HIF-2: STANDING GUARD AT THE CROSSROADS OF STRESS AND AGING

APPROVED BY SUPERVISORY COMMITTEE

Joseph A Garcia, M.D., Ph.D.

Richard Bruick, Ph.D.

Melanie Cobb, Ph.D.

Andrew Zinn, M.D., Ph.D.

Yi Liu, Ph.D.

DEDICATION

To my parents Fatou Demba and Babacar Dioum and to my wife Aissatou Tall Dioum and my daughters Fatou Demba, Fatima Kine and Ngone Amy Dioum

HIF-2: STANDING GUARD AT THE CROSSROADS OF STRESS AND AGING

By

El Hadji Mamadou Dioum

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas,

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

March 2009

ACKNOWLEDGEMENTS

This study was made possible by the great support and motivation provided by my mentor Dr. Joseph A. Garcia, whom I would like to sincerely thank for giving me the opportunity to work on such fascinating projects and helping me improve my critical thinking and my scientific skills.

I owe a great amount of gratitude to my dissertation committee members. My committee chairman, Dr. Richard Bruick has been very helpful and supportive. His suggestions helped me become more efficient and laid a more critical eye on my data. Dr Melanie Cobb, Dr Yi Liu and Dr Andrew Zinn have also tirelessly helped me with their pragmatic suggestions and encouragements.

I thank the Garcia lab members for the collaborative effort provided in this work. I particularly thank Dr. Rui Chen for providing such great technical skills; his significant contribution in helping complete the Sirt1/HIF-2α project is tremendously appreciated. I would also like to thank Dr Daniel Zhang for help provided in the RT-PCR data analysis and ChIP assay. I also thank Dr. Myong Ryou for help provided in reviewing my dissertation, Alok Das, Na Wang, and Kan Ding for technical assistance

Special thanks to Dr. Robert Gerard and the Gerard lab, especially Richard Hogg, for making the adenoviral reagents for us and for help in the mice tail vein injection experiments. I acknowledge Dr. Marie-Alda Gilles-

Gonzalez for giving me rigorous training in Biochemistry and for encouraging me to pursue a Ph.D. degree. I would like to personally thank my friend and mentor Dr. Diaga Diouf for introducing me to molecular biology techniques. We acknowledge the laboratory of Dr. Fred Alt for generously providing Sirt1 knockout mice and Dr. Robert Hammer for assistance with mouse husbandry. We thank the UTSWMC Protein Core Facility, particularly Dr. Yan Li for assistance on the mass spectrometry data acquisition and analysis.

Personal thanks is directed towards all my family and friends who have supported me through the good and bad times, particularly Mamadou Dioum, Mouhamed Hafez Dioum, Mame Fatou Dioum, Ana Isabel De Caceres, Jason Tuckerman, Amar and Desirae Campbell Khouma. Finally, I am very grateful towards my family to whom I dedicate this dissertation.

I would like to thank the internal medicine department and the cardiology division for their support provided through the National Heart, Lung and Blood Institute (NHLBI) predoctoral training grant. These studies were supported by funds provided by Amgen, the American Heart Association, the Department of Veteran Affairs, March of Dimes, and the National Institutes of Health.

HIF-2: STANDING GUARD AT THE CROSSROADS OF STRESS AND AGING

ELHADJI MAMADOU DIOUM, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2008

Mentor: JOSEPH A GARCIA, M.D., Ph.D.

ABSTRACT

The capacity of mammalian organisms to cope with hypoxic or ischemic stress is in part mediated by stress-induced transcription factors. Hypoxia-induced mediators include transcription factors, such as the α subunit of Hypoxia inducible factors (HIF-1α and HIF-2α). HIF-1α and HIF-2α have similar structural organization, and after forming an obligate heterodimer

with the common partner ARNT/HIF-1β, bind to the same recognition element located in target gene promoter or enhancer regions. However, despite these similarities, HIF-1α and HIF-2α regulate distinct target genes.

In previous studies from the Garcia laboratory using mouse knockout studies, we demonstrated the importance of HIF-2α in the *in vivo* regulation of genes involved in the cellular response to hypoxic and oxidative stress. These genes include Erythropoietin (*epo*), vascular endothelial cell growth factor (*Vegf*), superoxide dismutase 2 (*Sod2*) and other genes encoding major antioxidant enzymes (AOE). Novel roles for HIF-2α have been found not only in hematopoiesis, but also in the control of reactive oxygen species and mitochondrial homeostasis. The molecular mechanism by which HIF-2α selectively regulates its target genes remains an exciting area of research.

In the first part of my thesis, I identified a novel molecular mechanism regulating activity of the enhancer region in the Epo gene. First, by using bioinformatics to perform an unbiased sequence comparison of several mammalian 3' Epo enhancer region, we identified a previously unrecognized evolutionary conserved region. Second, we determined the functional significance of these conserved sequences using transient transfection and mutation analyses in cell culture studies and determined that these sequences contribute to HIF-2α selectivity. Finally, using a candidate factor strategy, we determined that members of the Egr transcription factor family bind to these elements and act synergistically with HIF-2α to augment Epo gene expression.

In the second part of my thesis, we demonstrate that the redox-sensing, NAD^+ dependent deacetylase enzyme Sirtuin 1, also known as Sirt1 or silent mating type information regulator 2 (Sir2) homolog 1, selectively stimulates HIF-2α signaling during hypoxia. In lower organisms and cell culture models, the FoxO family of transcription factor regulates the transcription of SOD2 and other major AOE. During oxidative stress, Sirt1 modulates FoxO transcriptional activity, promoting the protective cellular response to oxidative stress.

We hypothesized that Sirt1 would be activated by redox changes induced by hypoxia and that activated Sirt1 would in turn modulate HIF signaling. We determined that HIF-2α signaling is indeed increased by Sirt1 in transfection assays. Sirt1/HIF-2α signaling does not involve previously described oxygen-dependent HIF-2α modifications. Sirt1 augmentation of HIF-2α transcriptional activity involves direct binding to and deacetylation of HIF-2α. In cultured cells and in mice models, interventions that decrease or increase Sirt1 activity affect expression of the HIF-2α target gene *epo* accordingly. Thus, Sirt1 is a molecular switch that promotes HIF-2 signaling during hypoxia and likely other environmental stresses.

TABLE OF CONTENTS

Dedication ii

Copyright iv

Acknowledgements v

Abstract vii

Prior publications xiii

List of figures xiv

List of abbreviations xvii

Chapter One: General introduction 1

HIF family of transcription factors 1

Regulation of HIF transcriptional activity 5

HIF-1α and HIF-2α are not functionally redundant 8

Mouse knockout phenotypes 9

Dissertation overview 11

Chapter Two: Egr proteins confer HIF-2α selective activation of the erythropoietin gene, requirement of a novel regulatory sequence 13

INTRODUCTION 13

Biological function of epo 14

Regulation of the epo gene expression 16

HIIF-2α regulation of the epo gene expression *in vivo* 19

HIF-2α selective gene activation is associated with recruitment of transcriptional co-activator 21

Rational aim 26

RESULTS 27

Identification and functional analysis of evolutionarily conserved elements in the 3'epo enhancer region 27

Identification of candidate transcription factors that bind to conserved BS2 element 36

HIF complex formation with Egr1 and Egr2 42

Relevance of Egr in the regulation epo in cell culture 48

DISCUSSION 56

Chapter Three HIF-2 at the crossroads of hypoxic stress and aging **65**

INTRODUCTION 65

Post-translational modification of HIF as functional switch 66

RESULTS 72

Sirt1 selectively augment HIF-2α signaling but not HIF-1α 72

HIF-2α and Sirt1 form stable complex 77

Sirt1 gene expression increases under hypoxia 85

HIF-2α acetylation status is regulated by Sirt1 88

Sirt1 modulates HIF-2α dependent epo gene expression

in vivo 92

Acetylation of HIF-2α by p300 100

DISCUSSION 102

Chapter Four: Materials And Methods 110

Materials and methods for chapter two 110

Materials and methods for chapter three 118

Bibliography 142

Vitae

PRIOR PUBLICATIONS

Dioum E. M., , Quiyang Zhang, Robert D. Gerard, and Joseph A. Garcia. Molecular mechanism of epo gene regulation: Synergy between Hypoxia Inducible Factor 2 alpha (HIF-2α) and Egr factors. Manuscript in preparation

***Dioum E. M.**, *Rui Chen, Matthew S. Alexander, Quiyang Zhang, Richard T. Hogg, Robert D. Gerard, and Joseph A. Garcia. Hypoxia Inducible Factor 2 alpha (HIF-2α) signaling during hypoxia is selectively regulated by Sirtuin 1. Manuscript submitted.

Dioum E. M., Clarke, S. Ding, K., Repa, J. and Garcia J.A. HIF-2 haploinsufficient mice have blunted retinal neovascularization due to impaired expression of a proangiogenic gene battery. Invest. Ophthalmol. Vis. Sci., 07-1469 (2008)

Oktay Y., **Dioum E.M**, Matsuzaki S, Ding K, Yan LJ, Haller RG, Sweda LI, Garcia JA. Hypoxia-inducible factor 2alpha regulates expression of the mitochondrial aconitase chaperone protein frataxin. J Biol. Chem 283(16):11750-6 (2007)

Freitas TA, Hou S, **Dioum EM**, Saito JA, Newhouse J, Gonzalez G, Gilles-Gonzalez MA, Alam M. Ancestral hemoglobins in Archaea. *Proc Natl Acad Sci USA*,101:6675-80 (2004).

***Dioum EM**, *Dunham CM, Tuckerman JR, Gonzalez G, Scott WG, Gilles Gonzalez MA. A distal Arginine in oxygen-sensing heme-PAS domains is essential to ligand binding, signal transduction, and structure. *Biochemistry*, 42:7701-8 (2003).

Dioum EM, Rutter J, Tuckerman JR, Gonzalez G, Gilles-Gonzalez MA, McKnight SL. NPAS2: a gas-responsive transcription factor. *Science*, 298:2385-7 (2002).

Gonzalez G, **Dioum EM**, Bertolucci CM, Tomita T, Ikeda-Saito M, Cheesman MR, Watmough NJ, Gilles-Gonzalez MA. Nature of the displaceable heme-axial residue in the EcDos protein, a heme-based sensor from Escherichia coli. *Biochemistry*, 41:8414-21 (2002).

Tuckerman JR, Gonzalez G, **Dioum EM**, Gilles-Gonzalez MA. Ligand and oxidation-state specific regulation of the heme-based oxygen sensor FixL from Sinorhizobium meliloti. *Biochemistry*, 41(19):6170-7 (2002).

*Co- first author

LIST OF FIGURES

Figure 1-1	HIF family members domain organization	4
Figure 1-2	Oxygen-dependent regulation of HIF	7
Figure 2-1	Structural organization of the Epo gene	17
Figure 2-2.	Modular structure of Zinc-finger transcription Factors, Sp1/Sp2, WT1 and Egr1	23
Figure 2-3	Evolutionary conserved elements in the mammalian *epo* enhancer region	28
Figure 2-4	Contribution of conserved *epo* enhancer sequences to HIF-selective activation	31
Figure 2-5	BS2 contributes to hypoxia-induced *epo* enhancer activity	33
Figure 2-6	BS2 confers HIF-2 selective activation of the *epo* enhancer	35
Figure 2-7	Candidate approach of the evolutionarily conserved BS2 sequence	37
Figure 2-8	Egr1 and Egr2 act synergistically with HIF-2α to augment *epo* enhancer activity	39
Figure 2-9	NAB2 represses Egr1 and Egr2 activation of the *epo* enhancer	41
Figure 2-10	Egr1 selectively interact with HIF-2α, Egr2 and Egr4, but not Egr3, form a complex with HIF-1α and HIF-2α	43
Figure 2-11	Egr2 interacts with NATD domain of HIF-1α but with NTAD and CTAD domains of HIF-2α	45
Figure 2-12	Egr1 and Egr2 activation is conferred by the HIF-2α carboxy terminal region	47
Figure 2-13	HIF, Egr protein and *epo* mRNA expression profile in hypoxia-treated Hep3B cells	49

Figure 2-14	Egr1 and Egr2 over-expression regulate endogenous epo expression in Hep3B cells	51
Figure 2-15	Egr1 and Egr2 knockdown reduce endogenous epo expression in Hep3B cells under hypoxia	53
Figure 2-16	Trans-activation domain of Egr2 confers synergistic activation with HIF-2α.	55
Figure 3-1	The enzymatic activity of Sirt1	71
Figure 3-2	Sirt1 selectively augments HIF-2 signaling	73
Figure 3-3	Sirt1/HIF-2α augmentation is selectively modulated by Sirt1 enzymatic activity	75
Figure 3-4	HIF-2α forms stable complexes with Sirt1	78
Figure 3-5	The HIF-2α carboxy terminus confers Sirt1 synergy	80
Figure 3-6	Sirt1/HIF-2α complex formation requires HIF-2α NTAD-UR regions	82
Figure 3-7	SIRT1 does not affect HIF-2α protein level	84
Figure 3-8	Sirt1 mRNA levels are regulated by HIF-2α during hypoxia.	86
Figure 3-9	HIF-2α selectively activates the Sirt1 promoter	87
Figure 3-10	HIF-2α acetylation is regulated by Sirt1	89
Figure 3-11	The HIF-2α carboxy terminus is acetylated during hypoxia	91
Figure 3-12	Sirt1 modulates epo gene expression in cell culture	93
Figure 3-13	Sirt1 modulates epo gene expression in mice	95
Figure 3-14	Sirt1 affects HIF-2α-dependent regulation of hepatic *epo* expression in mice	97

Figure 3-15 Ectopic alteration of Sirt1 affects HIF-2α dependent increase of hematocrits in mice 99

Figure 3-16 p300 Acetylates HIF-2α, and trans-activation by p300 is further increased by Sirt1 101

Supplemental Figure 1. Egr1 and Egr2 protein sequence homology 61

Supplemental Figure 2. Egr1 and Egr3 protein sequence homology 62

Supplemental Figure 3. Egr2 and Egr3 protein sequence homology 63

Supplemental Figure 4. Model of HIF-2α and Egr1/Egr2 trans-activation Of the *epo* gene during hypoxia 64

Supplemental Figure 5. HIF-1α and HIF-2α protein sequence homology 108

Supplemental Figure 6. Model of Sirt1 activation of HIF-2α 109

LIST OF ABBREVIATIONS

AhR	Aryl Hydrocarbon Receptor
ARD1	Arrest-defective-1
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
Asn	Asparagine
Bax	BCL2-associated X protein
Bcl2	B-cell leukemia/lymphoma 2
bHLH	Basic helix-loop-helix
Bnip3	BCL2/adenovirus E1B 19kDa interacting protein 3
Cas1	Catalase 1
Cited 2	Cbp/p300-interacting transactivator, with Glu/Asp-rich
COOH-	Carboxyl
COX	Cytochrome oxidase
CTAD	C-terminal activation domain
DEC2	Differentially expressed in chondrocytes 2
DR2	Double Repeat sequence 2
E14.5	Embryonic day 14.5
Egr	Early growth response
EPAS1	Endothelial PAS Domain Protein 1
Epo	Erythropoietin
Ets1	v-ets erythroblastosis virus E26 oncogene homolog 1
FIH	Factor inhibiting HIF
Gpx1	Glutathione peroxidase 1
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H2O2	hydrogen peroxide
HIF	Hypoxia inducible factor
HNF4α	Hypatic nuclear factor 4 alpha

HRE	Hypoxia response element
IGF-II	Insulin-like growth factor II
lacZ	Gene coding for □-galactosidase
MAPK	Mitogen-activated protein kinase
MnSOD	Manganese superoxide dismutase, mitochondrial
NAB2	NGFI-A binding protein 2
NADH	Nicotinamide adenine dinucleotide hydrogen (reduced)
NEMO	NFkB Essential modulator
NH2-	Amino
NLS	Nuclear localization signal
NO	Nitric oxide
NTAD	N-terminal activation domain
NUR	NADH-ubiquinone reductase
ODD	Oxygen-dependent degradation domain
ORO	Oil red O
OZ	Organ of Zuckerkrandl
PAI-1	Plasminogen activator inhibitor-1
PAS	Period-ARNT-SIM
PDK1	Pyruvate dehydrogenase kinase 1
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PHD	Prolyl Hydroxylase Domain protein
PI3K	phosphatidylinositol 3-kinase
PMSF	Phenyl Methyl Sulfonyl Floride
pVHL	von Hippel-Lindau protein
ROS	Reactive oxygen species
RXR	Retinoic Acid Receptor alpha
SIM	Single-minded
Sir2	Silent information regulator protein 2
Sirt1	Sirtuin 1
SMAD	Mothers Against Decapentaplegic homolog

SO	Superoxide
Sod2	Superoxide dismutase 2, the gene coding for MnSOD
Sp1/Sp3	Specificity protein 1/ Specificity protein 3
VEGFR1	Vascular endothelial growth factor receptor 1
WT1	Wilm's tumor suppressor protein 1

CHAPTER ONE

General Introduction

The capacity to sense and respond to changes in oxygen tension is critical for cellular survival. This adaptive response includes altering fundamental processes short-term and long-term including a switch to anaerobic energy production and ensuring maintenance of oxygen homeostasis in mammals, respectively (Semenza, 1999). Physiological and/or pathological responses to hypoxic stress stimulate global gene expression (Bozzini et al., 1971; Wang and Semenza, 1993b). An important aspect of the transcriptional response to hypoxia is conferred by two members of the hypoxia-inducible transcription factor (HIF) family, HIF-1α and HIF-2α. These two homologous transcription factors are activated by similar stresses (Lando et al., 2000). However, their biological roles differ, in part due to induction of selective target genes. The molecular mechanism of target gene selectivity by HIF-α isoforms remains to be defined.

HIF family of transcription factors

The founding member of the hypoxia inducible transcription factor family, HIF-1 was discovered through characterization of the hypoxia-responsive element (HRE) located in the 3' enhancer region of the erythropoietin (*epo*) gene (Semenza, 1994; Semenza et al., 1990). Further studies revealed that the functional heterodimer complex binding to the HRE during hypoxia, called HIF-1,

consists of the hypoxia-inducible alpha subunit HIF-1α and a constitutively expressed subunit HIF-1β (Wang et al., 1995).

The HIF-1 subunits are members of the PAS domain superfamily of proteins whose founders include *D. melanogaster* period (Per) protein, aryl Hydrocarbon Receptor (Ahr), and single minded (Sim). PAS domain proteins (Taylor and Zhulin, 1999; Wang et al., 1995) are found throughout prokaryotic and eukaryotic organisms and function as sensors of environmental stimuli including light, redox state, energy state (i.e., voltage), xenobiotics and hypoxia.

HIF-1α is ubiquitously expressed in human as well as mouse tissues; HIF-1 plays a pivotal role in multiple physiological responses to hypoxia, including angiogenesis, erythropoiesis, iron and glucose metabolism in cell culture models (Wang et al., 2005; Warnecke et al., 2004; Wood et al., 1998). HIF-1β, also known as the aryl hydrocarbon nuclear translocator (Arnt) was identified as a binding partner for several transcription factors (Wang et al., 1995).

Shortly after the cloning of HIF-1α, a closely related protein HIF-2α (also called endothelial PAS protein 1 (EPAS1), HIF-like factor (HLF), HIF related factor (HRF) and member of PAS superfamily 2 (MOP2)) was discovered and cloned by several laboratories (Ema et al., 1997; Flamme et al., 1997; Hogenesch et al., 1997; Tian et al., 1997). HIF-1α and HIF-2α share a number of structural and biochemical properties.

A third member of the family, HIF-3α, was discovered later that lacks the carboxy terminal trans-activation domain (CTAD); it is also widely expressed (Gu

et al., 1998). A splice variant of HIF-3α called Inhibitory PAS domain protein (IPAS) lacks any activation domain and is able to interact with the HIF-1α amino terminal region. IPAS acts as a dominant negative regulator of HIF-mediated target gene expression (Makino et al., 2002; Maynard et al., 2003). All three HIF-α isoforms are stabilized by hypoxia, heterodimerize with ARNT, and bind to the HREs of target genes.

HIF-1α and HIF-2α proteins are highly homologous, displaying 48% overall amino-acid identity. In the amino terminus, the bHLH DNA-binding domain and the PAS dimerization domains share the highest level of similarity: 83% and 73% identity respectively (Ema et al., 1997; Tian et al., 1997 (Figure 1-1) (**Figure 1-1**). The carboxy terminus contains two trans-activation domains, the N-terminal trans-activation domain (NTAD) (Porntadavity et al., 2001) and the (CTAD) (O'Rourke et al., 1997; Pugh et al., 1997). These domains confer interaction with the co-activator protein p300.

The HIF-α subunits also contain an oxygen-dependent-degradation domain (ODD) imbedded within the NTAD that mediates their oxygen-dependent regulated stability (Pugh et al., 1997). The two trans-activation domains, NTAD and CTAD, are separated by an unstructured domain called the auto-inhibitory domain in HIF-1α (Jiang et al., 1997). However, in HIF-2α the function of this region is still the subject of discussion; we refer to it as the unique region (UR) since it is the least conserved region between HIF-1α and HIF-2α (19% sequence homology).

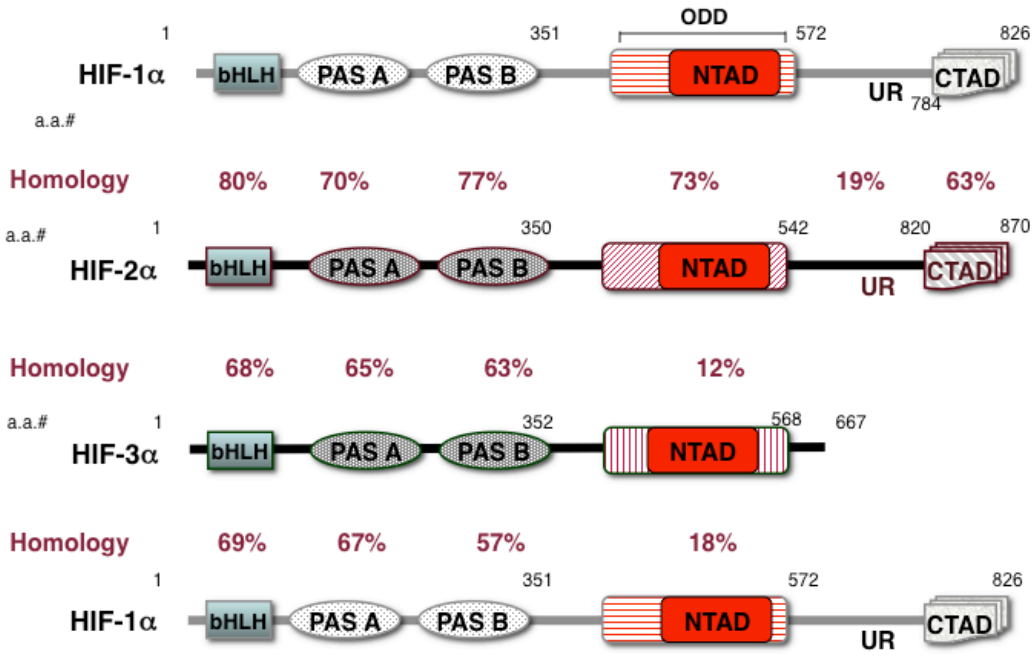


Figure 1-1. Domain organization of HIF family members. Homology comparisons of the conserved domains of human HIF-1α, HIF-2α, and HIF-3α. The similarity between two proteins is higher in basic helix-loop-helix (bHLH domain) DNA-binding and PAS domains in amino terminus. In the carboxy terminus, the conserved amino terminal activation domain (NTAD) located within the oxygen-dependent degradation domain (ODD) and the carboxy terminus activation domain (CTAD) are very homologous between HIF-1α and HIF-2α, whereas the splice variants of HIF-3α contain none of these domains or only the NTAD. The highly divergent unique region (UR) between HIF-1α and HIF-2α may confer selective activation.

Regulation of HIF transcriptional activity

Both HIF-1α and HIF-2α are regulated post-translationally by cellular oxygen tension. Under normoxia (20% O_2), conserved proline residues in HIF-1α and HIF-2α that are part of a consensus sequence LXXLAP in the ODD domain (P402, P577 and P405, P531 in HIF-1α and HIF-2α, respectively) are hydroxylated by a family of 2-oxoglutarate-oxygen-dependent prolyl hydroxylases (PHD1, PHD2, PHD3) (Bruick and McKnight, 2001; Epstein et al., 2001; Masson and Ratcliffe, 2003) (**Figure 1-2**). Upon prolyl hydroxylation, the von Hippel-Lindau tumor-suppressor protein (pVHL) is recruited to HIF-α (Salceda and Caro, 1997). The oxygen-dependent degradation of HIF-α is triggered by recruitment of pVHL, which, in a complex with the protein elongin C, recruits an E3 ubiqutin ligase complex that polyubiquitinates HIF-α and targets it to the 26S proteasome for degradation (Jaakkola et al., 2001; Mole et al., 2001).

