasma membrane in such a way that the active site of PTEN is brought in close proximity to its substrate, phosphatidylinositol trisphosphate. Determining the nuclear sub-localization (membrane or otherwise) of PTEN is difficult but may shed light on other nuclear functions of PTEN.

It is clear from this body of work as well as others that PTEN has critical activities outside of its canonical lipid phosphatase role. The ability of PTEN to positively regulate the hypoxia response has direct therapeutic implications for the treatment of ischemic cardiovascular diseases, such as peripheral, cerebral, and coronary artery disease. In these settings, strategies targeting PTEN may aid in the recovery of stroke and myocardial infarction, in which neuronal and cardiomyocyte survival is dependent on a normal hypoxia response and is critical to the ultimate clinical outcome. The ability to separate PTEN's lipid phosphatase and hypoxic functions might allow delivery of an inactive PTEN

(PTEN-C/S) that maintained its ability to promote hypoxic gene expression while simultaneously enhancing cell survival without adversely affecting cell growth. By characterizing PTEN's role in positively regulating the hypoxia response, results from this work suggest that PTEN overexpression and subsequent induction of HRE-containing genes, such as VEGF and EPO, might actually *increase* perfusion in ischemic diseases. This hypothesis is in direct opposition to the currently accepted model of PTEN's regulation of angiogenesis, which is based solely on PTEN's role as a negative regulator of PI3K/Akt. Clearly, the novel role of PTEN in the hypoxic nucleus carries important therapeutic implications and thus deserves further investigation. In this regard, the results of this thesis have provided an important direction for studies to further investigate this novel function of PTEN.

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PUBLICATIONS

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