In addition to oxygen-dependent prolyl hydroxylation, a conserved asparagine residue in the CTAD, N803 in HIF-1α and N851 in HIF-2α (Lando et al., 2002), can be hydroxylated by a HIF-specific oxygen-dependent N-hydroxylase called factor inhibiting HIF-1 (FIH). Asparaginyl-hydroxylated HIF-α prevents the recruitment of the transcriptional co-activator CBP/p300 (Koivunen et al., 2004; Mahon et al., 2001).

During hypoxia, the activity of the PHDs and FIH are suppressed, resulting in stabilization and accumulation of HIF-α, followed by its dimerization with ARNT and translocation into the nucleus, where it activates hypoxia-responsive genes

(Bruick and McKnight, 2002; Zagorska and Dulak, 2004). Mutation of the oxygen-modified proline and asparagine residues to alanine residues disrupts the interaction of HIF-1α with pVHL and prevents inhibition of p300 binding in the presence of normal oxygen, thereby constitutively increasing HIF-α stability as well as activity (Masson et al., 2001)

Emerging evidence indicates that reactive oxygen species (ROS) play a prominent role in HIF activation. ROS generated by mitochondrial complex III are required for hypoxic activation of HIF (Klimova and Chandel, 2008). Agents that generate ROS (Chandel et al., 2000) or over-expression of NAD(P)H oxidase 1 (Goyal et al., 2004) result in normoxic HIF-1α stabilization.

In addition to the negative regulation of HIF-α by pVHL-mediated proteosomal degradation, other mechanisms contribute to down-regulation of HIF-1α transcriptional activity and/or stability. Cited2, a p300-interacting protein, has been described as a competitive inhibitor of HIF that abrogates the recruitment of p300, an essential HIF co-activator (Freedman et al., 2003). Cited2 expression is up-regulated by hypoxia, suggesting that it can act in a negative feedback loop to down-regulate HIF-mediated transcription. The HIF response can also be potentiated by the recruitment of a variety of co-activator proteins (Ets1, SRC-1, p300) (Carrero et al., 2000; van den Beucken et al., 2007)

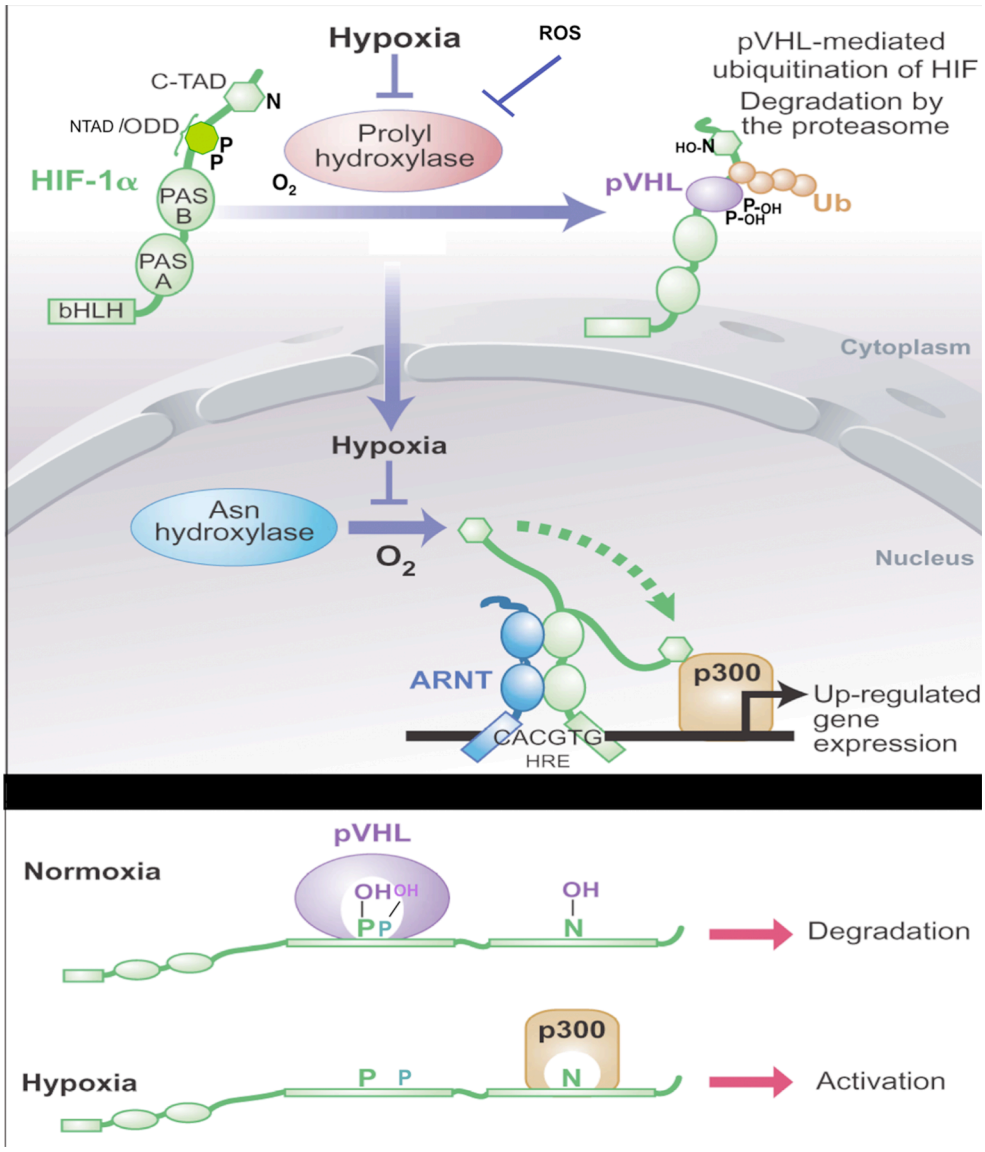


Figure 1-2. **Oxygen-dependent regulation of HIF**. HIF-1α prolyl (PHDs) and asparaginyl hydroxylases (FIH) act as cellular oxygen sensors. HIF-alpha oxygen-dependent proline hydroxylation in the ODD results in pVHL binding and poly-ubiquitination of HIF-α with subsequent proteosomal degradation. Hydroxylation of a conserved asparagine in the CTAD prevents recruitment of the transcriptional co-activator CBP/p300. Modified from Bruick and McKnight 2002. Reprint with permission from AAAS.

HIF-1α and HIF-2α are not functionally redundant

HIF-1α and HIF-2α proteins share a requirement for ARNT/HIF-1β heterodimerization. HIF heterodimers up-regulate a large number of target genes containing *cis*-acting HRE (consensus binding sequences: 5'RCGTG-3') (Semenza et al., 1991). HIF biological specificity is conferred by the alpha subunit. This raises the question of the relative role of HIF-1α or HIF-2α in the *in vivo* regulation of selective target gene expression. Data from systematic comparative analyses between HIF-1α and HIF-2α are scarce. However, there is increasing evidence that HIF-1α and HIF-2α have different biological functions, and their *in vivo* spatial as well as temporal pattern of expression are quite different (Jain et al., 1998; Tian et al., 1997).

HIF-1α is expressed ubiquitously. HIF-2α, which was initially thought to be expressed only in endothelial cells during embryonic development (Tian et al., 1997), is found in a number of adult cell types including kidney fibroblasts, hepatocytes, intestinal epithelial cells, interstitial cells, cardiomyocytes, type II pneumocytes, bone marrow, testis, pancreas, and retina (Ding et al., 2005; Ema et al., 1999; Jurgensen et al., 2004; Wiesener and Maxwell, 2003). Unlike HIF-1α, HIF-2α was shown to be transcriptionally inactive in MEF cells treated with hypoxia (Park et al., 2003). HIF-2α is very weakly regulated by FIH asparagine hydroxylase *in vitro* (Bracken et al., 2006).

HIF-1α mediates the transcriptional response to hypoxia by inducing several genes involved in glycolysis (glucokinase, aldolase A, phosphoglycerate

kinase 1, lactate dehydrogenase A, phosphofructokinase (PFKB3), enolase 1, pyruvate dehydrogenase kinase 1 (PDK1), glucose uptake glucose transporter 1 (Glut1), angiogenesis vascular endothelial growth factor (VEGF) and VEGF receptor 1 (Flt1), oxygen supply (globin 2, plasminogen activator inhibitor 1 (PAI-1), nitric oxide synthase (iNOS and eNOS), iron metabolism (transferrin, transferrin receptor, ceruloplasmin), cell proliferation/apoptosis (insulin like growth factor binding protein 1, Bcl2/adenovirus E1B 19kDa interacting protein 3) and others including differentially expressed in chondrocytes protein 1 (DEC1), Cited2, and Ets1 (Campo et al., 2005; Wenger et al., 2005). The ubiquity of the HIF-1α target genes implies a major role for HIF-1α in mediating the hypoxia response pathway at systemic, local, and intracellular levels (Semenza, 2004). Due to the lack of a viable HIF-1α knockout mouse model, however, these studies were performed mostly in cell culture models and therefore their physiological relevance is uncertain.

Mouse knockout phenotypes

Given the similarities of HIF-1α and HIF-2α, a question arises as to whether HIF-2α is simply a redundant protein that activates the same genes as HIF-1α or if it has a unique role, either in activating a different subset of genes or in regulating these genes in a cell type-dependent or stimulus-specific manner. HIF-2α has been shown to regulate some HIF-1α target genes (VEGF, Glut1) (Krieg et al., 2000; Tian et al., 1997), but also induces a distinct repertoire of

target genes (*epo*, superoxide dismutase 2 (*Sod2*), plasminogen activator inhibitor-1 (*Pai*-1) (Carroll and Ashcroft, 2006; Scortegagna et al., 2003a; Scortegagna et al., 2003b; Warnecke et al., 2004). Moreover, HIF-1α- and HIF-2α-deficient mice manifest distinct phenotypes. Disruption of the HIF-1α gene in mice results in embryonic lethality, and these embryos display defective vasculature, prominent cardiovascular malformation, neural tube defects and mesenchymal cell death (Iyer *et al*. 1998, Kosh et al 1999). Disruption of the HIF-2α gene results in embryonic lethality that has been attributed to bradycardia induced by lower fetal catecholamine production and/or to an improper remodeling of the primary vasculature (Tian et al., 1997).

The generation of a viable adult HIF-2α null mouse provided investigators with what is still today a unique model of mice globally lacking any HIF-α family member ct (Scortegagna et al., 2003a). Studies from our laboratory of viable HIF-2α-deficient juvenile mice revealed multiple organ pathology consistent with a mitochondrial defect (Scortegagna et al., 2003a; Scortegagna et al., 2005). The pathologic features include retinopathy, cardiac hypertrophy and hematopoietic deficiency as a result of blunted Epo expression, mitochondrial defects, and hepatic oxidative stress. The biochemical basis for the phenotype in liver involves increased oxidative stress due to reduced expression of anti-oxidant enzyme (AOE) genes, including *Sod2* (Scortegagna et al., 2003a) encoding a potent ROS detoxifying agent that is an essential modulator of mitochondrial homeostasis (Rabilloud et al., 2001), and *frataxin* (*frx*), encoding the oxidative stress-regulated

chaperone protein essential for stabilization of mitochondrial aconitase (Oktay et al., 2007).

Concomitant with the *in vivo* studies, there is a growing body of evidence demonstrating different biological responses and roles for HIF-1α and HIF-2α in relation to cellular localization, hypoxic responsiveness and target gene selectivity. HIF-1α appears to be more important in the early phase of hypoxia, when the transition from aerobic to anaerobic metabolism is essential for cell survival. In contrast, HIF-2α appears to regulate processes that are important for long-term adaptive or anticipatory responses including the desired, yet potentially perilous, reoxygenation phase.

Dissertation overview

The first aim of my thesis was to understand the molecular mechanism of HIF-2α selective activation of the *epo* gene. I hypothesized that under hypoxia, HIF-2α, not HIF-1α, is capable of recruiting a transcriptional co-activator to the Epo enhancer. First, by using bioinformatic analysis and DNA sequence comparison of several mammalian 3' Epo enhancer regions, I identified a novel evolutionarily conserved region adjacent to the HRE that we named BS2 (binding site 2). To determine the functional relevance of this conserved sequence, I tested candidate transcription factors using cell culture techniques. I determined that the BS2 sequence contributes to HIF-2α selectivity through interaction with the zinc-finger transcription factors Egr1 and Egr2. Synergistic activation of the

Epo enhancer by Egr1/HIF-2α or Egr2/HIF-2α requires intact HRE and BS2 sequences.

The second aim of my thesis was to identify a regulator that confers HIF-2α selective activation of target genes. My hypothesis is that target gene selectivity between HIF-1α and HIF-2α is dictated in part by the capacity of each HIF-α to differentially recruit co-activators. Transcriptional co-activators, unlike transcription factors, do not bind directly to DNA, but rather bind to DNA-binding factors which impacts larger set of target genes. In our HIF-2α knockout mouse model, HIF-2α regulates Sod2 and other major AOE (Scortegagna et al 2003a). In cell culture models, the FoxO family of transcription factors regulates the transcription of Sod2 and other major anti-oxidant enzymes (AOE). During oxidative stress, silent mating type information regulator 2 homolog 1 gene or Sirtuin 1 (Sirt1), a redox-sensing, NAD^+ dependent deacetylase enzyme, modulates FoxO transcriptional activity to favor the protective cellular response to oxidative stress. I demonstrate here that Sirt1 selectively stimulates HIF-2α, but not HIF-1α, signaling during hypoxia. Sirt1/HIF-2α signaling does not involve previously described oxygen-dependent HIF-2α modifications. Sirt1 augmentation involves direct binding to and deacetylation of HIF-2α. In cultured cells and in mice, interventions that decrease or increase Sirt1 activity affect expression of the HIF-2α target gene Epo. Thus, Sirt1 is a molecular switch that promotes HIF-2α signaling during hypoxia and likely other environmental stresses.

CHAPTER TWO

Egr proteins confer HIF-2α-selective activation of the erythropoietin gene: requirement of a novel regulatory sequence.

INTRODUCTION

Erythropoietin (Epo), a member of the superfamily of cytokines, is the major endocrine regulator of erythropoiesis or red blood cell production. Epo acts synergistically with other cytokines to promote progenitor cells of the erythroid lineage to proliferate and differentiate, thus maintaining the circulating red blood cell (erythrocyte) mass in a feedback controlled fashion (Lacombe and Mayeux, 1999). Epo mediates the adaptation of an erythroid fate by early pluripotent hematopoietic stem cells, and also acts later on colony forming unit (CFU) erythroid cells to prevent their apoptosis and induce at the same time the expression of erythroid-specific proteins (Lacombe and Mayeux, 1999; Scortegagna et al., 2005; Scortegagna et al., 2003b). Recombinant Epo has been used in clinical settings as an erythropoietic stimulating agent in the treatment of anemia associated with various pathological conditions (cancer, chronic renal insufficiency) and is being evaluated as a tissue protective mediator in response to ischemia reperfusion injuries (Konstantinopoulos et al., 2007).

The importance of identifying homeostatic mechanisms regulating *epo* gene expression has become evident from recent clinical studies suggesting that supra-therapeutic levels of exogenous Epo have detrimental effects on patient

outcomes (Milano and Schneider, 2007; Yoon et al., 2008). Understanding how epo gene expression is normally regulated will provide novel opportunities for therapeutic intervention aimed at modulating endogenous Epo production using existing regulatory mechanisms.

Biological functions of epo

Epo produced in the kidney of adult mammals is the quintessential hypoxia inducible gene; it is up-regulated up to 1000-fold during hypoxia or chronic anemia (Ebert and Bunn, 1999). The predominant action of Epo is to induce red blood cell formation by regulating pluripotent hematopoietic stem cell maturation. Epo appears to accelerate the commitment of colony-forming unit erythroid (CFU-E) cells toward proliferation and further maturation, an action that is primarily due to the anti-apoptotic effect of Epo.

Binding of Epo leads to conformational changes in the Epo receptor (EpoR) and the associated intracellular Janus family tyrosine protein kinase 2 (JAK-2). These events lead to activation of signal transducer and activator of transcription 5 (STAT-5), a major mediator of Epo action, as well as other signaling proteins including protein kinase B/Akt (PKB/Akt). Activated STAT-5 within the nucleus induces the transcription of several genes, including Bcl-X_L, which codes for an anti-apoptotic protein of the Bcl-2 family (Sylva et al 1999 JBC). Activation of Akt enables it to inactivate pro-apoptotic proteins such as Bad and caspase 9 (Chong et al., 2003; Kashii et al., 2000).

The biological function of epo extends well beyond its erythropoietic effects and encompasses diverse physiological processes including angiogenesis, vasculogenesis and cytoprotection. Expression of the epo receptor is not restricted to hematopoietic cells, and includes neural cells, vascular endothelium, and muscle progenitor cells (Anagnostou et al., 1990; Brines and Cerami, 2006; Digicaylioglu et al., 1995). Similar to erythropoiesis, the tissue protective effects of epo are contingent in part upon its anti-apoptotic properties (Cerami et al., 2002; Ghezzi and Brines, 2004).

Epo expression is very tightly regulated by developmental, tissue-specific, and physiological cues (Ebert and Bunn, 1999). The liver is the principle site of epo synthesis during the prenatal and neonatal stages in mice, but in the adult mouse Epo expression shifts from the liver to the kidney (Koury and Bondurant, 1988; Semenza et al., 1990). Epo production has also been found in organs where it has cytoprotective functions. Epo is expressed in the brain, specifically in neurons and astrocytes (Arai et al., 2002; Masuda et al., 1994) where it promotes cellular survival and differentiation.

The *in situ* production of endogenous epo and EpoR transcripts (Awogu and Abohweyere, 2006; Brines and Cerami, 2005) is regulated in a hypoxia-dependent manner, suggestive of a paracrine or autocrine action of epo not only in normal brain development (Chen et al., 2006; Yu et al., 2002), but also as a cytoprotective agent in response to ischemia/reperfusion injuries (Marti et al., 2000; Marti et al., 1996). Epo also has a paracrine function in the kidney, where

EpoR is widely expressed (Westenfelder et al., 1999) and where it has a cytoprotective effect on interstitial cells and reduces cortical renal blood flow and urinary solute excretion (Coleman et al., 2006).

Regulation of *epo* gene expression

In vivo data from our laboratory (Scortegagna et al., 2003a; Scortegagna et al., 2005) and *in vitro* data studies from others (Rankin et al., 2007; Warnecke et al., 2004) suggested that Epo is a HIF-2α specific target gene. Defining the molecular mechanism that underlies HIF-2α preferential regulation of Epo gene expression in comparison to HIF-1α, in spite of the fact that both factors are capable of binding to the HRE of the epo enhancer, is an active area of investigation.

Comparison of the human and mouse epo genes provided valuable information on the location of key regulatory elements in the promoter and aided in defining a hypoxia-inducible enhancer in the 3'-flanking sequence of the *epo* gene located 120 bp downstream of the polyadenylation site (Beck et al., 1991; Semenza et al., 1991; Wang and Semenza, 1993a). This regulatory region is referred to as the 3' Epo enhancer region. With the help of hepatoma cells lines (Hep3B and HepG2), putative hypoxia-activated regulatory DNA sequences of the epo gene were tested.

Detailed characterization of the Epo 3' enhancer by transient transfection experiments and studies with a transgenic mice model defined conserved sites

critical for hypoxia regulation. Several studies (Beck et al., 1991; Blanchard et al., 1992; Pugh et al., 1991; Semenza et al., 1991) defined a minimal enhancer region that confers inducibility by cobalt chloride, a chemical mimetic of hypoxia, by using reporters consisting of Epo enhancer restriction fragments. Based on these studies, a minimal 3' enhancer core element of the Epo gene consisting of a 43 bp restriction fragment extending from the Apa I site to the second Hpa II site, was described. This region encompasses critical *cis*-regulatory elements responsible for epo hypoxia-dependent responses (**Figure 2-1**).

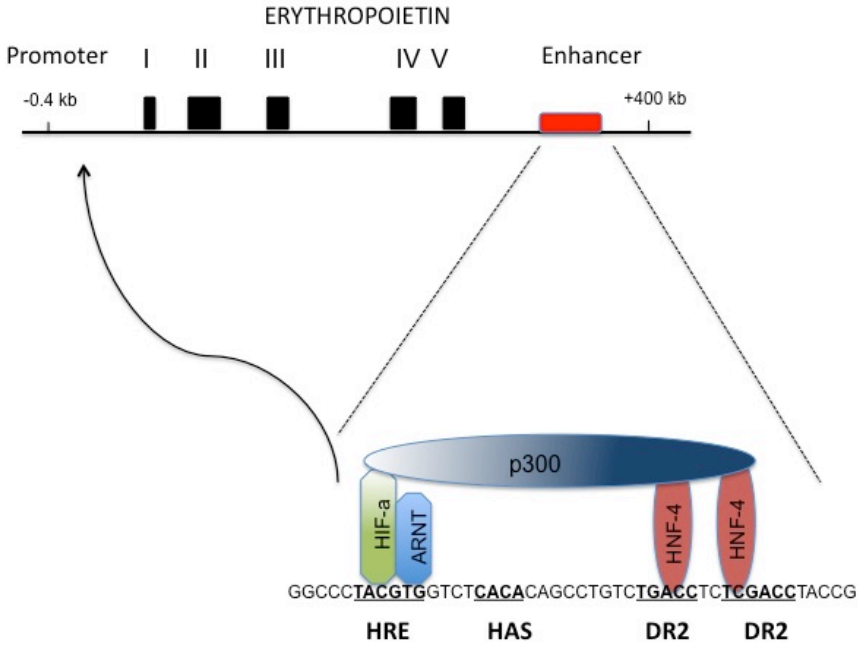


Figure 2-1. Structural organization of the Epo gene. Exons are indicated by solid black boxes. The 3' enhancer region highly homologous between human and murine genes indicated by the red rectangle is expanded for greater detail. Sites that are functionally critical for hypoxic induction are underlined. Binding of HIF to the hypoxia responsive element (HRE), HNF4α (hepatocyte nuclear factor 4-α) binding to DR2 (direct repeat 2 sequences) and HIF recruitment of p300 is illustrated. The trans-activator p300 is capable of interacting with the basal transcriptional machinery in the promoter and induced *epo* gene transcription.

From the 5' end of the minimal epo enhancer region, the epo HRE (CACGTGCT) was the first hypoxia-responsive element described for a HIF target gene. Binding of HIF to the HRE site, after hypoxia stabilization, mediates the hypoxic response of HIF target genes. An intact HRE sequence and HIF binding are indispensable for hypoxia-induction of epo expression. Downstream of the HRE, a CACA repeat found in the human epo enhancer, referred to as the HIF ancillary sequence or HAS, is very important for HIF action, although no protein has been described that binds to this region (Firth et al., 1995). The sequence of a third region consists of a direct repeat (DR) of two tandem nuclear steroid receptor half sites termed the DR2 site. Hepatocyte nuclear factor-4α (HNF4α) binds to the DR-2 site, making it absolutely required for hypoxic regulation of epo expression (Blanchard et al 1992). The HNF4α interaction is believed to contribute to the tissue specificity of Epo gene expression (Ebert and Bunn, 1999).

During mouse embryonic development,E9.5 to E12.5, another member of the nuclear receptor family, the retinoic acid receptor-α (RXRα), binds to the DR2 site and induces HIF-independent epo expression in fetal liver. After E12.5, HNF-4α activity predominates, competitively blocks RXR-α binding, and may initiate the switch from retinoid acid control to hypoxic control of epo expression (Makita et al., 2001). Between E12.5 and E14.5, hepatic epo gene expression is likely regulated in a HIF-1α dependent manner (Koury et al., 1988; Koury et al., 1991; Semenza, 1994).

HIF-2α regulation of epo gene expression *in vivo*

HIF-1α was initially characterized as a transcription factor binding to the 3' enhancer of the Epo gene *in vitro* (Semenza, 1994; Wang and Semenza, 1995) suggesting a dominant role of HIF-1α in epo gene regulation (Yu et al., 1999). However, subsequent *in vivo* and *in vitro* data indicate that HIF-2α, not HIF-1α, is the major regulator of Epo gene expression. Systematic comparison between HIF-1α and HIF-2α *in vivo* has been scarce due to the lack of a viable HIF-1α-deficient animal model. Increasing evidence (Rankin et al., 2007; Scortegagna et al., 2005) has demonstrated the importance of HIF-2α in the regulation of Epo *in vivo*.

In HIF-2α knockout mice maintained in room air or short-term intermittent hypoxia, there is a significant decrease in renal Epo expression accompanying the observed anemia compared to wild type littermate mice. Although there are no significant differences in Epo gene expression for haploinsufficient HIF-2α mice maintained at room air, Epo expression is blunted in HIF-2α haploinsufficient mice maintained under short-term intermittent or short-term continuous hypoxia exposure. For mice treated with hypoxia, there is a significant decrease in serum Epo protein levels in HIF-2α haploinsufficient compared to wild type mice (Afsar et al., 2006; Yin et al.) that results in a significant decrease of the hematocrit. These results indicate that HIF-2α is a critical mediator of renal Epo production in mice.

Cell culture experiments demonstrate functional differences between the HIF-α subunits by knockdown of endogenous HIF-1α or HIF-2α expression using RNA interference (Warnecke et al., 2004). RNAse protection assays showed that in HeLa and Hep3B cells, HIF-1α knockdown significantly reduced the hypoxic response of Glut-1, lactate dehydrogenase A (LDH A) carbonic anhydrase *IX* (CA*IX*) and VEGF mRNA expression, whereas HIF-2α knockdown had no effect on these genes. The above results supported the importance of HIF-1α in the regulation of glucose metabolism genes (Glut-1, LDH-A), angiogenic factors (VEGF) as well as other hypoxia inducible genes (PHD3 and CA *IX*). In contrast, Epo mRNA induction is almost completely abolished by knockdown of HIF-2α in the hepatoma cell line Hep3B and in neuroblastoma Kelly cells, a neuronal cell line known to express and induce Epo upon hypoxic stimulation. In both Hep3B and Kelly cells, HIF-1α siRNA has no significant effect on Epo induction.

The above data suggest that epo regulation *in vivo* is not regulated at the level of DNA binding or spatial expression of HIF-1α. Rather, HIF-2α has an inherent capacity to activate epo gene expression in the kidney and in cell culture models. I hypothesized that HIF-2α, but not HIF-1α, possesses an intrinsic capacity to assemble a more transcriptionally active complex by recruiting synergistic co-activators. I proposed that such a mechanism would confer upon HIF-2α the ability to selectively activate the *epo* gene.

HIF-2α selective gene activation is associated with the recruitment of a transcriptional co activator

Studies in which HIF-1α and HIF-2α hybrid proteins were over-expressed followed by chromatin immunoprecipitation studies have shown that the selective activation of HIF target genes is not based solely on the DNA-binding ability of these factors (Hu et al., 2007). Both HIF-1α and HIF-2α are capable of binding to the HRE of endogenous target genes, suggesting that binding is not sufficient for selective target gene activation. Instead, selectivity involves a post-DNA-binding mechanism mediated by the more heterogeneous carboxy terminal regions of the HIF-α proteins (Hu et al., 2007; Lau et al., 2007). As defined for the HIF-1 target gene carbonic anhydrase *IX* (CA *XI*), the HIF-1α NTAD region is sufficient to confer target gene selectivity (Hu et al., 2007). In contrast, for the HIF-2α target gene prolyl-hydroxylase 3 (PHD3), selectivity requires the HIF-2α NTAD together with the UR-CTAD (Lau et al., 2007).

Cooperation with other transcription factors may also be required for maximal or cell type-specific up-regulation of HIF target genes. Ets transcription factor family members have been implicated in hypoxic gene regulation (Aprelikova et al., 2006; Kojima et al., 2007). Ets-1 preferentially cooperates with HIF-2α, but not HIF-1α, to synergistically activate transcription of the VEGF receptor 2 gene (Flk-1) (Elvert et al., 2003). Interestingly, the physical interaction between HIF-2α and Ets1 is mediated through the HIF-2α carboxy terminus. Another Ets family member, Elk-1, cooperates with HIF-2α to activate the target

genes CITED-2, epo, insulin-like growth factor binding protein-3 (IGFBP3) and PAI-1 in RCC4 or MCF-7 cancer cells lines (Aprelikova et al., 2006; Hu et al., 2007).

The NF-kB essential modulator (NEMO) has been identified as a HIF-2α, but not a HIF-1α, interacting protein from a mammalian two-hybrid screening (Bracken et al., 2005). In a cell culture model, the specific interaction with NEMO enhances normoxic HIF-2α transcriptional activity by facilitating p300/CBP recruitment. However, a requirement for NEMO in the activation of endogenous HIF-2α target genes has not been described.

Hypoxia triggers the activation of several transcription factors in mammalian cells besides HIF proteins. Several HIF target genes possess GC-rich DNA binding motifs in their promoter or enhancer regions (Aprelikova et al., 2006). Zinc finger transcription factors (**Figure 2-2**) bind to GC-rich sequences regulatory regions. Specificity proteins Sp1/Sp3, a ubiquitously expressed family of zinc finger transcription factors, have been implicated in the hypoxic regulation of epo and VEGF gene expression (Lee et al., 2004; Sanchez-Elsner et al., 2004). The Epo promoter is activated by Sp1 in conjunction with HIF-1α during hypoxia through retinoic acid-activated SMAD3/4. Wilm's tumor suppressor protein 1 (WT-1), another transcriptional activator of basal expression of epo under normoxia, was shown to bind to the epo proximal promoter (Dame et al., 2006).

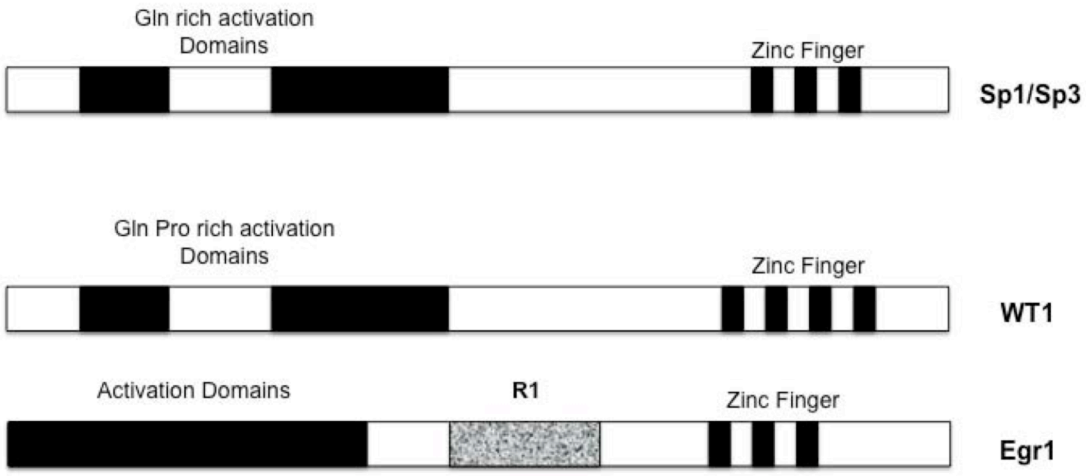


Figure 2-2. **Modular structure of Zinc-finger transcription factors WT1, Sp1/Sp3 and Egr1**. These proteins have zinc-finger DNA binding domains located in the carboxy terminus. WT-1 and Sp1/Sp3 contain respectively a bipartite glutamine/proline and glutamine rich trans-activation domain on the amino terminus. The Egr1 protein contains an extended transcriptional activation domain on the amino terminus and a repression domain (R1) between the activation and the DNA binding domain that function as a binding site for the transcriptional repressors NAB1 and NAB2.

The Egr transcription factor family is comprised of four members in mice and humans, all of which can bind GC-rich recognition elements. Egr1, Egr2 (Krox20), Egr3 (Pilot), and Egr4 (NGFI-C) are closely related transcription factors with highly homologous (92%) Cys2-His2 zinc-finger DNA binding domains in the carboxy terminus and a loosely conserved activation domain in the amino terminus (**Supplemental Figure 1)** (Beckmann and Wilce, 1997; O'Donovan et al., 1999). The Egr proteins recognize similar GC-rich consensus DNA motifs 5'-CGCCCCCGC-3', suggesting that they may bind to *cis*-regulatory regions of at least a subset of the same target genes which are required for cellular proliferation and differentiation (Chavrier et al., 1990; Swirnoff and Milbrandt, 1995).

Several lines of evidence identified the zinc finger transcription factor early growth response 1 factor (Egr1), also called ZIF268, NGFI-A (nerve growth factor inducible gene A), Krox24 and TIS8 (Lemaire et al., 1988; Lim et al., 1987; Milbrandt, 1987) as a growth factor- and hypoxia-regulated transcription factor (Lim et al., 1998; Milbrandt, 1987). Hypoxia up-regulated Egr1 mRNA expression, as well as its DNA binding activity to the IGF-II promoter, in HepG2 cells (Bae et al., 1999). Egr1 is also up-regulated in the kidney (the main site of epo expression in the adult) in response to hypoxia or ischemia induced by renal artery occlusion (Heiniger et al., 2003; Ouellette et al., 1990). Egr1 mRNA levels are increased in the embryonic liver as well during early postnatal days (Yang et

al., 2004). These times correlate with the developmental expression of epo in the liver.

Egr trans-activation capacities can be inhibited by NGFI-A binding co-repressor proteins 1 and 2 (NAB1 and NAB2) (Russo et al., 1995; Svaren et al., 1996). NAB proteins possess two regulatory domains called NAB conserved domains 1 (NCD1) and NAB conserved domain 2 (NCD2) that are necessary for repression. Repression depends on direct protein interaction between NCD1 and a conserved region within Egr1, Egr2 and Egr3 called the R1 domain (Swirnoff et al., 1998). Egr1 binds to the NAB2 promoter and induces its transcription, thereby establishing a negative feedback loop (Kumbrink et al., 2005). Egr2 is capable of inducing both NAB1 and NAB2 expression in Schwann cells (Srinivasan et al., 2007).

Egr1-dependent gene expression involves negative or positive cooperativity with a growing number of transcription or regulatory factors including NFAT, SF-1, AP-2, p300, RelA (p65), p53, Sp1 and NAB1 (Chapman and Perkins, 2000; Decker et al., 1998; Lee et al., 1996; Liu et al., 2001; Mouillet et al., 2004). It is therefore likely that Egr1 does not act alone in response to hypoxic stress, but instead works in concert with other factors. Egr2 is highly expressed in the nervous system; it is required for normal nervous system development and peripheral nerve maturation (Topilko et al., 1994). Egr2 expression outside of neural tissues has not been thoroughly evaluated; it

displays low basal expression in rat liver and human hepatoma cells lines (Kang et al., 1999).

Rationale and aim

HIF-1α and HIF-2α are both capable of binding to the HRE in the 3' Epo enhancer and equally induce minimal Epo reporter activity. However, HIF-2α is the main regulator of Epo *in vivo*. I hypothesized that HIF-2α, not HIF-1α, is capable of recruiting a more transcriptionally active complex through interaction with specific co-activators. In order to test this hypothesis, I first thoroughly reevaluated the 3' *epo* enhancer region and identified an evolutionarily conserved sequence conferring the hypoxic response and HIF-2α selectivity using modern bioinformatic tools. I defined the biological relevance of these conserved DNA elements in the regulation of *epo* gene expression in relation to HIF-1α and HIF-2α. Next, using a candidate factor approach and bioinformatic analyses of transcription binding sites, I identified Egr factors capable of binding to one of these elements. I determined that HIF-2α-selective activation of the *epo* gene expression during hypoxia could be explained by a novel and selective interaction of Egr1 and Egr2 with HIF-2α.

.

RESULTS

Identification and functional analysis of evolutionarily conserved elements in the 3' epo enhancer region

The minimal *epo* enhancer region was defined solely by virtue of its responsiveness to hypoxia, a requirement that led to the identification of the HRE and subsequently that of HIF-1. However, the minimal epo enhancer reporter is non-selectively activated by both HIF-1α and HIF-2α in over-expression reporter assays. I reasoned that the discrepancy between the selective regulation of endogenous *epo* gene expression by endogenous HIF-2 and the non-selective activation of the minimal 3' *epo* enhancer in transfection reporter assays by both HIF-1 and HIF-2 was not due to over-expression artifacts. I instead hypothesized that this minimal 3' Epo enhancer region lacked *cis*-elements important for HIF-2α selective activation.

To identify putative novel cis-elements involved in HIF-2 selective activation of the *epo* enhancer region, we performed an alignment of several mammalian *epo* enhancer regions to identify evolutionarily conserved sequences using bioinformatics and manual inspection (**Figure 2-3**). Using rVISTA (Loots and Ovcharenko, 2005), we identified three blocks of evolutionarily conserved sequences, designated boxes 1 through 3, in the *epo* enhancer region. The 5' block, box 1, contains the HRE whereas the 5' portion of box 2 contains the recognition site for the HNF-4α nuclear factor, designated as DR2, which has previously been implicated in the function of the minimal 3' *epo* enhancer region.

Manual and BLAST inspection identified an additional stretch of conserved sequence located 5' to box 1. These regions were arbitrarily designated as putative binding sites (BS) 1 through 3 with the 3' aspect of BS3 encompassing the HRE, thus overlapping with box 1.

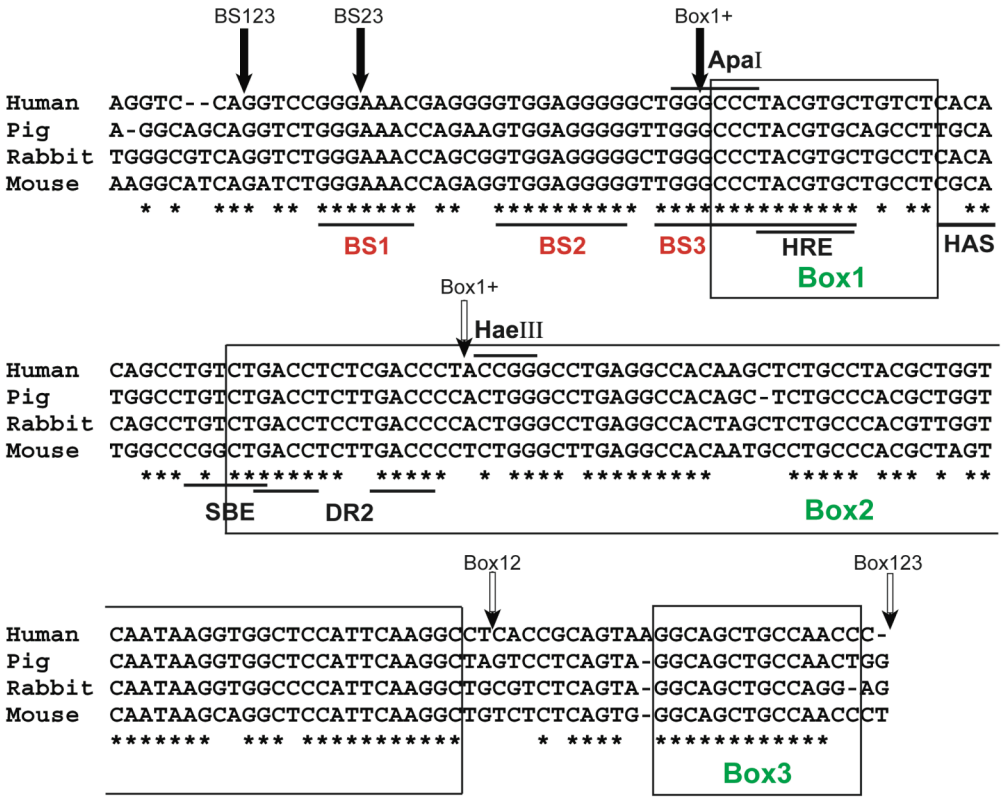


Figure 2-3. Evolutionarily conserved elements in the mammalian *epo* enhancer region. Using bioinformatics and manual inspection, alignments were made between human, pig, rabbit and mouse 3' *epo* enhancer regions. The regions designated as Box1, 2, or 3 were identified by rVISTA whereas the underlined elements designated as binding sites (BS) 1, 2, or 3 were identified by nucleotide BLAST and manual inspection. The ApaI and HaeIII restriction sites define the borders of the 3' minimal *epo* enhancer region used in the majority of epo enhancer reporter constructs. This region contains the HIF recognition element (HRE), the HIF accessory region (HAS), the SMAD binding element (SBE), and the dyad recognition element (DR2). The HRE is contained within Box1 whereas the DR2 element is located at the 5' aspect of Box2. The open arrows indicate the boundaries for the 3' aspect of the deletion constructs whereas the solid arrows indicate the boundaries for the 5' aspect of the deletion constructs.

To define the functional contribution of the evolutionarily conserved domains in the 3' epo enhancer in HIF-2α selective activation, I constructed a series of 3' and 5' deletion constructs of the *epo* enhancer region and fused them to a minimal *epo* promoter placed upstream of the firefly luciferase reporter gene. These constructs were introduced into either a human embryonic kidney epithelial cell line, HEK293 or a human hepatoma cell line, Hep3B, along with expression plasmids encoding constitutively active forms of HIF-1α or HIF-2α, hereafter designated as P1P2N HIF-1α or P1P2N HIF-2α (**Figure 2-4A**). HEK293 cells are commonly used in transient transfection studies, including those investigating *epo* enhancer function, whereas Hep3B cells, which show endogenous epo expression in response to hypoxia, are a model cell line for endogenous *epo* regulation in response to hypoxia.

The Epo enhancer reporter transfection data reveal differential contributions of the conserved sequences to HIF-dependent regulation of *epo* enhancer function. First, the parental construct encompassing the entire conserved region of the *epo* enhancer, BS123-Box123, has the lowest absolute activity with HIF activation and exhibits minimal differences with respect to activation by over-expressed HIF-1α versus HIF-2α. Deletion of box 3 (BS123-Box12) or both boxes 2 and 3 (BS123-Box1) results in an increase in HIF-2α versus HIF-1α activation in both HEK293 and Hep3B cells, likely as a result of loss of a repressor binding to box 3. This preferential activation of the *epo* enhancer by HIF-2α is maintained when BS1 is deleted (BS23-Box1). This

construct is the first demonstration of an *epo* enhancer construct showing preferential activation by over-expressed HIF-2α compared to HIF-1α. The preferential activation of the *epo* enhancer is lost when BS2 is removed (BS3-Box1). We note the BS3-Box1 construct is virtually identical to the minimal human *epo* enhancer region, delineated by an ApaI-HaeIII restriction endonuclease fragment, also activated to a similar extent by over-expressed HIF-1α or HIF-2α.

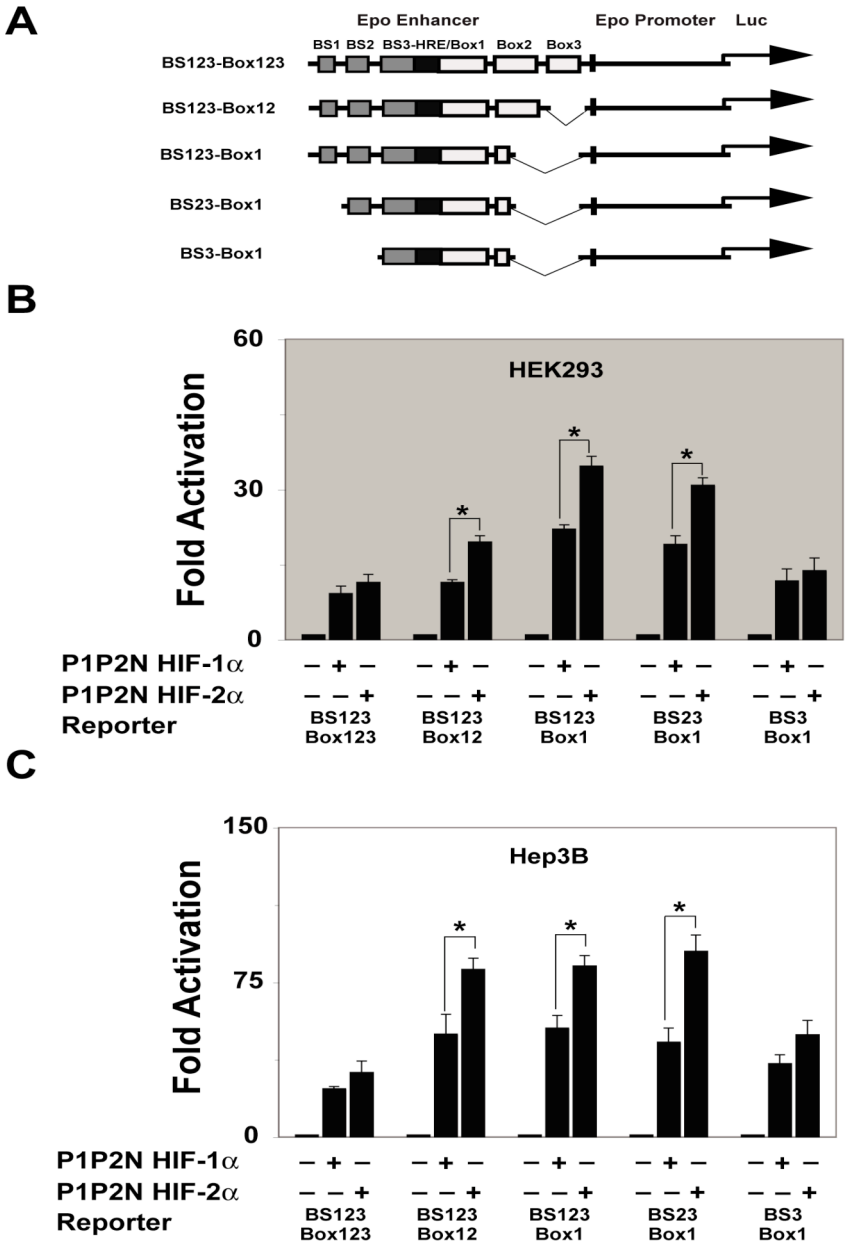


Figure 2-4. Contribution of conserved *epo* enhancer sequences to HIF-selective activation. (A) Schematic representation of the mouse *epo* enhancer reporter constructs used in these transfection studies. The reporter constructs were made by fusing 3' followed by 5' deletions of the mouse *epo* enhancer to a mouse minimal *epo* promoter inserted upstream of the firefly luciferase gene. The reporter constructs were transfected into (B) HEK293 or (C) Hep3B cells along with expression plasmids encoding constitutively active forms of HIF-1α or HIF-2α generated by alanine substitutions for the two proline (P1P2) and the asparagine (N) residues that are normally hydroxylated in an oxygen-dependent manner.

The transfection data for the deletion *epo* enhancer constructs support the existence of repressive *cis*-element(s) in box 3, and presumably *trans*-acting repressive factors, that modulate HIF-2 activation of the *epo* enhancer region. The deletion reporter data also support the existence of an activating *cis*-element(s) in BS2, and presumably *trans*-acting activating factors, that selectively potentiate HIF-2 activation of the *epo* enhancer region. To determine if BS2 contributes to hypoxia dependent activation of the *epo* enhancer, I used the reporter that was preferentially activated by HIF-2α (BS23-Box1) and constructed a site-directed mutation of the *epo* enhancer region that alters the sequence of BS2 (mutBS2-BS23-Box1). I compared the hypoxia inducibility of the parental BS23-Box1 and the BS2 point mutant, muBS2-BS23-Box1, to a similar construct in which I altered the sequence of the HRE (mutHRE-BS23-Box1) or a construct in which BS2 was deleted (BS3-Box1) (**Figure 2-5).**

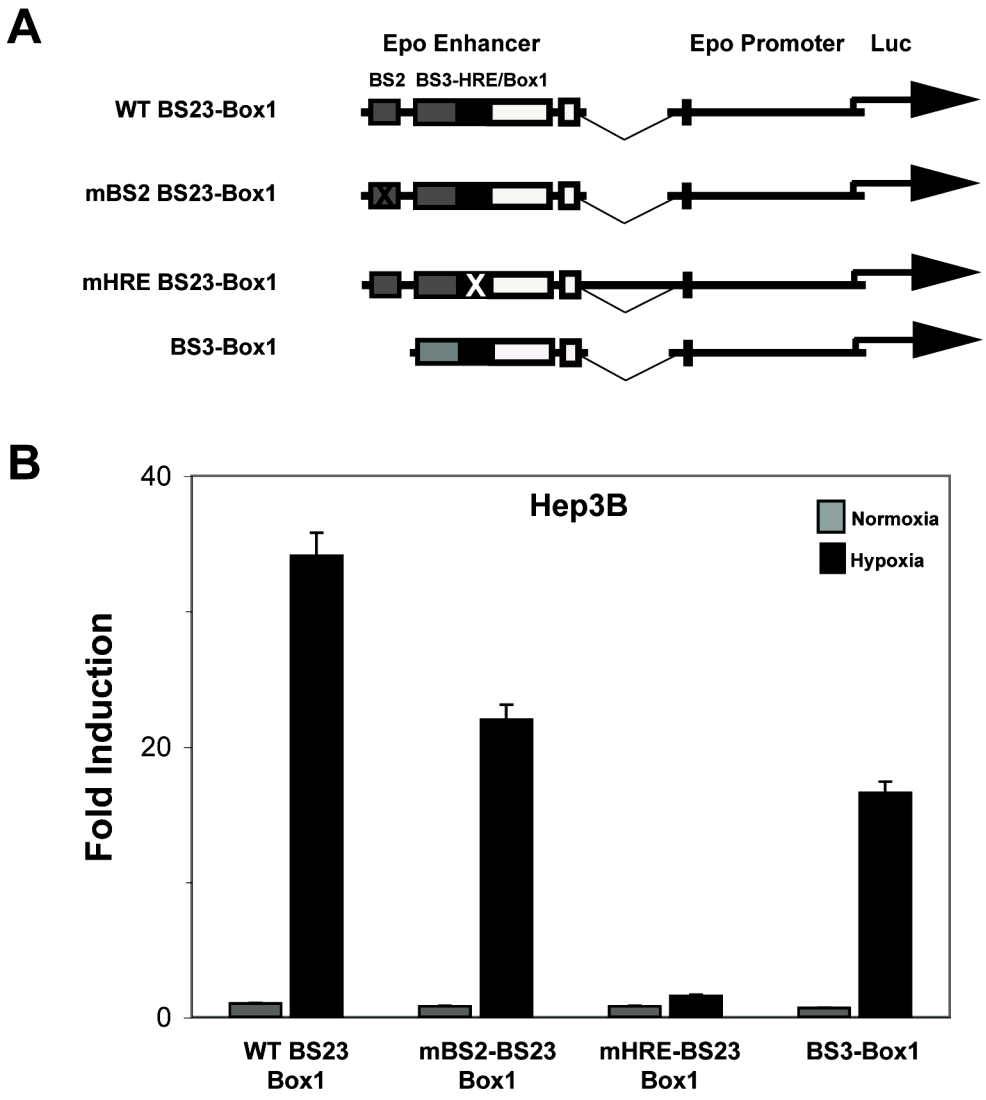


Figure 2-5. BS2 contributes to hypoxia-induced *epo* enhancer activity.
(A) Schematic representation of the mouse *epo* enhancer reporter constructs used in these transfection studies. The reporter constructs were either deletion (BS3-Box1) or point mutations (mutBS2 BS23-Box1, mutHRE BS23-Box1) of the mouse *epo* enhancer reporter that is efficiently and preferentially activated by HIF-2 (WT BS23-Box1). The reporter constructs were transfected into (B) Hep3B cells and then exposed to continuous hypoxia (1% oxygen) for 16 hours.

The effects of BS2 deletion or mutation upon the BS23-Box1 Epo enhancer reporter construct function indicate that BS2 is important for optimal hypoxia inducibility. Both of these constructs exhibited a significant reduction in hypoxia inducibility relative to the parental *epo* enhancer construct (BS23-Box1). Although alteration or elimination of BS2 sequence reduced hypoxia-induced activation of the *epo* enhancer by up to half, BS2 is not sufficient for hypoxia inducibility as evident by the effect of the HRE site-directed mutation (mutHRE-BS23-Box1). I note that similar basal levels of activity were present for all *epo* enhancer reporters, indicating that BS2 contributes substantially to the absolute responsiveness of the *epo* enhancer to hypoxia.

To evaluate if BS2 sequence was also important for HIF-2α preferential activation of epo enhancer reporter, I over-expressed P1P2N HIF-1α or P1P2N HIF-2α together with the *epo* enhancer reporter containing the site-directed mutation of BS2 (mutBS2-BS23-Box1) compared to the parental construct (BS23-Box1) (**Figure2-6A**). As shown, mutation of BS2 abrogates preferential activation of the *epo* enhancer by HIF-2α relative to HIF-1α in both HEK293 (data not shown) and Hep3B cells (**Figure 2-6B**). Immunoblots of epitope tag over-expressed P1P2 HIF-1α and P1P2N HIF-2α show similar level of HIF-α **(Figure 2-6B).** These data confirm results observed with the BS2 deletion construct (BS3-Box1) in the presence of HIF over-expression (**Figure 2-4**).

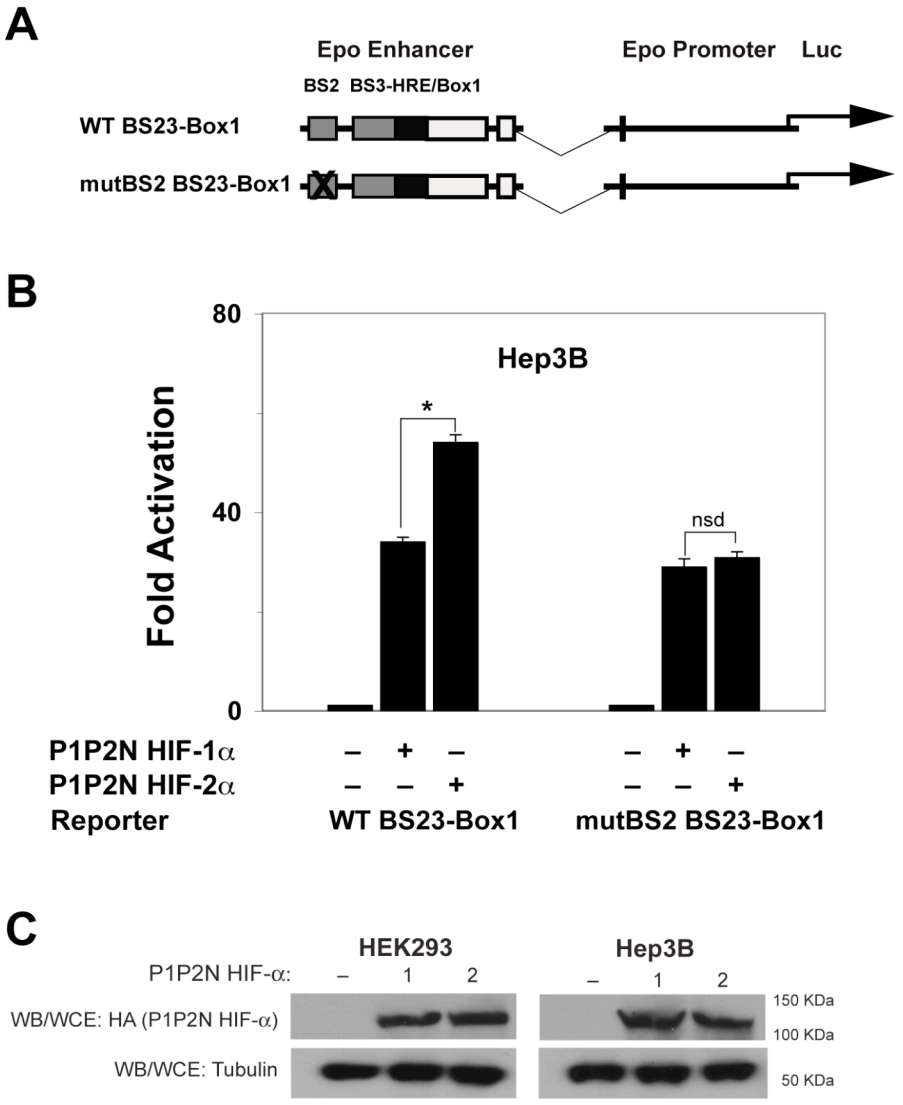


Figure 2-6. BS2 confers HIF-2 selective activation of the *epo* enhancer
(A) Schematic representation of the mouse *epo* enhancer reporter constructs used in these transfection studies. The reporter constructs were either a mouse *epo* enhancer reporter that is efficiently and preferentially activated by HIF-2 (WT BS23-Box1), or the same construct with point mutations introduced into BS2 (mutBS2 BS23-Box1). The reporter constructs were transfected into (B) Hep3B cells along with expression plasmids encoding constitutively active forms of HIF-1α or HIF-2α. **(C)** Exogenous P1P2N HIF-1α and HIF-2α proteins exhibit similar stability. Whole cell extracts (WCE) from HEK293 or Hep3B cells expressing P1P2N HIF-1α:HA or P1P2N HIF-2α:HA were immunoblotted using antibodies recognizing the HA epitope tag or α-tubulin.

Identification of candidate transcription factors that bind to conserved BS2 element

I hypothesized that the evolutionarily conserved BS2 sequence dictates HIF-2α selective activation of the Epo enhancer. To test this hypothesis, I used a transcription factor binding site analysis database (TRANSFAC TFscan) of eukaryotic *cis*-acting regulatory DNA elements and trans-acting factors to interrogate mammalian epo enhancer regions (ftp://ftp.ebi.ac.uk/pub/databases/transfac/transfac32.tar.Z). This database includes yeast to human transcription factors and their recognition sites. The BS2 GC-rich sequence fits the consensus sequence of zinc-finger transcription factors Sp1 /Sp2, WT-1 and Egr family members **(Figure 2-7A)**, and a weak Ets binding site consensus.

To determine whether Sp1/Sp3 and WT1 can confer HIF-2α selective activation of the epo enhancer BS23 epo promoter reporter through the BS2 element, I compared activation of the parental *epo* enhancer reporter (BS23-Box1) with the site-directed mutation of BS2 (mutBS2-BS23-Box1) (**Figure 2-7B**). I transfected HEK293 (data not shown) and Hep3B cells with the BS23-Box1 or the mutBS2-BS23-Box1 reporter in conjunction with either P1P2N HIF-1α or P1P2N-HIF-2α. Sp1 protein over-expression has no significant effect upon HIF-1α or HIF-2α activation of the epo enhancer reporters, whereas Sp3 and WT1 reduces or increases HIF-1α and HIF-2α activation in a non-selective manner respectively (**Figure 2-7C**). Mutation of BS2 does not abrogate Sp3 or WT1 effects on HIF-1α or HIF-2α.

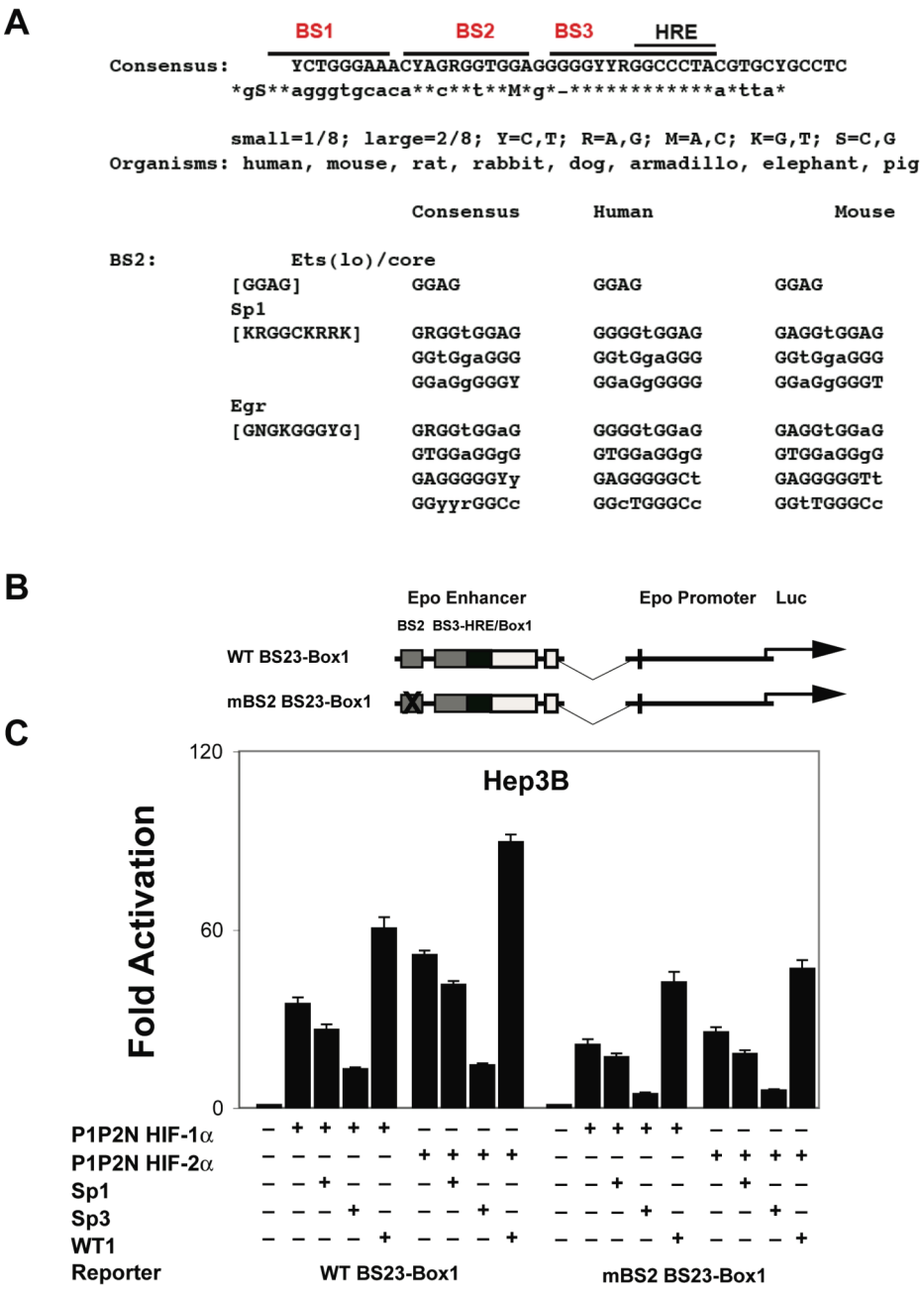


Figure 2-7. Candidate approach of the evolutionarily conserved BS2 sequence. (A) Candidate transcription factor binding site in the conserved epo enhancer sequence BS2 adjacent to the hypoxia responsive element (HRE). A consensus sequence was generated based on the conservation in mammalian species and evaluated for transcription binding site using software analysis programs (TFscan, rVISTA) and manual inspection (Akuzawa et al., 2000; Al-Sarraj et al., 2005). (B) Set of reporters used in transfection assays in which mBS2-BS23-Box1 have point mutations in the BS2 region. C) Over-expression of P1P2N HIF-1α or P1P2N HIF-2α together with Sp1, Sp3 or WT1 in Hep3B in the presence of the indicated reporters.

The Egr transcription factor family members are potential candidates that may bind to the BS2 region. I investigated whether Egr members could act synergistically with HIF-2α to activate the BS23 Epo enhancer. Over-expression of Egr1, Egr2, Egr3, and Egr4 **(Figure 2-8A)** together with the P1P2N HIF-1α or P1P2N HIF-2α revealed synergistic activation by Egr1 and Egr2 with HIF-2α, but not HIF-1α, in both HEK293 as well as Hep3B cells (**Figure 2-8B**). Egr3 had no significant effect on either HIF-1α or HIF-2α activation of the Epo enhancer construct, whereas Egr4 repressed both HIF-1α as well as HIF-2α activation of the *epo* enhancer. These data revealed a selective role for Egr1 and Egr2 on HIF-2α activation of the BS23-Box1 Epo enhancer construct.

To determine if Egr1 and Egr2 act through the BS2 element, we examined the activation of the Epo enhancer reporter containing the site-directed mutation of BS2 (mutBS2-BS23-Box1), compared to activation of the parental construct (BS23-Box1) (**Figure 2-8C)**. Egr1 or Egr2 augmentation of the epo enhancer reporter with HIF-2α is abrogated by BS2 mutation. Egr1 and Egr2 require an intact BS2 to synergistically activate the *epo* enhancer with HIF-2α.

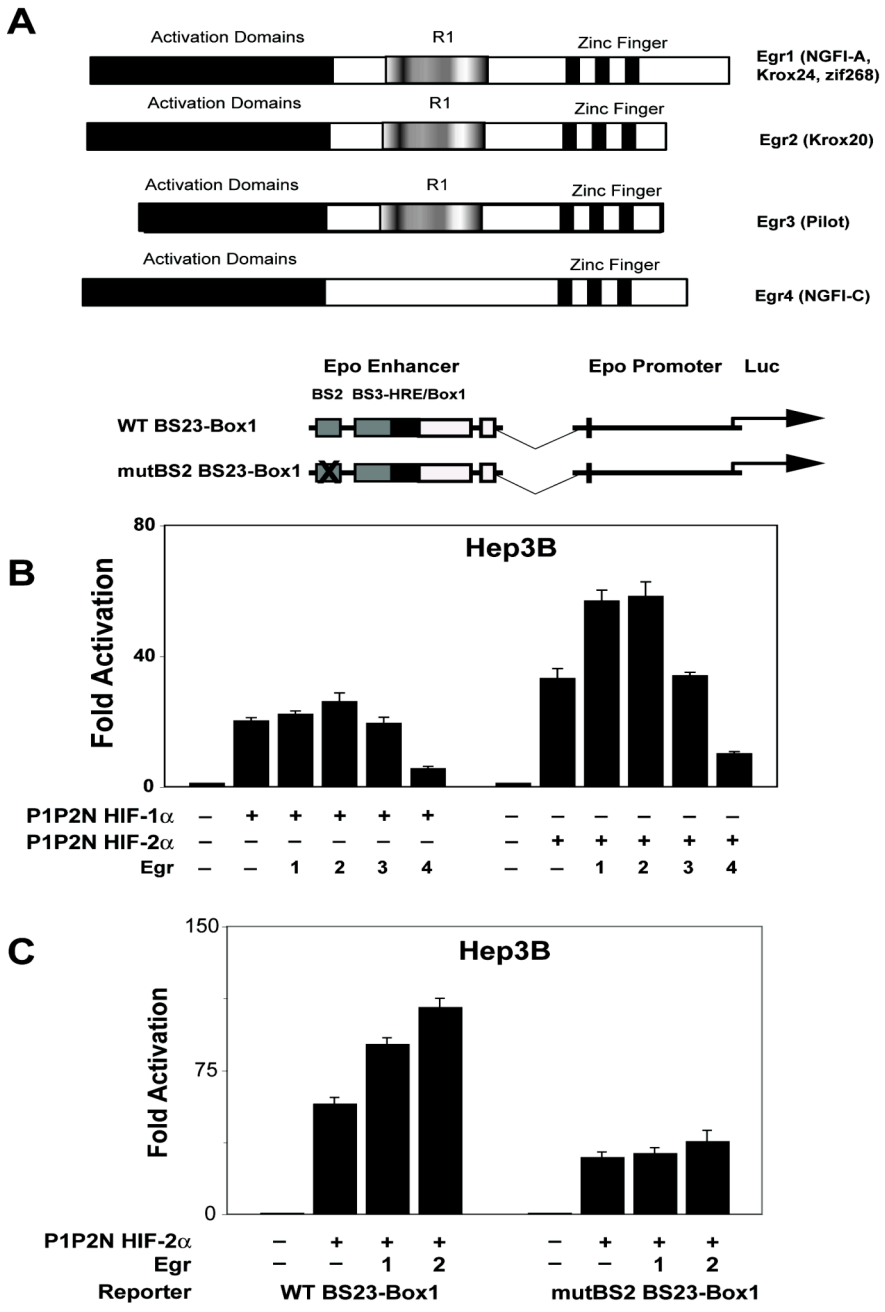


Figure 2-8. Egr1 and Egr2 act synergistically with HIF-2 to augment *epo* enhancer activity. (A) Schematic representation of the mouse *epo* enhancer reporter construct used in these transfection studies. The reporter construct was a mouse *epo* enhancer reporter that is efficiently and preferentially activated by HIF-2 (WT BS23-Box1). The reporter construct was transfected into (B) Hep3B cells along with expression plasmids encoding constitutively active forms of HIF-1α or HIF-2α with or without expression plasmids encoding each of the four Egr family members (Egr1, 2, 3, 4). (C) Over-expression of Egr1 or Egr2 together with HIF-2α using the BS2mut BS23-Box1 or the BS23-Box1 parental reporter.

The NAB2 protein inhibits Egr transcriptional activity by direct protein interaction between the NAB2 NCD1 domain and the conserved R1 domain present in Egr1, Egr2 and Egr3, but absent in Egr4 (Houston et al., 2001; Svaren et al., 1996). The repressive interaction of NAB2 to Egr can be abrogated by point mutation of a conserved glutamate (E82K) in the NAB2 conserved domain 1 (NCD1) making it dominant negative form (dnNAB2). A point mutation within the R1 domain of Egr1 (I 292F) or Egr2 (I 218F), which abrogates interaction with NAB proteins renders, Egr1 and Egr2 constitutively active (CA Egr1, CA Egr2).

To further examine the influence of Egr signaling in *epo* enhancer function, we over-expressed together with P1P2N HIF-2α the wild type (WT) or dominant negative (dn) forms of NAB2, in either the presence or absence of Egr1 or Egr2 (**Figure 2-9**). The over-expression of WT NAB2 or dnNAB2 has no effect on HIF-2α transcriptional activity *per se*. WT NAB2 over-expression blocks Egr1 and Egr2 synergy of the *epo* enhancer reporter with P1P2N HIF-2α. CA Egr1 and CA Egr2 are insensitive to the repressive effect of WT NAB2. The over-expression of dn-NAB2 did not affect HIF-2α/Egr1 and HIF-2α/CA Egr1 synergy; however, it further stimulated HIF-2α/WT Egr2 and HIF-2α/CA Egr2 activation of the BS23-Box1 epo enhancer reporter.

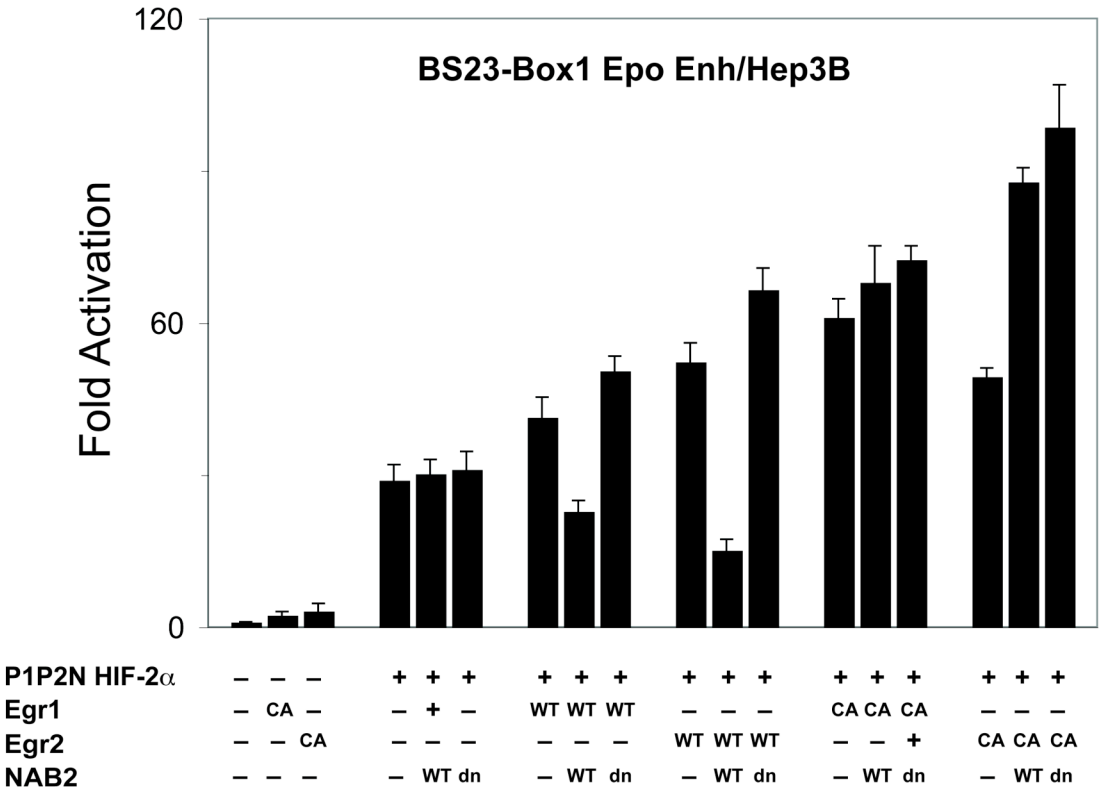


Figure 2-9. NAB2 represses Egr1 and Egr2 activation of the *epo* enhancer. The BS23-Box1 epo enhancer epo promoter reporter construct was transfected into Hep3B cells along with expression plasmids encoding P1P2N HIF-2α with or without expression plasmids encoding Egr1 or Egr2 with or without wild type (WT) or dominant negative (dn) forms of the Egr repressor protein NAB2.

HIF complex formation with Egr1 and Egr2

To determine if Egr synergy is conferred by selective complex formation with HIF-2α, we performed a co-immunoprecipitation assay by co-expressing P1P2N- HIF-1α:HA or P1P2N- HIF-1α:HA together with WT vsv-g tagged Egr1, Egr2, Egr3, or Egr4 proteins in HEK 293 cells (**Figure 2-10**). Egr1 protein is extremely unstable under normoxic conditions. To prevent its degradation, I treated Egr1 transfected cells with the proteasome inhibitor MG132 (Bae et al., 2002). In HA pull down assays followed by immunoblots against HA and vsv-g, Egr1 selectively interact with HIF-2α, but not HIF-1α. In contrast, Egr2 interacts with both HIF-1α and HIF-2α, in spite of the fact that it only augments HIF-2α trans-activation. Egr3 binds to neither HIF-1α nor HIF-2α whereas Egr4, which inhibits both HIF-1α and HIF-2α, formed stable complexes with both HIF-α isoforms (**Figure 2-10**).

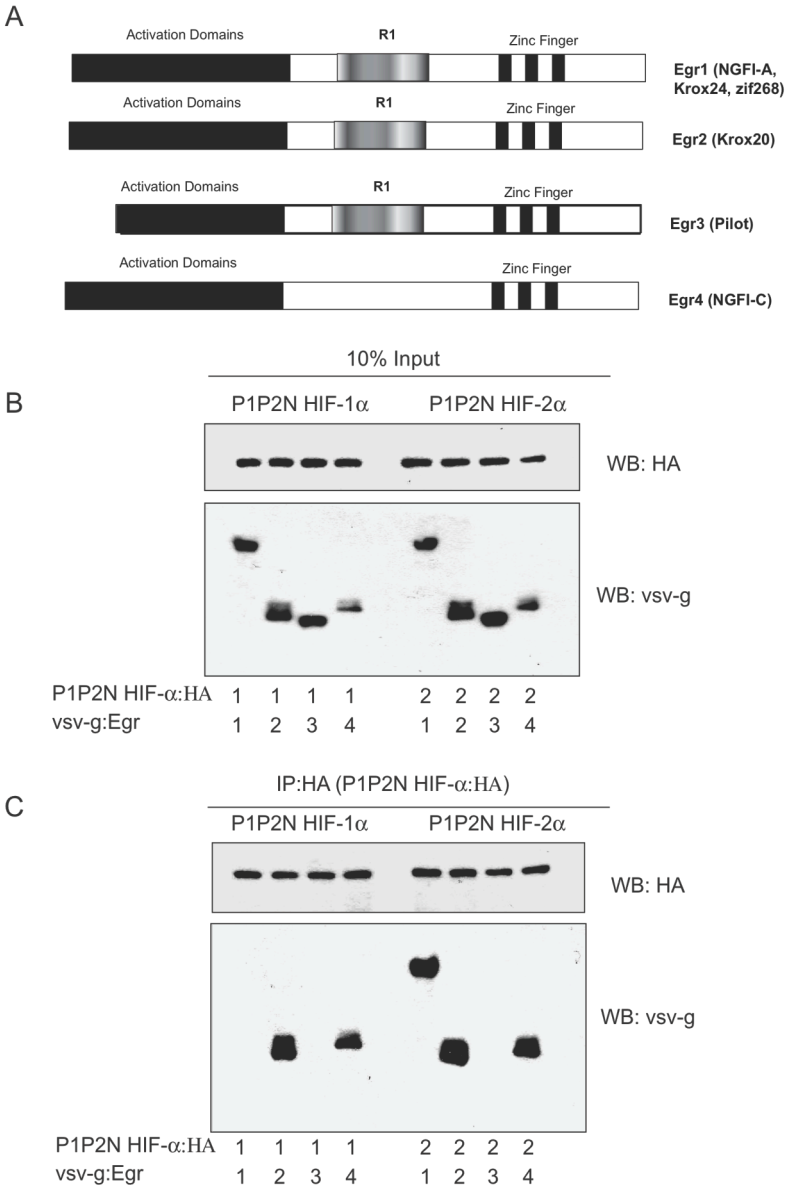


Figure 2-10. Egr1 selectively interact with HIF-2α, Egr2 and Egr4, but not Egr3; form a complex with HIF-1α and HIF-2α. (A) Western blots (WB) using whole cell extracts from HEK293 cells transfected with expression plasmids encoding epitope-tagged P1P2N HIF-1α, P1P2N HIF-2α:HA along with vsv-g:Egr1, Egr2, 3, or Egr4 representing 10% input. (B) Western blots (WB) of the HA (P1P2N HIF-α) co-immunoprecipitation (IP) fraction from the whole cell extracts described above with a monoclonal antibody against the indicated epitope tag.

Physical interactions between Egr2 and full length HIF-1α or HIF-2α is necessary, but not sufficient, to induce synergistic activation of the epo enhancer reporter. To further define the nature of the HIF-1α/Egr2 and HIF-2α/Egr2 interaction, I constructed a series of HIF-1α and HIF-2α deletion constructs that were over-expressed in HEK293 cells together with WT vsv-Egr2. The P1P2N HIF-1α:HA carboxy terminus deletion NTAD-UR-CTAD (aa# 351-826) co-immunoprecipitates with Egr2 as well as the amino terminus deletion (aa# 1-572) containing the bHLH-PAS-NTAD domains. However, the HIF-1α N-terminus bHLH PAS (aa# 1-351) and the UR CTAD (aa# 573-826) deletions failed to form stable complexes with Egr2.

Using similar P1P2N HIF-2α deletions, only the amino terminus bHLH-PAS (aa# 1-350) failed to interact with Egr2 in HA pull-down assays. The carboxy terminus NTAD-UR-CTAD region (aa# 351-870) as well as the UR-CTAD region (aa 543-870) form stable complexes with Egr2 (**Figure 2-11B)**. To further delineate the domain in HIF-2α required for stable interaction with Egr2, I made several deletions of the carboxy terminus region of P1P2N HIF-2α:HA (**Figure 2-11B**). In co-immunoprecipitation assays followed by HA and vsv-g epitope immunoblots, Egr2 stably interacts with the NTAD and UR-CTAD domains of HIF-2α (**Figure 2-11D)**. The HIF-2α UR domain alone is not sufficient to interact with Egr2. Whether it participates in the HIF-2α CTAD interaction with Egr2 remain to be explored.

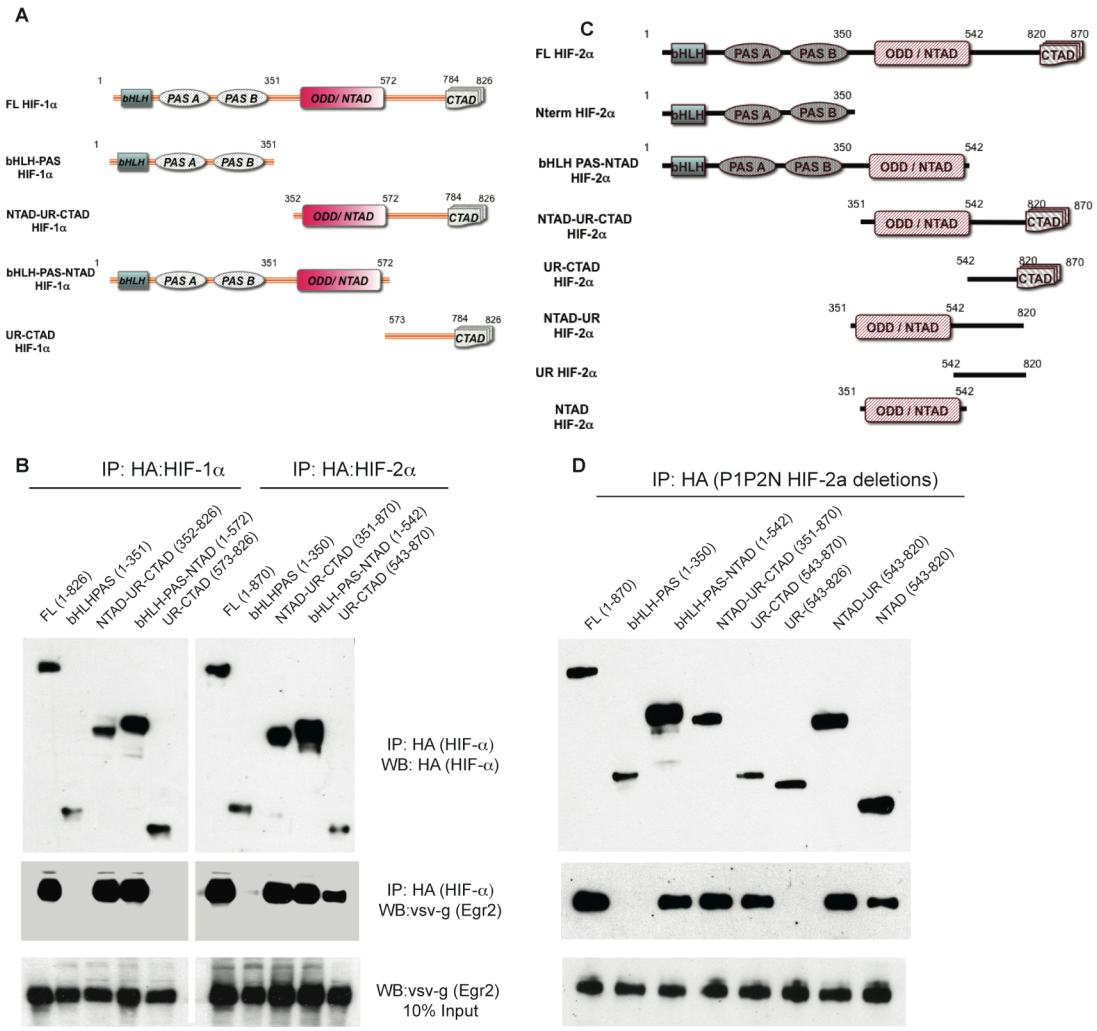


Figure 2-11: Egr2 interacts with NTAD domain of HIF-1α but with NTAD and CTAD domains of HIF-2α. (A) Schematics of the HIF-1α/HIF-2α deletions used for co-immunoprecipitation (IP). (B) Western blot (WB) of the immunoprecipitated (IP) HA–tagged HIF-α deletion along with vsv-g:Egr2 from HEK293 whole cell lysate over-expressing either the P1P2N HIF-1α orP1P2N HIF-2α deletion constructs together with vsv-g:Egr2. (C) HIF-2α:HA deletions used in co-IP with vsv-g:Egr2. (D) Western blot (WB) of immunoprecipitated (IP) HIF-2α:HA deletions together with the vsv-g:Egr2. Input is whole extract representing 10% of the IP protein.

To identify the region of HIF-2α conferring Egr1 and Egr2 synergistic activation, I constructed hybrid forms of HIF-1α and HIF-2α in which the UR-CTAD carboxy terminal regions of the proteins were exchanged **(Figure 2-12 A)**. The HIF-1α/HIF-2α hybrids and the parental HIF-α forms were over-expressed in the absence or presence of Egr1 or Egr2 **(Figure 2-12B**) in Hep3B cells together with the BS23-Box1 epo enhancer reporter. The data indicate that Egr1 or Egr2 synergistic activation of HIF-2α signaling is conferred by the HIF-2α carboxy terminal UR-CTAD region. Exchange of the HIF-1α UR-CTAD region with the HIF-2α UR-CTAD region results in a hybrid protein that exhibits Egr synergistic signaling whereas the converse results in a hybrid HIF protein without synergistic Egr signaling. The hybrid results, taken together with the interaction domain results, suggests that the HIF-2α UR-CTAD domain is necessary for Egr synergy with HIF-2α.

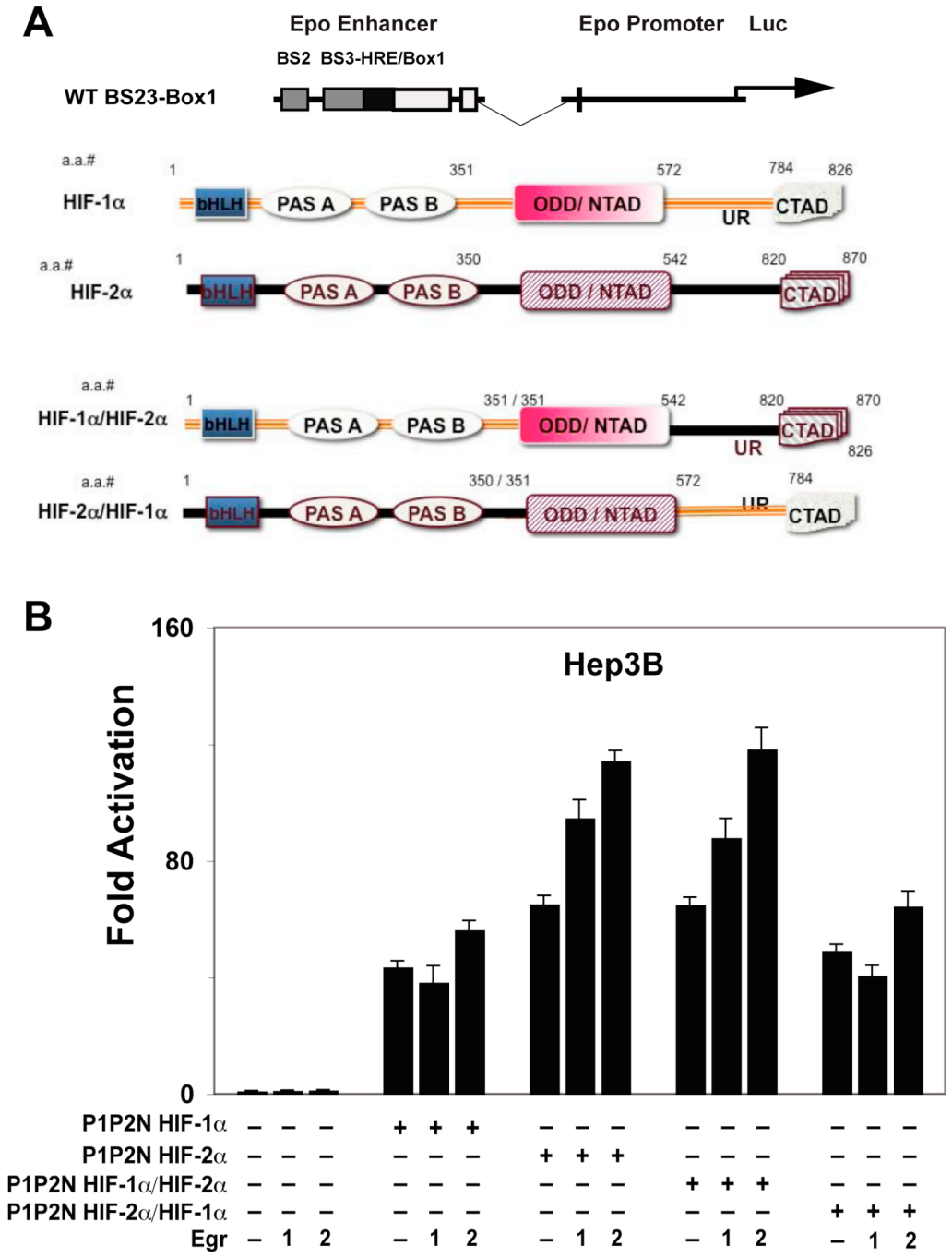


Figure 2-12: Egr1 and Egr2 activation are conferred through the HIF-2α carboxy terminal region. (A) Schematic representations of the mouse *epo* enhancer reporter construct (WT BS23-Box1) and the parental constitutively active forms of HIF-1α or HIF-2α as well as hybrid constructs in which the unique region and carboxy terminal activation domain (UR-CTAD) where exchanged between HIF-1α and HIF-2 in Hep3B cells (B) reporter assay with BS23-Box1 epo enhancer reporter along with expression plasmids encoding the P1P2N HIF-α parental and hybrid proteins in the absence or presence of Egr1 or Egr2.

Relevance of Egr in the regulation epo in cell culture

Next, I examined levels of endogenous HIF-1α, HIF-2α, Egr1, Egr2, and NAB2 protein level in Hep3B cells treated with hypoxia at various times. From nuclear extracted proteins, (**Figure 2-13A**) western blotting analyses indicate an early transient peak of HIF-1α as well as Egr1 and NAB2 protein level within the four first hours of treatment whereas HIF-2α and Egr2 protein levels showed a delayed and sustained induction with hypoxia. To assess the *in vivo* relevance of Egr signaling to endogenous Epo mRNA expression, I purified total RNA from Hep3B cells treated with hypoxia and determined Epo, Egr1 and Egr2 mRNA expression by real-time RT-PCR experiments**.** The data revealed a sustained exponential increase of Epo mRNA expression in response to hypoxia for the duration of the experiment (**Figure 2-13B)**. Egr1 mRNA increased gradually with hypoxia and peak at 8 hrs whereas Egr2 mRNA expression level remains invariable (**Figure 2-13C D)**.

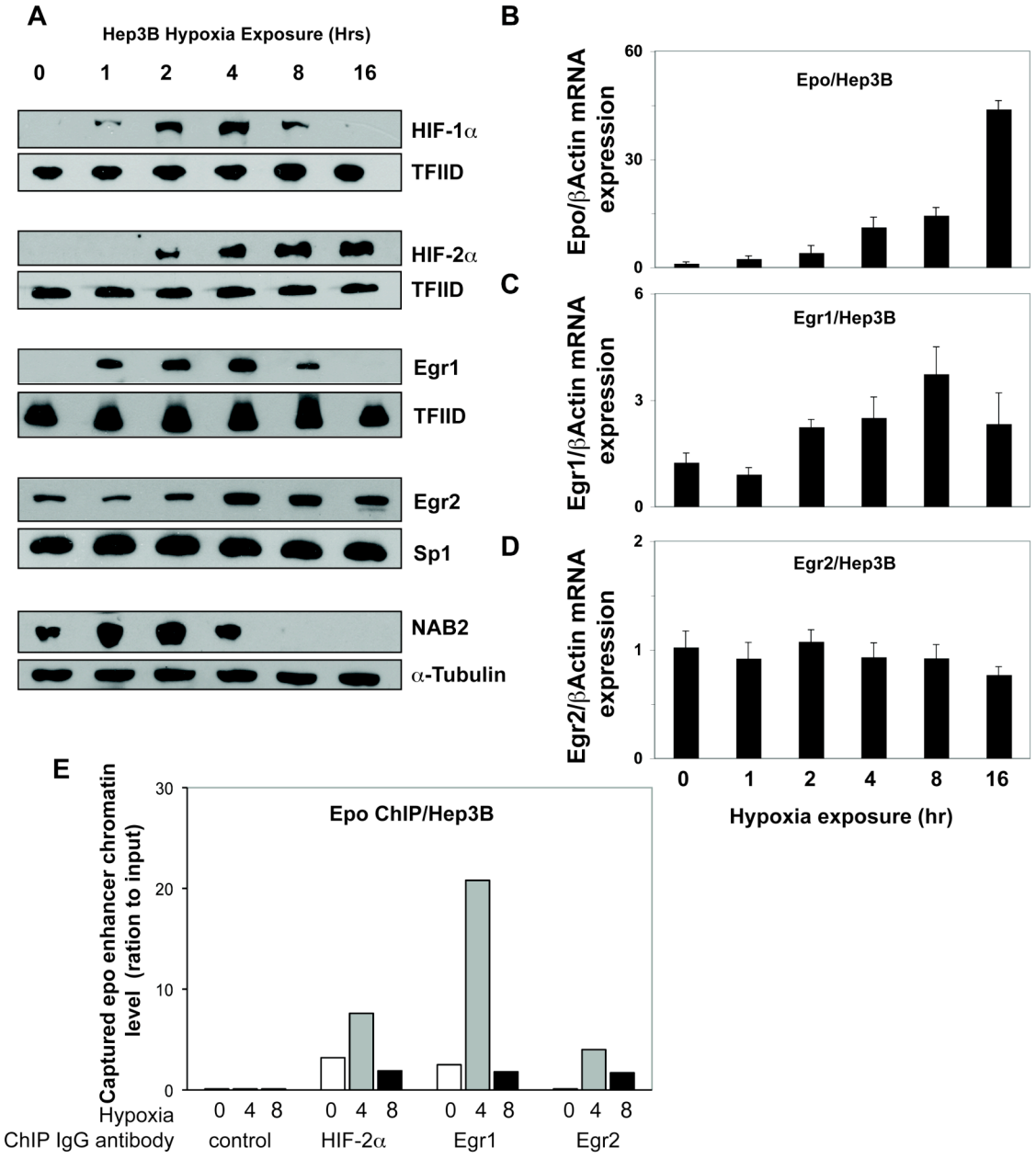


Figure 2-13: HIF, Egr protein and *epo* mRNA expression profile in hypoxia-treated Hep3B cells. (A) Western blots of endogenous HIF-1α, HIF-2α, Egr1, Egr2 and NAB2 as well as the loading controls TFIID, Sp1 and α-tubulin. Extracts were prepared from Hep3B cells at 0, 1, 2, 4, 8, and 16 hours following continuous hypoxia exposure (1% oxygen). (B) *Epo,* Egr1 (C) or Egr2 (D) mRNA levels as determined from a portion of the same cells exposed to continuous hypoxia as above. The measurements were made by semi-quantitative real-time RT-PCR determined relative to β-actin normalized

Recruitment of transcription factor to target gene regulatory sequences is a pre-requisite for activation. To determine if Egr1 and/or Egr2, as well as HIF-2α, are associated with the endogenous *epo* enhancer chromatic region following hypoxia exposure in Hep3B cells, we performed chromatin immunoprecipitation (ChIP) experiments after 4 hr and 8 hr following the onset of hypoxia compared to normoxia control (0 hr) (**Figure 2-13 E)**. As seen below, both Egr1 and Egr2 binding to the endogenous *Epo* enhancer region is increased following hypoxia exposure. Binding of Egr1, Egr2, and HIF-2α are maximal at 4 hr relative to the room air or 8 hr hypoxia time-point.

To determine if endogenous *Epo* gene expression can be affected in Hep3B cells by either positively or negatively altering of Egr signaling proteins, I over-expressed P1P2N HIF-1α or P1P2N HIF-2α in the presence or absence of WT Egr1, WT Egr2, or dnNAB2. The level of Epo expression induced by P1P2N HIF-1α is dramatically lower than that of P1P2N HIF-2α. Over-expression of P1P2N HIF-1α together with WT Egr1, WT Egr2 or dnNAB2 did not further increase the level of epo induction **(Figure 2-14A)**. WT Egr2 or dnNAB2 over-expression with P1P2N HIF-2α resulted in synergistic activation of endogenous *epo* gene expression. The magnitude of Epo induction was close to or exceeded the level of induction observed upon hypoxia exposure. Wt Egr1 over-expression with P1P2N HIF-2α failed to increase Epo mRNA to levels higher than that of HIF-2α alone. This might be due to the low level of Egr1 proteins in normoxia (**Figure 2-14D**).

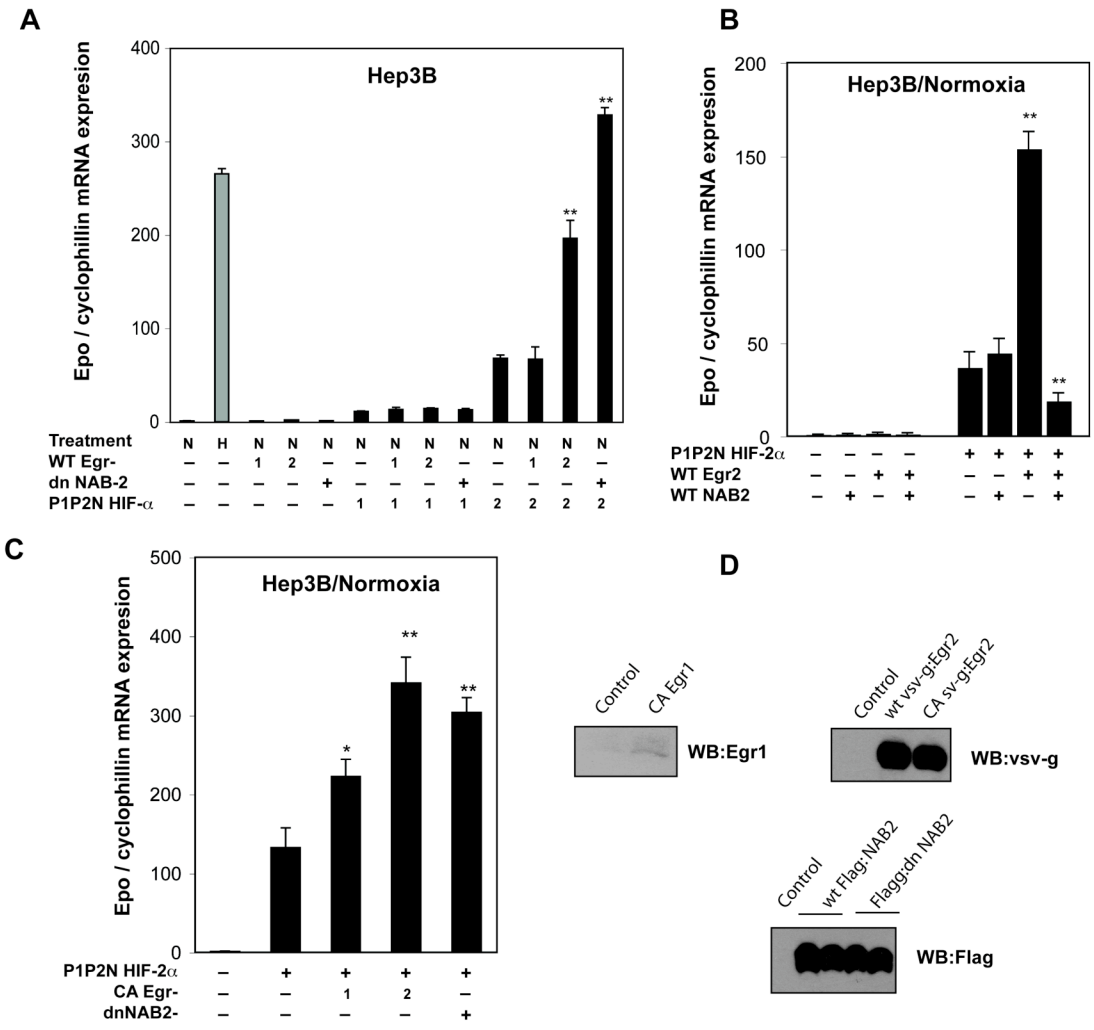


Figure 2-14: Egr1 and Egr2 over-expression regulate endogenous epo expression in Hep3B cells. (A) Real-time RT-PCR of endogenous *epo* expression in Hep3B cells over-expressing P1P2N HIF-1α or P1P2N HIF-2α together with WT Egr1, WT Egr2 or dn NAB2 under normoxia (N). Hypoxic treated Hep3B cells for 16 hrs (H) was used a positive control. WT Egr1, WT Egr2 and dn NAB2 over-expressed alone are used as control. (B) Real-time RT-PCR of endogenous epo in normoxic Hep3B cells over-expressing P1P2N HIF-2α in the presence or absence of wtEgr2 and/or WT NAB2, (C) CA Egr1, CA Egr2 or dnNAB2. (D) Western blot (WB) analysis of 20μg of total protein from over-expressed CA Egr1, Wt and CA vsv-g:Egr2, WT Flag:NAB2 or dn Flag:NAB2 in Hep3B cells *p<0.05, **p<0.0001).

Over-expression of the Egr co-repressor NAB2 has no effect on P1P2N HIF-2α activation of *epo* gene expression in Hep3B cells. However, when over-expressed with P1P2N HIF-2α and WT Egr2, WT NAB2 dramatically reduced endogenous epo expression to levels substantially lower than P1P2N HIF-2α alone induction (**Figure 2-14B).** Endogenous NAB proteins are capable of inhibiting the activity of the low level of Egr1 protein over-expressed in our cell culture experiment. To circumvent NAB inhibitory effect, over-expression of CA Egr1, similar to CA Egr2, significantly increased P1P2N HIF-2α mediated induction of endogenous Epo (**Figure 2-14C)**.

Egr1 and Egr2 protein level can be knocked down by short hairpin RNA (shRNA) over-expression in Hep3B cells treated with hypoxia **(Figure 2-15A)**. Epo mRNA expression is significantly reduced after knockdown of either endogenous Egr1 or Egr2, and is further reduced by combined knockdown of both Egr1 and Egr2 (**Figure 2-15B**). Combined knockdown of Egr1 and Egr2 show an additive affect upon reduction of epo mRNA levels below 50% of control. Levels of a classical HIF target gene, vascular endothelial growth factor (VEGF) mRNA expression was not affected by Egr1, or Egr2 knockdown, or by the combination of Egr1 and Egr2 shRNA knockdown (**Figure 2-15C)**, suggesting the specificity of Egr1 and Egr2 signaling to *epo* gene expression.

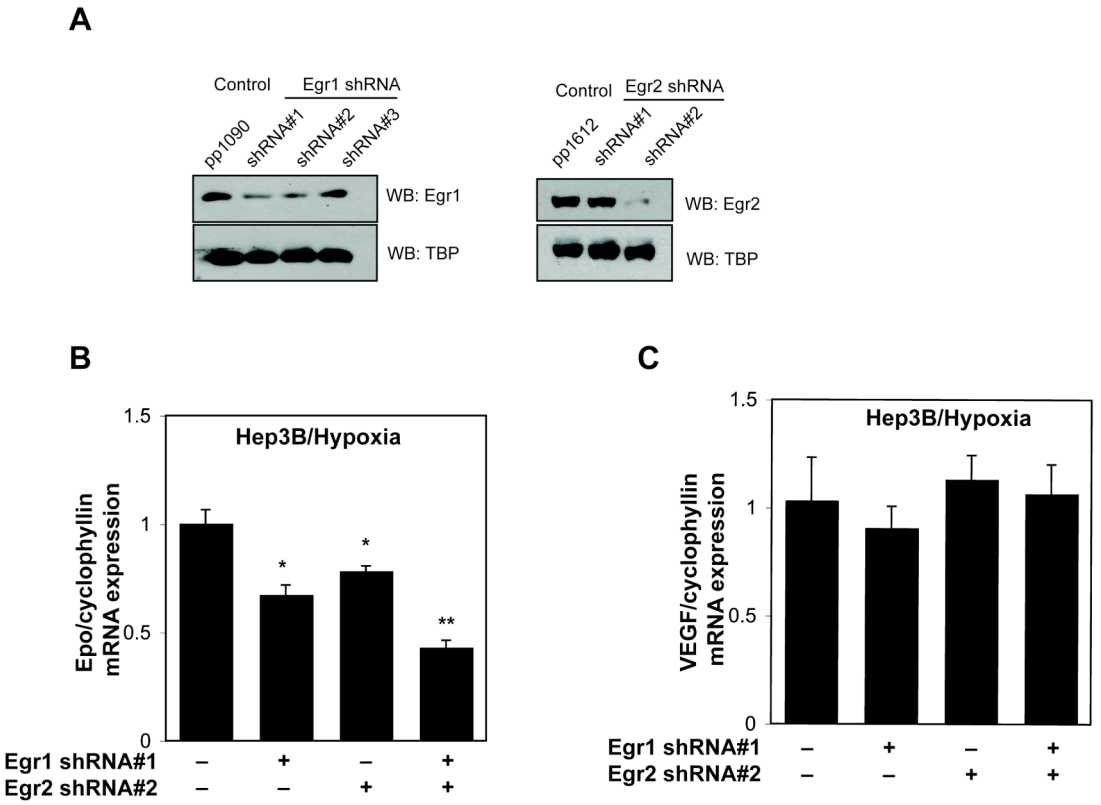


Figure 2-15: Egr1 and Egr2 knockdown reduce endogenous epo expression in Hep3B cells under hypoxia. (A) Western blot (WB) analysis of Egr1 and Egr2 nuclear proteins following knock down with short hairpin RNA (shRNA) compared to control non-silencing shRNA in Hep3B cells treated with hypoxia TBP is used a loading control. (B) Real time RP-PCR analysis of endogenous epo expression in Hep3B cells treated with hypoxia after Egr1, Egr2 or Egr1+Egr2 shRNA knockdown. (C) As a control VEGF mRNA expression is assessed from the same samples. (*$p<0.05$, **$p<0.0001$ compared to control shRNA).

Egr transcription family members share a homologous zinc-finger carboxy-terminal DNA-binding domain (**Supplemental Figure 2)**. Egr3 does not interact with HIF-2α, nor does it augment HIF-2α transcriptional activity. To determine the molecular mechanism by which Egr2 augments HIF-2α trans-activation of the BS23-Box1 Epo enhancer reporter, I made Egr2/Egr3 hybrids by swapping the Egr2 trans-activation domain (TAD) alone, or Egr2 TAD and repressor R1 domain with, respectively, the Egr3 zinc-finger domain or Egr3 R1 and zinc-finger domain, and *vice-versa* (**Figure 2-16A**). The parental as well as the Egr2/Egr3 and Egr3/Egr2 hybrids expression vectors were transfected together with the P1P2N HIF-2α and the BS23-Box1 epo enhancer reporter in Hep3B cells. The reporter assays data show that the TAD domain of Egr2 (aa# 1-99) confers synergistic activation with HIF-2α; the TAD domain of Egr3 fused to the Egr2 carboxy terminus R1 and zinc-finger domains failed to augment HIF-2α trans-activation (**Figure 2-16B)**.

In order to identify the Egr2 domain that confers complex formation with HIF-2α, I performed a co-immunoprecipitation experiment between P1P2H HIF-2α:HA and vsv-g:Egr2/Egr3 hybrids as well as the Egr parental constructs (**Figure 2-16C).** HA (HIF-α) immuno-precipitation (IP) followed by western blot (WB) against HA and vsv-g epitope tag showed that Egr2 TAD domain is required for interaction between Egr2 and HIF-2α. These data suggest that binding of Egr2 to BS2, together with interaction with HIF-2α, are necessary for Egr2 synergistic activation of the Epo enhancer.

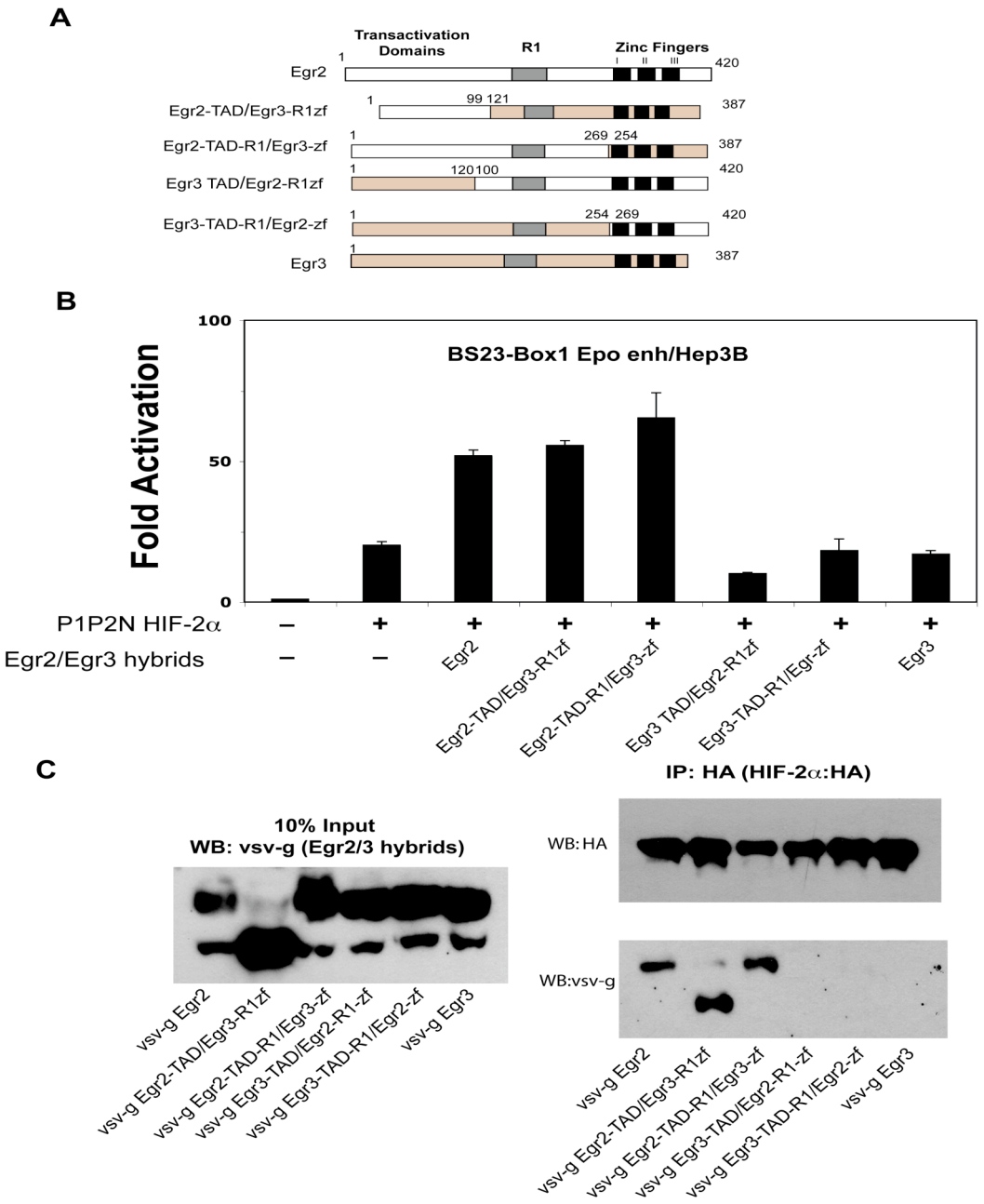


Figure 2-16. Trans-activation domain of Egr2 confers synergistic activation with HIF-2α. (A) Schematics of Egr2/Egr3 (Egr2/3) hybrid constructs where the trans-activation domain (TAD), the R1 repressive domain or the zinc-finger DNA-binding domains (zf) were swapped between vsv-g:Egr2 and vsvg:Egr3. (B) Transient transfection reporter assay in Hep3B cells with BS23-Box1 epo enhancer reporter along with expression plasmids encoding the P1P2N HIF-2α in the absence or presence of Egr2/Egr3 parental and hybrid proteins. (C) Co immuno-precipitation of assays (IP) between over-expressed P1P2N HIF-2α:HA and vsv-g:Egr2/Egr3 after HA IP followed by western blots (WB) against designated tags.

DISCUSSION

Epo gene expression is dynamically and tightly regulated. The identification of a novel evolutionarily conserved regulatory element, BS2, introduces another level of complexity to the regulation of *epo* gene expression (**Figure 2-3**). Deletion and site-directed mutagenesis data indicate that BS2 is an essential *cis*-acting element required for optimal induction of the Epo enhancer reporter with HIF-2α, but not HIF-1α over-expression or under hypoxia (**Figure 2-4B, Figure 2-5B).** Deletion or point mutation of the BS2 abrogates HIF-2α selective activation of Epo enhancer reporter in transient transfection assay. These observations are suggestive of a *trans*-acting factor that binds to BS2 involved in preferential activation of the Epo enhancer region by HIF-2α.

Using a candidate approach, we identified Egr1 and Egr2 as factors that synergistically activate the Epo enhancer with HIF-2α through the BS2 region. Egr1 activation under hypoxia is HIF-independent. Egr1 expression is induced by members of the protein kinase C (PKC) family, in particular PKC β (Yan et al., 2000) and PKCα upstream in the RAF/RAS/ERK1/2 signaling pathway (Lo et al., 2001). Egr1 or Egr2 synergistically activates HIF-2α, but not HIF-1α, signaling. However, Egr1 or Egr2 over-expression alone has no effect upon Epo enhancer activity or endogenous epo expression (**Figure 2-14A)** indicating a synergistic, rather than an additive, effect of Egr1 and Egr2 upon HIF-2α activation of the BS23-Box1 epo enhancer reporter. Endogenous Egr1 and or Egr2 knockdown significantly reduced *epo* gene expression, but did not alter *Vegf* gene expression

in Hep3 cells treated with hypoxia (**Figure 2-15C)**. These data suggest that the HIF-2α/Egr signaling might be exclusive to a limited HIF target gene regulation *in vivo*.

The HIF-2α/Egr synergy can be altered by NAB2, a known inhibitor of Egr1 and Egr2 signaling (Sharp et al., 2004; Srinivasan et al., 2007), and expectedly the NAB-repression of Egr1 and Egr2 signaling is eliminated by dnNAB2. Over-expression of dnNAB2 together with P1P2N HIF-2α significantly augments endogenous *epo* expression compared to P1P2N HIF-2α alone in normoxic Hep3B cells **(Figure 2-14)**. NAB2 protein does not alter Egr protein DNA binding capacity, but rather forms a complex with Egr and inhibits its transcriptional activity (Svaren, 1996 #12846). NAB2 proteins can homodimerize and heterodimerize with NAB1 protein. The dnNAB2 protein retain the capacity to form multimers and can titrate away endogenous NAB proteins. DnNAB2 prevents binding of the endogenous NAB1 and NAB2 to Egr proteins (Venken et al., 2002), thereby allowing Egr to be constitutively active. We are actively investigating the molecular mechanism of Egr/NAB repression of the epo transcription.

Egr1/NAB2 and Egr2/NAB2 complexes might prevent Egr1 and Egr2 interaction with HIF-2α, reducing the efficient activation of *epo* gene expression. Another mechanism of NAB2 repression of HIF-2α/Egr synergy is that upon binding to Egr1 or Egr2, NAB2 protein can recruit through its NCD2 domain the CHD4 (chromodomain helicase DNA-binding protein 4) subunit, an integral

component of the NuRD (nucleosome remodeling and deacetylase) chromatin remodeling complex which represses transcription using both histone deacetylation and nucleosome mobilization (Mager et al., 2008; Srinivasan et al., 2006). In future studies, we will determine whether over-expressed NAB2 recruits the transcriptional repressive NuRD complex to the Epo enhancer in Hep3B cells.

Egr3 did not activate or complex with HIF-1α or HIF-2α. Egr4 interacts with and inhibits both HIF-1α and HIF-2α. Egr4 can repress both HIF-1α and HIF-2α mediated activation of the Epo enhancer, independently of BS2. *In vivo*, Egr4 expression is predominantly at high levels in the forebrain in areas where Egr1 and Egr2 are simultaneously expressed (Crosby et al., 1992) and at very low levels in maturing male germ cells. In Hep3B cells, Egr3 and Egr4 levels are borderline detectable and remain unchanged during hypoxia (data not shown). It is quite possible that Egr4 is a relevant inhibitor of HIF signaling in select cell types and might compete with Egr1 and Egr2 in the regulation of HIF-2α regulated Epo expression in the brain.

The mechanism by which Egr2 interacts with HIF-1α or HIF-2α and synergistically activates of the *epo* gene is presumably more complex. Egr2 interacts with both HIF-1α and HIF-2α; however, the nature of the interaction is different. Egr2 interacts with HIF-1α through the HIF-1α NTAD domain **(Figure 2-11)** and with HIF-2α through both trans-activation domains NTAD and CTAD. These data are suggestive of Egr2 anchored to HIF-2α *via* the NTAD and CTAD. In contrast, Egr2 interacts with HIF-1α only through the NTAD. This conclusion is

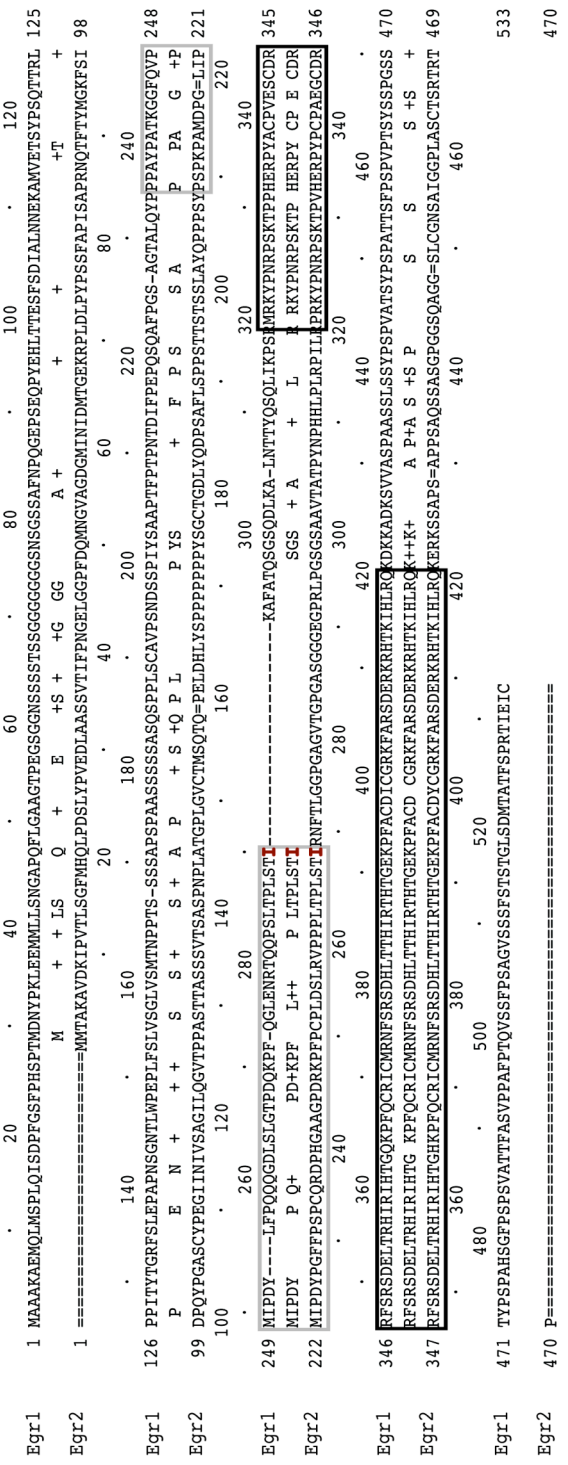
similar to other studies indicating that HIF-1α gene activation is dictated by the HIF-1α NTAD/ODD domain whereas HIF-2α gene selectivity is conferred through both the NTAD/ODD and CTAD domains (Hu et al., 2007). Whether HIF-1α/Egr2 interaction also regulate HIF-1α specific gene activation *in vivo* remain unknown.

Over-expressed Egr1 forms a stable complex with HIF-2α after inhibition of protein degradation using MG132 (Bae et al., 2002). Egr1 can only interact with HIF-2α, but not HIF-1α. In HIF hybrids transfection assay, Egr1 synergistic activation of the Epo enhancer requires the HIF-2α UR-CTAD region. In future immunoprecipitation experiments, I will determine the nature of the Egr1/HIF-2α interaction using P1P2N HIF-2α:HA deletion constructs over-expressed with WT vsv-g:Egr1 in the presence of MG132. Furthermore, the use of Egr1/Egr3 hybrids constructs will allow me to determine Egr1 domains that confer not only posttranslational instability, but also confer interaction with HIF-2α in co-immunoprecipitation assay.

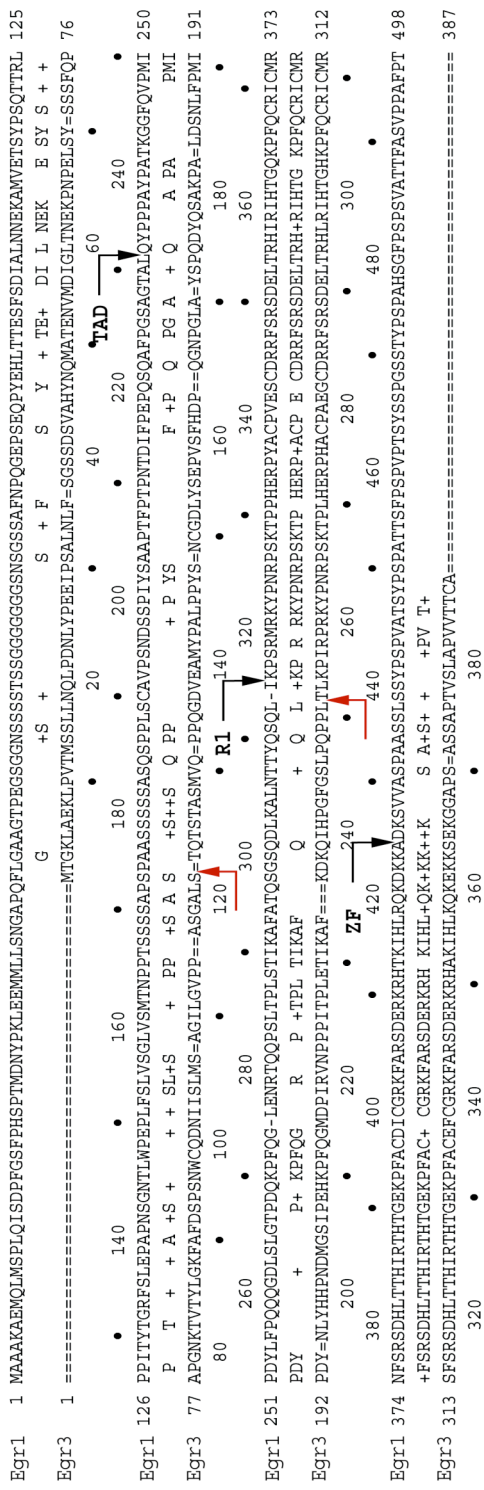
Egr1 and Egr2 proteins are induced during hypoxia with different kinetics in Hep3B cells. Egr1 is characterized by an early and transient induction similar to HIF-1α whereas Egr2 is induced with a late and more sustained induction similar to HIF-2α (**Figure 2-13A**). This induction of Egr2 is not directly a resultant of mRNA expression, since Egr2 mRNA was unchanged during hypoxia. Egr1 and Egr2 proteins might display increased nuclear translocation during hypoxia as described during viral infection (Silva et al., 2006). Alternatively, Egr2 protein levels may increase passively by interaction with HIF-2α as it is stabilized during

hypoxia. HIF-2α, together with Egr1 and Egr2, are recruited to the Epo enhancer during hypoxia (**Figure 2-13 E**) and synergistically induce endogenous epo expression (**Supplemental Figure 4 model**).

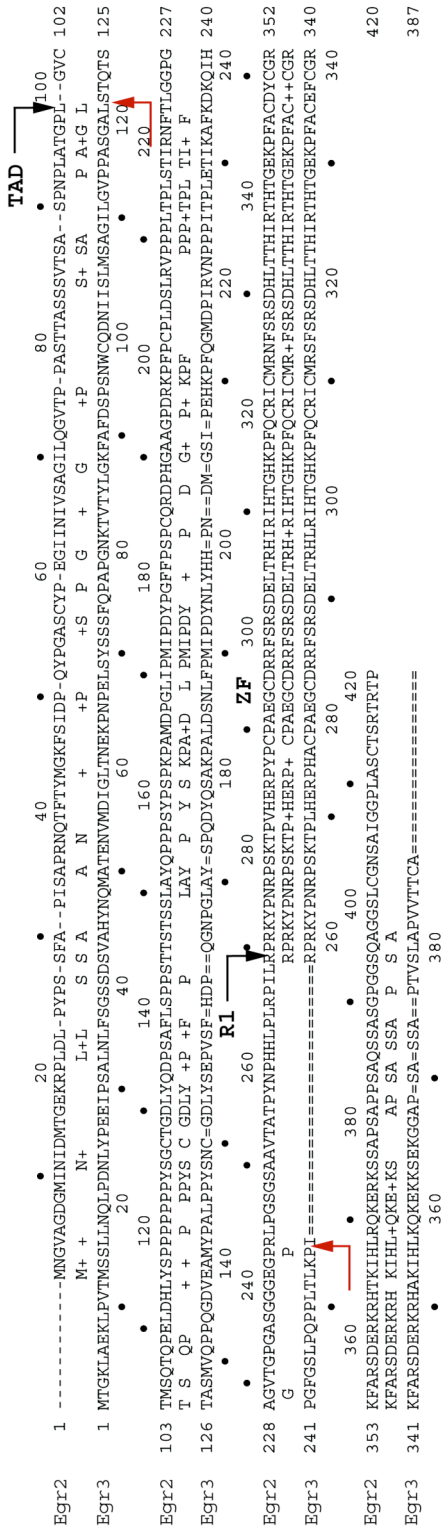
The cell culture experiments have laid the groundwork for subsequent *in vivo* experiments using mouse models to assess the role of Egr signaling during *in vivo* epo production. Matings of Egr2/cre-recombinase knock-in mice with floxed HIF-2α mice will allow us to determine whether cell co-expressing Egr2 and HIF-2α in mice are important for control of *epo* gene expression *in vivo*. Egr1 knockout mice treated with short-term continuous hypoxia will allow us to determine the biological function of Egr1 in the regulation of Epo expression. The induction of hepatic epo production can be modulated using adenoviral delivery systems with combinations of P1P2N HIF-2α, WT Egr1/2, CA Egr1/2 and/or WT/dnNAB2. We expect that over-expression of P1P2N HIF-2α alone will modestly increase hepatic Epo production, but expect substantial induction of hepatic Epo when combined with either CA Egr2 or dnNAB2. We predict the increased Epo mRNA levels will lead to substantial increase in the hematocrits of these experimental mice.



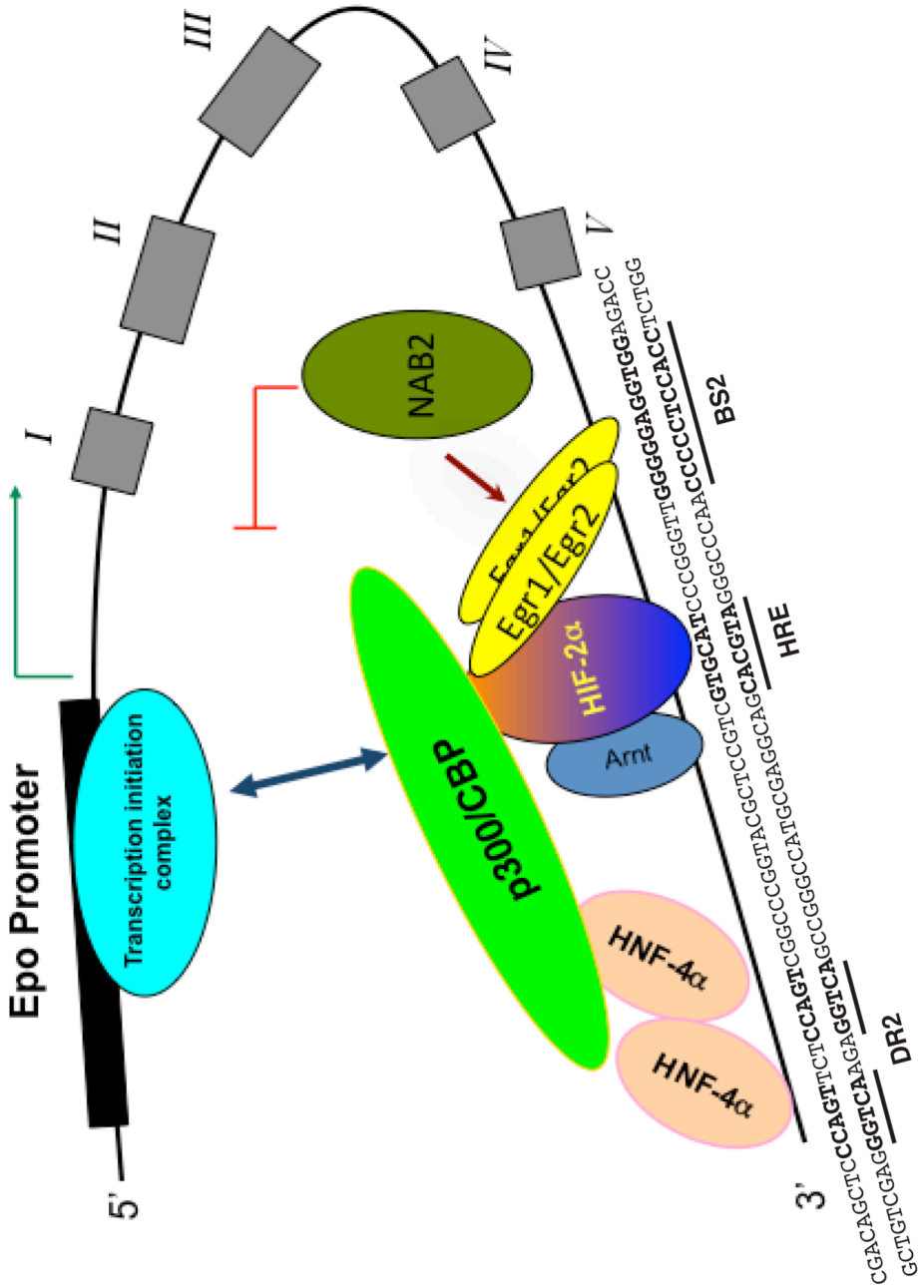
Supplemental Figure 1. Egr1 and Egr2 protein sequence homology. The black box represents the conserved C terminus zinc finger DNA-binding domain, whereas the grey box represents R1 repressor domain. In red letter we have the conserved isoleucine (I 290 in Egr1, and I 218 in Egr2) in the R1 domain essential for NAB2 interaction.



Supplemental Figure 2. Egr1 and Egr3 protein sequence homology. Arrows represent the borders (black is Egr1 and red is Egr3) between the trans-activation domains (TAD), R1 domain and zinc finger DNA binding domain (ZF) exchanged to make the Egr2/3 hybrid constructs



Supplemental Figure 3. Egr2 and Egr3 protein sequence homology. Arrows represent the borders (black is Egr2 and red is Egr3) between the transactivation domains (TAD), R1 domain and zinc finger DNA binding domain (ZF) exchanged to make the Egr2/3 hybrid constructs.



Supplemental Figure 4. Model HIF-2α and Egr1/Egr2 trans-activation of the *epo* gene during hypoxia. Recruitment of Egr1/Egr2 to the BS2 regions in the *epo* enhancer and interaction of HIF-2 bound to the HRE form an optimal transcription complex and induce transcription of the *epo* gene during hypoxia. The transcriptional activity of the HIF-2α/Egr1-2 complex can be inhibited by NAB2 binding to the R1 domain in Egr1 or Egr2.

CHAPTER THREE:

HIF-2: Standing Guard at the Crossroads of Hypoxic Stress and Aging

INTRODUCTION

HIFs are obligate heterodimeric transcription factors that regulate genes involved in hypoxic and other stress responses (Semenza, 2000). The alpha components of the three HIF transcription factors are unique and dictate biological specificity. Although the second HIF alpha member, Endothelial PAS domain protein 1 (*EPAS1*), also known as HIF-2α (Tian et al., 1997), is closely related to and shares some target genes with HIF-1α(Wang et al., 1995), HIF-1α and HIF-2α exhibit unique biological roles in part conferred by non-overlapping target genes.

Phenotypic analyses of HIF-2α null (Oktay et al., 2007; Scortegagna et al., 2003a; Scortegagna et al., 2005) and knockdown (Kojima et al., 2007) mice revealed that HIF-2α regulates expression of a number of pro-survival factors. HIF-2α target genes include the major antioxidant enzymes (AOE) *Cas1* and *Sod2* encoding catalase and the mitochondrial-localized manganese superoxide dismutase. HIF-2α also activates *VegfA* encoding the pro-angiogenic vascular endothelial growth factor and *Epo* encoding erythropoietin, a potent pleiotropic cytoprotective factor whose downstream effects include augmentation of the protective cellular response to oxidative stress (Diaz et al., 2005; Lui et al., 2006)

in addition to its function as the principal regulator of erythropoiesis (Lacombe and Mayeux, 1999).

Post -translational modifications of HIF as functional switch

The tight regulation of HIF transcriptional activity involves post-translational modifications of the HIF-α subunit. HIF-α members undergo post-translational modifications by oxygen-dependent prolyl (Bruick and McKnight, 2001; Epstein et al., 2001) and asparaginyl (Mahon et al., 2001) hydroxylases that modulate protein stability and transcriptional activity. Despite similarities in hypoxia-mediated induction and proteasomal-mediated degradation pathways, HIF-1α and HIF-2α exhibit differences in several aspects of their respective activation mechanisms. HIF-1α, but not HIF-2α, is transcriptionally regulated during hypoxia by NFκB signaling, (Rius et al., 2008; Sperandio et al., 2008; Wang et al., 1995). Additionally, HIF-1α protein levels during hypoxia generally mirror trans-activation, while increases in HIF-2α protein levels are more blunted during hypoxia. Yet, HIF-2α dependent trans-activation markedly increases (O'Rourke et al., 1999) as a result of co-activator recruitment or redox-dependent modifications (Bracken et al., 2005; Gradin et al., 2002; Lando et al., 2000). HIF-2α, but not HIF-1α, DNA binding activity may be affected by a reduced redox state by recruitment of reducing factor-1 (Ref-1) to the amino terminus of HIF-2α protein (Lando et al., 2000).

Phosphorylation is a well-established mechanism for regulation of transcription factor activity (Holmberg et al., 2002). Direct phosphorylation of the UR-CTAD region of HIF-1α has been reported under hypoxia. The activation of p42/44 and p38 mitogen-activated protein kinase (MAPK) results in phosphorylation of HIF-1α/HIF-2α *in vitro* (Richard et al., 1999; Sodhi et al., 2000) and increased HIF transcriptional activity without affecting its stability and DNA-binding activity, seemingly by increasing HIF interaction with ARNT (Suzuki et al., 2001). Moreover, HIF-1α/HIF-2α trans-activation during hypoxia required p42/44 MAPKs (Conrad et al., 1999; Hofer et al., 2001; Hur et al., 2001; Minet et al., 2000) on the threonine 796 in HIF-1α and threonine 844 residues in HIF-2α.

Small ubiquitin-like modifier (SUMO) conjugation regulates diverse cellular functions including transcription, nuclear localization, and stress response (Girdwood et al., 2004). HIF-1α SUMOylation has been reported, and decreases HIF-1α trans-activation without affecting its half-life (Berta et al., 2007; Brahimi-Horn et al., 2005). HIF-1α possesses three SUMOylation consensus sequences containing lysines 391, 477 and 532; two of the consensus sequences, lysines 391 and 532, are conserved in HIF-2α (Berta et al., 2004). These results contrast with a previous report that indicated that HIF-1α SUMOylation, in cells transfected with SUMO-1, increased its transcription activity (Bae et al., 2004; Cheng et al., 2007). S-nitrosation of cysteine 800 in the HIF-1α CTAD increases its trans-activation by favoring interaction with co-activator p300/CBP (Yasinska and Sumbayev, 2003).

Post-translational acetylation and deacetylation of the HIF-α subunit by an acetyltransferase called Arrest-defective-1 (ARD1) and by histone deacetylases (HDACs) have been suggested as a major mechanism for regulating HIF transcriptional activity. Lysine residue 532 in the ODD region of HIF-1α was reported to be acetylated by ARD1 (Jeong et al., 2002) promoting interaction with pVHL and to destabilization of HIF-1α protein. However, recent studies question the foundation of the ARD1-mediated mechanisms (Murray-Rust et al., 2006; Yoo et al., 2006), and the precise role of these proteins in respect to HIF activation remains unclear.

The histone deacetylases HDAC1 and HDAC3 interact directly with the ODD domain of HIF-1α, resulting in an increase in HIF-1α stability and transcriptional activity under hypoxia (Kim et al., 2007). HDACs are divided into three classes based on their homology with yeast histone deacetylase: Rpd3 (class I), Hda1 (class II) and Silencing Information Regulator 2, Sir2 (Class III) (de Ruijter et al., 2003; North and Verdin, 2004). While the class I and II HDACs are sensitive to trichostatin A (TSA), class III HDACs are not, and their deacetylase activity uniquely relies on the cofactor nicotinamide adenine dinucleotide (NAD^+).

Sir2 is a redox-sensitive, NAD+ dependent deacetylase initially identified from aging studies in yeast (Tissenbaum and Guarente, 2001). Sir2 homologues in higher eukaryotic organisms are known as Sirtuins (Sirt) with Sirt1 being most closely related to Sir2 (Michishita et al., 2005). Mammalian homologues of Sir2

comprise a family of seven proteins termed Sirt1 through Sirt7, which have gained considerable attention for their impact on several important physiological processes associated with metabolism and stress resistance. Changes in NAD+ levels or NAD+/NADH ratios control the enzymatic activity of Sir2 and its homologues (Zhao et al., 2008).

In mammalian cell culture models, nuclear-localized Sirt1 modulates activity of co-activator proteins (Bouras et al., 2005; Fulco et al., 2003), represses select transcription factors (Langley et al., 2002; Luo et al., 2001; Vaziri et al., 2001), and activates other transcription factors including FoxO members (Brunet et al., 2004; Chua et al., 2005; Daitoku et al., 2004; Kobayashi et al., 2005; Liu et al., 2005; van der Horst et al., 2004). The activating and repressing actions of Sirt1 in these cases are mediated by its deacetylase activity.

Globally, cell regulatory systems can modify cell stress resistance, DNA repair, and apoptotic signaling (Hyun et al., 2006; Morris, 2005). It is of interest to understand how these regulatory systems respond to external or internal signals and how cells adapt to improve cell survival. Redox changes during nutrient/oxidative stress activate Sirt1 and thereby influence FoxO-dependent gene expression. In response to growth factor deprivation and increased oxidative stress, Sirt1 alters FoxO target gene selection to favor induction of pro-survival factors (Brunet et al., 2004).

Recent data have supported a pro-survival effect of increased HIF-2α signaling in response to hypoxic or oxidative stress (Ralph et al., 2004). Hypoxia

induces a metabolic stress that alters cellular redox state *in vivo* (Garofalo et al., 1988; Vannucci and Brucklacher, 1994). We reasoned that redox changes during hypoxic stress would activate Sirt1, concomitantly with HIF activation. We further reasoned that activated Sirt1 would influence HIF-2 signaling, similar to that observed with Sirt1 and FoxO signaling. In this study, we defined the role of Sirt1 in HIF-2α signaling and in regulation of the HIF-2α target gene *epo* during hypoxia.

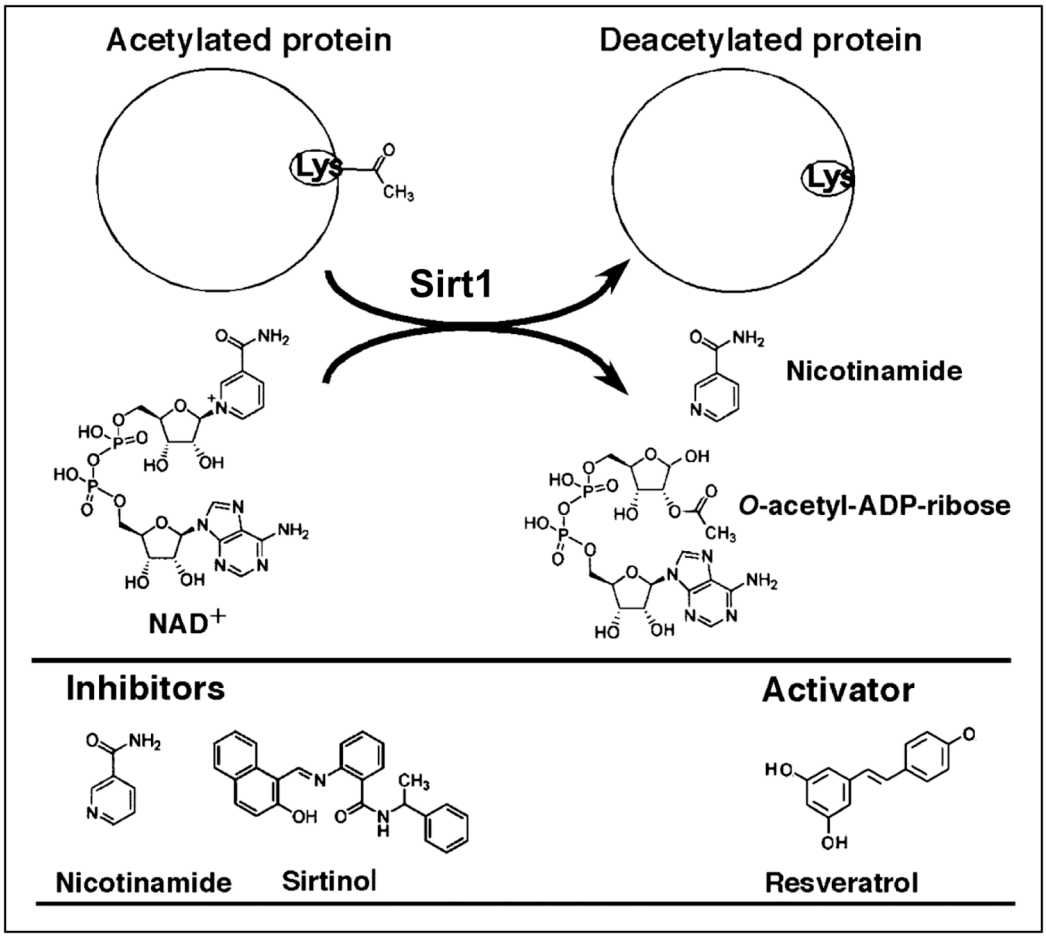


Figure 3-1. The Sirt1 enzymatic activity. Sir2 deacetylates an acetylated protein substrate in the presence of NAD^+ or increased NAD^+/NADH ratio. For each deacetylation of an acetyl lysine residue, one NAD molecule is decomposed into 2'-O-acetyl-ADP-ribose and nicotinamide. Nicotinamide, and sirtinol, are potent inhibitors of the deacetylation reaction, while Resveratrol, a polyphenol compound found in red wine, activates SIRT1 enzymatic activity. Resveratrol lowers the K_m values for the substrate and NAD by 35- and 5-fold, respectively *in vitro*. Adapted from Hisahara et al., 2005 authorization granted by the editor of the journal of Pharmacological Sciences.

RESULTS

Sirt1 selectively augment HIF-2α signaling but not HIF-1α

We first asked if HIF-responsive promoter regions respond to Sirt1 over-expression. In Hep3B cells, wild-type (WT) HIF-2α over-expression activated the isolated mouse *Sod2* promoter, also activated by HIF-2α over-expression in HEK293 cells (Scortegagna et al., 2003a). Sirt1 over-expression in conjunction with WT HIF-2α augments this activation **(Figure 3-2A**). In contrast, Sirt1 did not augment WT HIF-1α activation of the isolated mouse *Sod2* promoter. Alanine substitution of the normoxia-hydroxylated proline and asparagine residues results in constitutively active HIF forms, referred to as P1P2N HIF-1α and P1P2N HIF-2α. Sirt1 augmented P1P2N HIF-2α, but not P1P2N HIF-1α, trans-activation of the *Sod2* promoter (**Figure 3-2-A**). Sirt1 also augmented WT or P1P2N HIF-2α activation of isolated regulatory regions from *VegfA*, a hypoxia-responsive gene activated by either HIF-1α or HIF-2α, or *epo,* a prototypical hypoxic response gene preferentially regulated by HIF-2α in cells (Warnecke et al., 2004) and adult mice (Scortegagna et al., 2005) (**Figure 3-2-A**). Constitutively active (nuclear) FoxO over-expression modestly activated the *Sod2* promoter and had no significant effect on the *VegfA* promoter or *epo* enhancer/promoter reporters with or without Sirt1 (**Figure 3-2-A**).

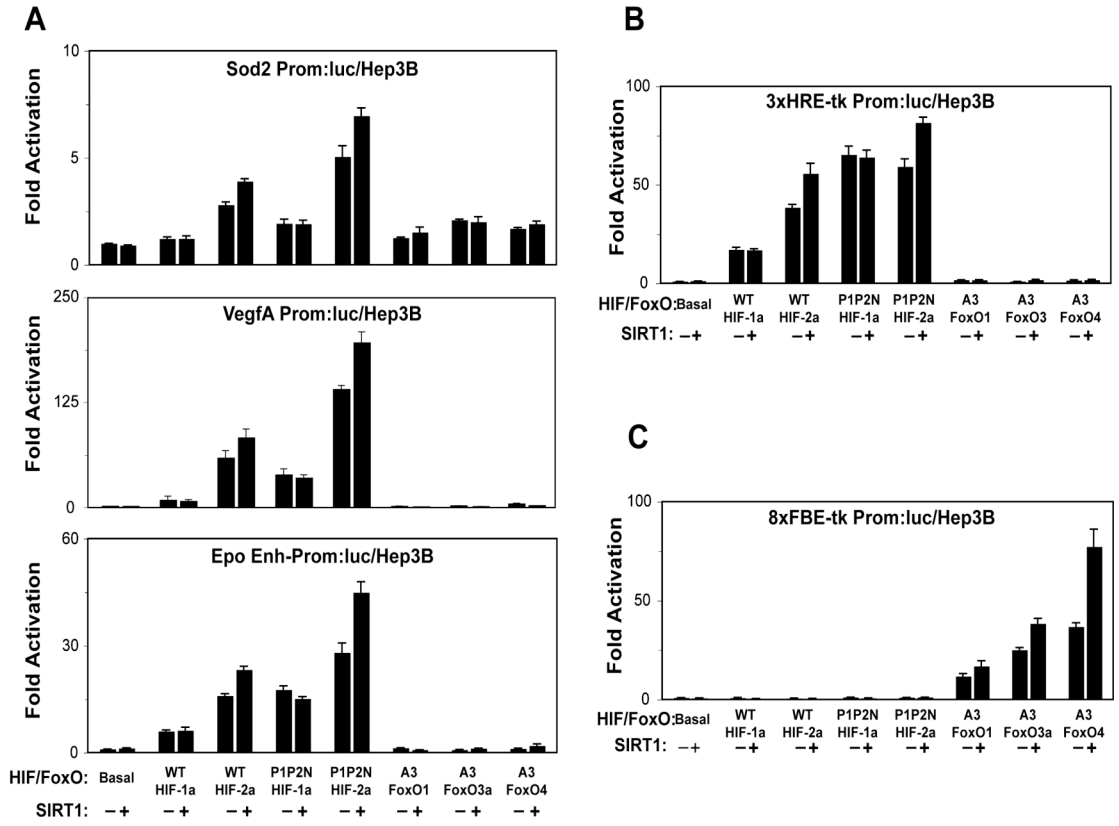


Figure 3-2: Sirt1 selectively augments HIF-2 signaling.
(A) Sirt1 selectively augments HIF-2α trans-activation independent of oxygen-regulated HIF modifications. Activation of a mouse *Sod2* promoter (Sod2 Prom/luc), *VegfA* promoter (VegfA Prom/luc), or *epo* enhancer-promoter (Epo Enhancer-Prom/luc) reporter in Hep3B cells was highest for wild type (WT) or constitutively active (P1P2N) HIF-2α compared to HIF-1α ($p<0.01$ relative to basal). Sirt1 augmented HIF-2α induction for all reporters ($p<0.05$ relative to HIF-2α alone) and had no effect upon HIF-1α trans-activation. Constitutively active (A3) FoxO1, FoxO3a, or FoxO4 with or without Sirt1 modestly activated (Sod2 Prom/luc) or had minimal effect (VegfA Prom/luc, Epo Enh-Prom/luc). **(B)** Exogenous HIF-1α and HIF-2α do not cross-activate FoxO reporters. Reporter plasmids containing a synthetic HIF-responsive (3xHRE-tk/luc) promoter were transfected into Hep3B cells along with wild type (WT) HIF-1α, WT HIF-2α, constitutively active (P1P2N) HIF-1α, or P1P2N HIF-2α expression plasmids. Sirt1 selectively augmented WT ($p=0.0036$) or P1P2N ($p=0.012$) HIF-2α activation of 3xHRE-tk/luc. **(C)** Exogenous FoxO1, 3a, or 4 do not cross-activate HIF reporters. The FoxO-responsive reporter 8xFBE-tk/luc was transfected into Hep3B cells along with constitutively active (A3) FoxO1, FoxO3a, or FoxO4. Sirt1 augmented activation of 8xFBE-tk/luc by FoxO3a ($p=0.003$) and FoxO4 ($p=0.01$).

The *Sod2* promoter, *VegfA* promoter, and *epo* enhancer are complex regulatory regions. We next addressed if Sirt1 could activate a minimal HIF-responsive synthetic promoter. Activation of *3xHRE-tk/luc,* consisting of three HREs fused upstream of a minimal thymidine kinase (tk) promoter, was comparable with P1P2N HIF-1α or P1P2N HIF-2α over-expression. Sirt1 augmented induction of *3xHRE-tk/luc* with WT or P1P2N HIF-2α, but not with WT or P1P2N HIF-1α (**Figure 3-2B**). Sirt1 augmentation of HIF-2α activity was not due to activation of FoxO signaling; the synthetic FoxO-responsive reporter *8xFBE-tk/luc* responded only to constitutively nuclear FoxO, and not to WT or P1P2N HIF-2α over-expression with or without Sirt1 (**Figure 3-2C**).

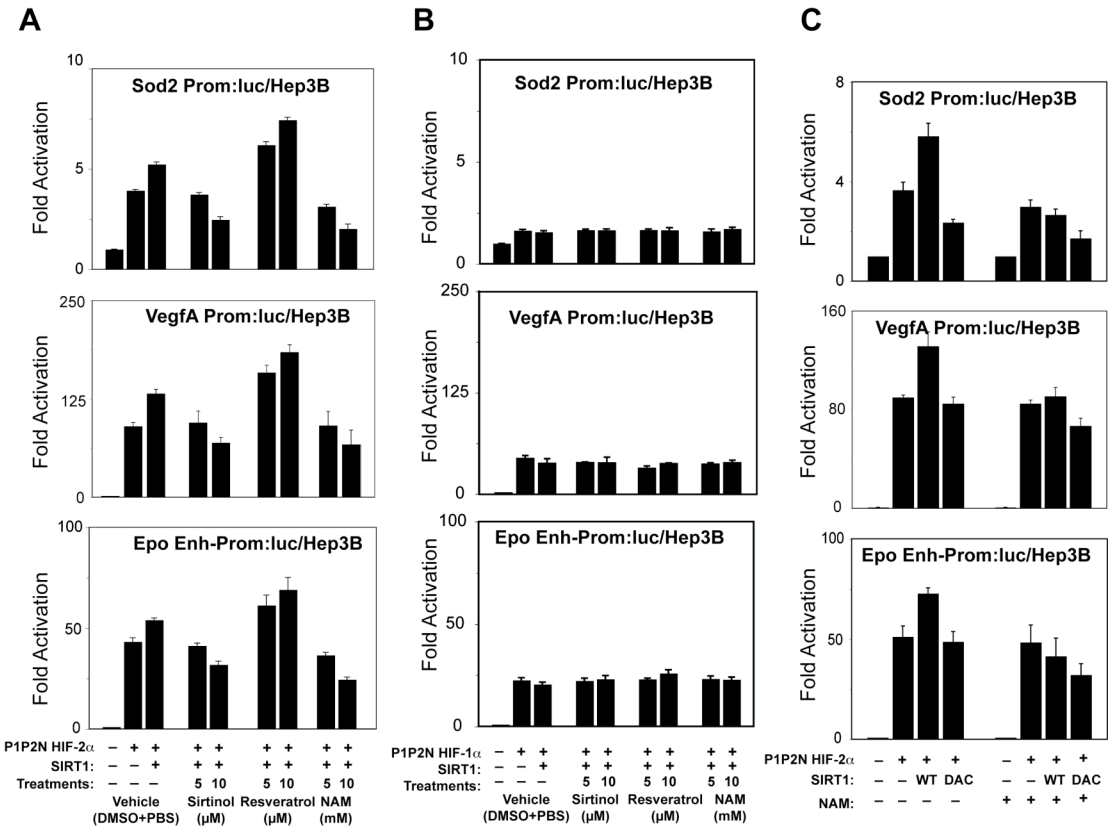


Figure 3-3: Sirt1/HIF-2α augmentation is selectively modulated by Sirt1 enzymatic activity. (**A)** Modulation of Sirt1 activity alters HIF-2α trans-activation. Treatment with the Sirt1 inhibitor sirtinol, activator Resveratrol, or end product inhibitor nicotinamide (NAM) affects Sirt1/HIF-2α synergy of the Sod2 Prom/luc, VegfA Prom/luc, or Epo Enh-Prom/luc reporter. Sirt1 augmented HIF-2α activation for all three reporters ($p<0.05$); this augmentation significantly decreased with the highest concentration of sirtinol ($p<0.02$) or NAM ($p<0.01$), and increased with the highest dose of Resveratrol ($p<0.01$). **(B)** Modulation of Sirt1 activity does not affect HIF-1α trans-activation. P1P2N HIF-1α and/or Sirt1 expression plasmids were transfected along with Sod2 Prom/luc, VegfA Prom/luc, or Epo Enh-Prom/luc reporter plasmids into Hep3B cells treated with sirtinol, Resveratrol, or nicotinamide (NAM). The bars for all transfection assays represent the mean ± SEM of three independent transfections with each transfection performed in triplicate. **(C)** Sirt1 deacetylase activity is required for augmentation of HIF-2α signaling. Wild type (WT) or deacetylase mutant (DAC) Sirt1 was expressed with P1P2N HIF-2α in the absence or presence of nicotinamide (NAM). WT Sirt1 augmented HIF-2α dependent activation of the Sod2 Prom/luc, VegfA Prom/luc, or Epo Enh-Prom/luc reporter ($p<0.05$); DAC Sirt1 was unable to augment HIF-2α activation with any of the reporters. NAM prevented WT Sirt1 augmentation of HIF-2α trans-activation for all three reporters.

We asked if Sirt1 augmentation of HIF-2 signaling is affected by pharmacological or genetic modulation of Sirt1 activity. Nicotinamide (NAM), an end product generated after Sir2/Sirt1-mediated deacetylation, inhibits Sir2/Sirt1 action (Bitterman et al., 2002) as does the synthetic compound sirtinol (Grozinger et al., 2001), whereas the natural polyphenol Resveratrol selectively stimulates Sir2/Sirt1 activity (Howitz et al., 2003). NAM or sirtinol reduced, whereas Resveratrol increased, the ability of Sirt1 to augment HIF-2α trans-activation for the *Sod2* Prom*, VegfA* Prom*,* and *Epo* Enh-Prom reporters (**Figure 3-3A**). These compounds had no effect on HIF-1α trans-activation of any of these reporters (**Figure 3-3B**). A site-directed mutation that inhibits Sirt1 deacetylase activity (DAC) eliminated the Sirt1 stimulatory effect for the *Sod2* Prom*, VegfA* Prom*,* and *Epo* Enh-Prom reporters. NAM inhibited the stimulatory effect observed with WT Sirt1 over-expression (**Figure 3-3C**).

HIF-2α and Sirt1 form stable complex

To assess whether SIRT1/HIF-2α synergy is conferred by complex formation, exogenous tagged forms of P1P2N SP:HIF-2α:HA or P1P2N SP:HIF-2α:HA together with WT or DAC vsv-g:Sirt1 were over-expressed in HEK293 cells under normoxia or hypoxia and subjected to S-peptide (SP) pull-down analyses followed by immuno-blot against HA (HIF) or vsv-g (SIRT1). SIRT1/HIF-2α, but not HIF-1α, complexes remains stable under normoxia, and after hypoxia exposure with WT, but not DAC, Sirt1 (**Figure 3-4A**). Sirt1 complexes with P1P2N HIF-2α, but not with P1P2N HIF-1α, in normoxia-maintained cells irrespective of whether an Class I/II (trichostatin A, TSA) or Class I/II+III (TSA + NAM) HDAC inhibitor are present (**Figure 3-4B**). To determine the biological relevance of SIRT1/HIF-2α interaction *in vivo,* in Hep3B cells treated with hypoxia, endogenous Sirt1 forms complexes with HIF-2α, but not HIF-1α, during hypoxia as assessed by *in vivo* pull-down assays (**Figure 3-4C**). We assessed HIF-2α SIRT1 direct interaction by *in vitro* pull-down studies between bacterially purified GST-fusion of carboxy-terminal HIF-1α or HIF-2α (HIF CTERM) and *in vitro* translated/transcribed 35S radiolabeled WT or DAC SIRT1. GST-HIF pull-down revealed stable and direct binding of WT Sirt1, but not DAC Sirt1, with HIF-2α CTERM (**Figure 3-4D**).

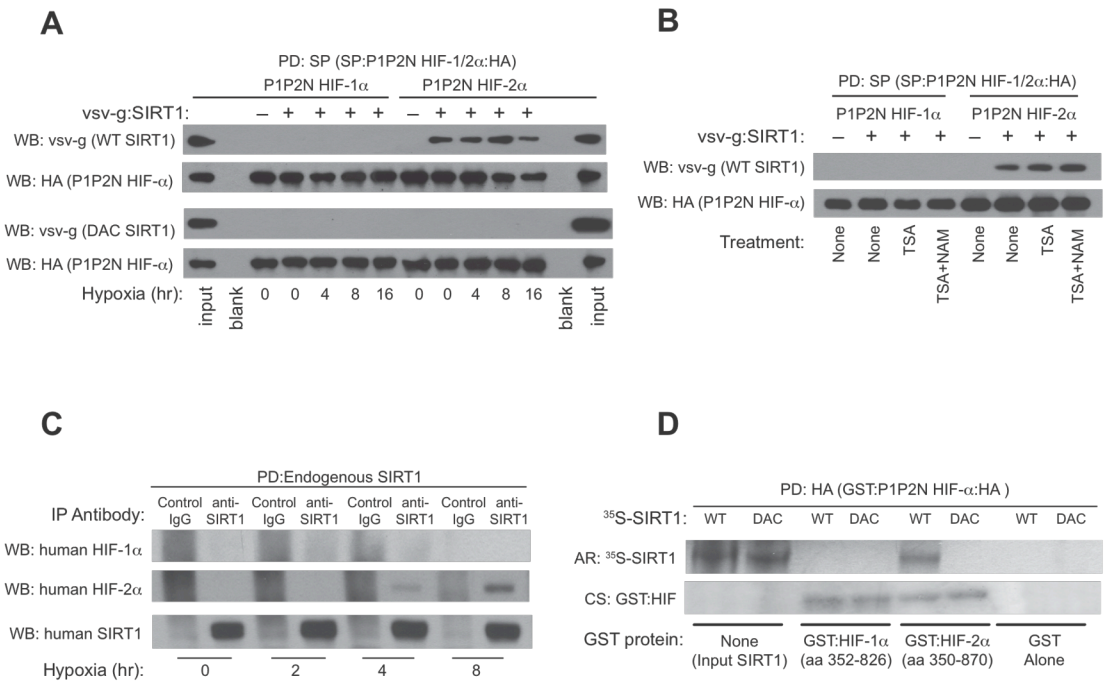


Figure 3-4: HIF-2α forms stable complexes with Sirt1.

(A) Exogenous Sirt1/HIF-2α complexes are stable during hypoxia. SP:P1P2N HIF-1α:HA or SP:P1P2N HIF-2α:HA was pulled down from normoxia- or hypoxia-exposed HEK293 extracts in which WT or DAC vsv-g:Sirt1 proteins were expressed. Sirt1 or HIF proteins were visualized by immunoblotting. **(B)** Exogenous Sirt1/HIF-2α complex formation does not require HDAC inhibition. SP:P1P2N HIF-1α:HA or SP:P1P2N HIF-2α:HA was pulled down from normoxic HEK293 extracts in which WT vsv-g:Sirt1 was also expressed. Cells were treated with no, class I/II (trichostatin A, TSA), or Class I/II + Class III (TSA + sirtinol) histone deacetylase (HDAC) inhibitors. Sirt1 and HIF proteins were visualized by immunoblotting. **(C)** Endogenous Sirt1/HIF-2α complexes form during hypoxia. Pull-down of Sirt1 from Hep3B nuclear extracts brings down HIF-2α, but not HIF-1α after hypoxia exposure. **(D)** Sirt1 and the HIF-2α carboxy terminus bind directly to each other. *In vitro* translated, ^{35}S-labelled WT, but not DAC, Flag:Sirt1 is pulled down with a bacterial-produced GST-HIF-2α carboxy terminus:HA, but not GST-HIF-1α, carboxy terminus:HA.

The pull-down studies indicate Sirt1 augmentation of HIF-2α signaling may be mediated in part by protein-protein interactions. As assessed by NMR studies (Erbel et al., 2003), the HIF alpha amino terminal region, encompassing the basic helix-loop-helix (bHLH) and PAS domains, is highly structured whereas the carboxy terminal region containing the most divergent regions between HIF isoforms is unstructured. The HIF-2α carboxy terminus suffices for Sirt1 augmentation, as evident by transfection studies with hybrid forms of HIF-1α and HIF-2α that exchange the carboxy terminal regions (**Figure 3-5A**) or by mammalian Gal4/VP16 two-hybrid transfection studies with Gal4:Sirt1/VP16:HIF-α carboxy terminus constructs (**Figure 3-5B**). The HIF-2α carboxy terminus confers stable interactions with Sirt1 as shown by pull-down studies with Gal4:Sirt1 and VP16:HIF-α fusion proteins (**Figure 3-5C**).

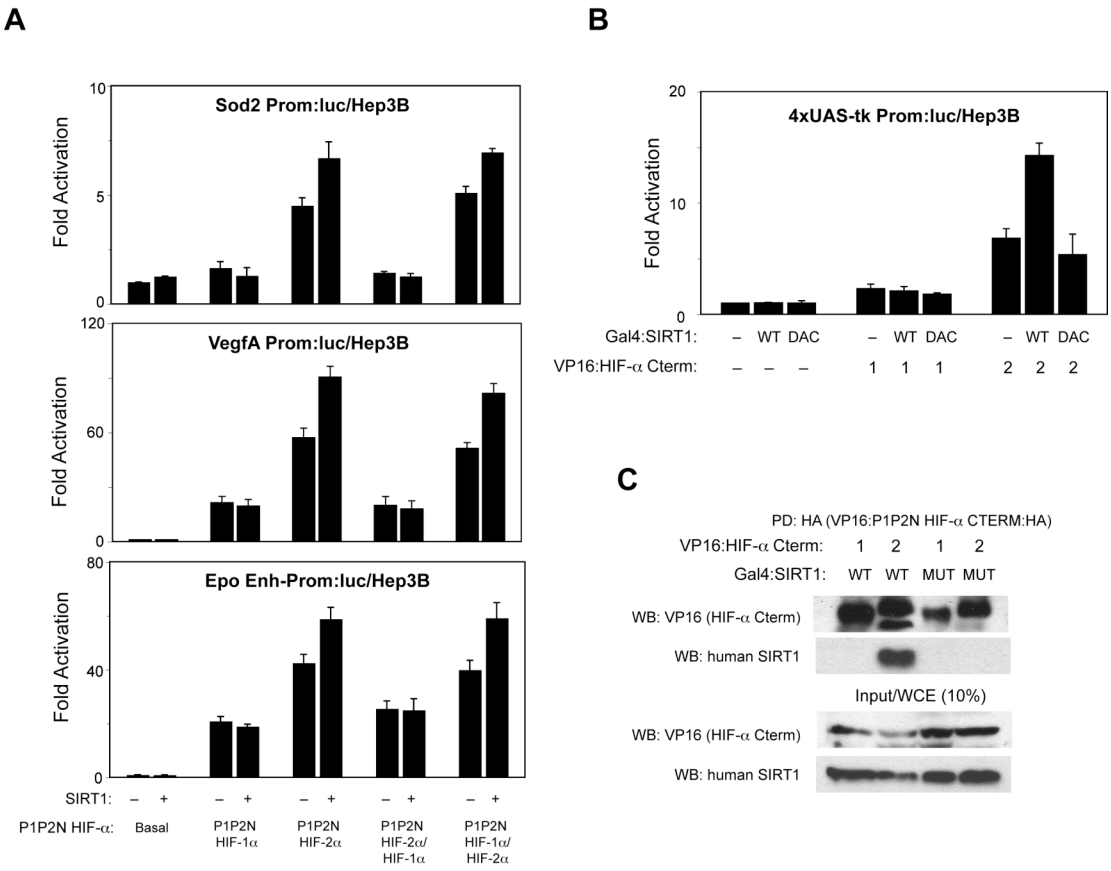


Figure 3-5: The HIF-2α carboxy terminus confers Sirt1 synergy. **(A)** Sirt1 augmentation is mediated through the HIF-2α carboxy terminus. Hybrid constructs were generated that exchange the carboxy termini of P1P2N HIF-1α with P1P2N HIF-2α and vice-versa. Sirt1 and either P1P2N HIF-1α, P1P2N HIF-2α, P1P2N HIF-2α/HIF-1α, or P1P2N HIF-1α/HIF-2α expression plasmids were transfected into Hep3B cells along with the Sod2/luc, VegfA/luc, or Epo Enh/luc reporter. Sirt1 only augmented activation with P1P2N HIF-2α or with P1P2N HIF-1α/HIF-2α for all three reporters ($p<0.05$). All transfection data represent the mean ± SEM of three independent transfections with each transfection performed in triplicate. **(B)** The HIF-2α carboxy terminus interacts with Sirt1 in a mammalian two-hybrid assay. VP16:P1P2N carboxy terminus (Cterm) HIF-1α:HA or HIF-2α:HA fusion proteins as well as Gal4:WT or DAC Sirt1 fusion proteins were expressed in Hep3B cells with a Gal4 reporter (4xUAS-tk Prom/luc). VP16:HIF-2α, but not VP16:HIF-1α, activation is augmented by Gal4:WT Sirt1. Gal4:DAC Sirt1 does not augment activation of VP16:HIF-2α. **(C)** Sirt1/HIF-2α mammalian two-hybrid proteins form stable complexes. Pulldown (PD) assays reveal Gal4:WT Sirt1 interacts stably with VP16:HIF-2α and not with VP16:HIF-1α. Gal4:DAC Sirt1 does not interact stably with either VP16:HIF-1α or VP16:HIF-2α.

To determine what domain of HIF-2α is required for Sirt1/HIF-2α complex formation, I made and overexpressed in HEK293 cells P1P2N HIF-2α:HA carboxy terminus (**Figure 3-6A**) or amino terminus (**Figure 3-6B)** deletion constructs together with vsv-g:Sirt1 and performed co-immunoprecipitation with an anti-HA antibody. Sirt1 binding to HIF-2α requires both the amino terminal activation domain/oxygen dependent degradation domain (NATD/ODD) as well as the unique region (also known as the inhibitory region) located in the carboxy terminus and encompassed by amino acids 350 to 826 (**Figure 3-6A B).** The NTAD alone is able to weakly interact with SIRT1.

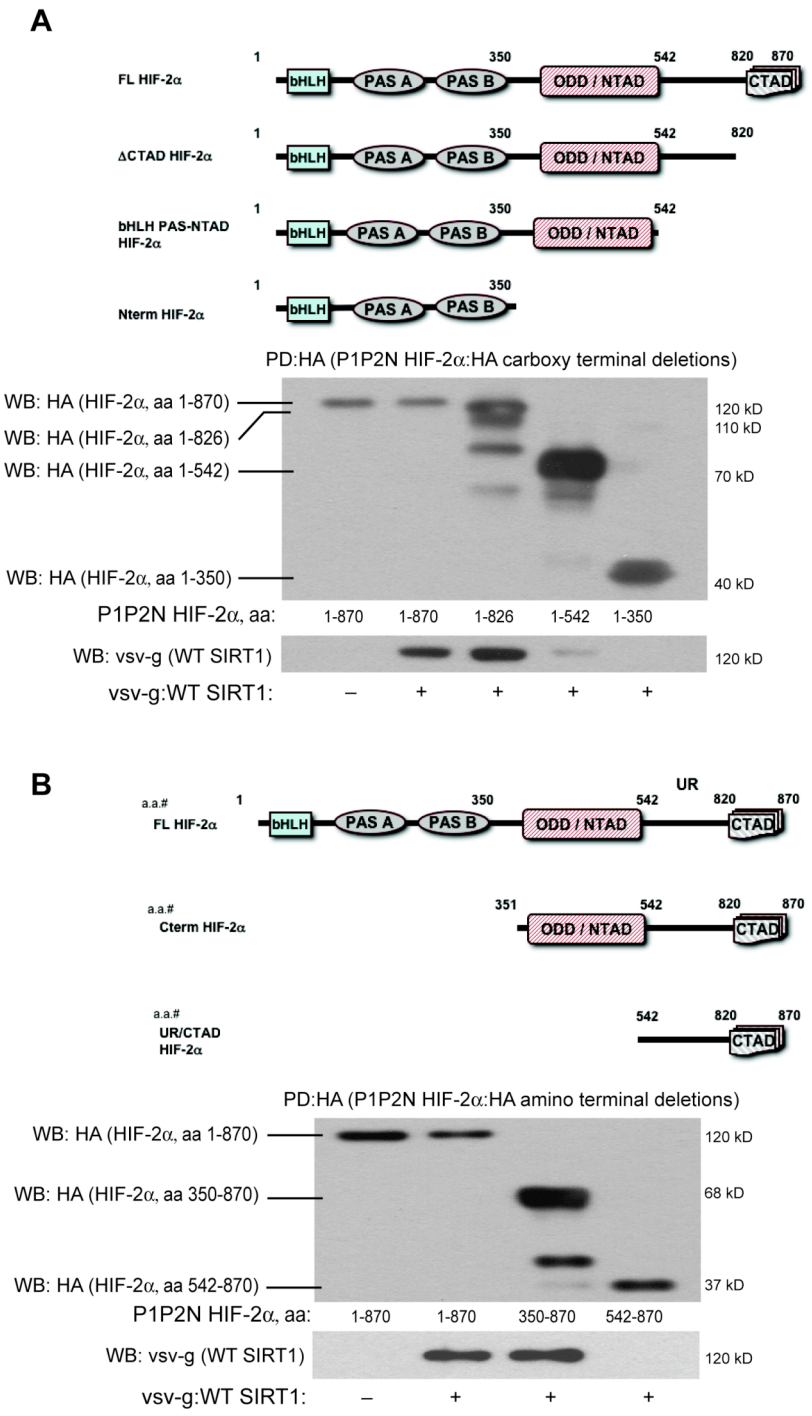


Figure 3-6. Sirt1/HIF-2α complex formation requires HIF-2α NTAD-UR regions. **(A)** Over-expressed carboxy-terminal truncations or **(B)** amino-terminal of P1P2N HIF-2α:HA together with vsv-g:Sirt1 proteins were immunoprecipitated with HA antibody and visualized by western blotting (WB) with an anti-HA antibody. Pull-down (PD) of vsv-g:Sirt1 was assessed by immunoblotting with anti-vsv-g antibody. The indicated amino acids (aa) for each HIF-2α:HA construct is indicated.

Dynamic acetylation/deacetylation by Ard1 and HDACs regulate HIF-1α stability under normoxia and hypoxia (Jeong et al., 2002; Kim et al., 2007). To determine whether Sirt1/HIF-2α synergy involves alteration of HIF-2α protein level, we over-expressed P1P2N HIF-1α and HIF-2α together with WT SIRT1 or deacetylase mutant DAC SIRT1. In whole cell extract P1P2N HIF-1α and P1P2N HIF-2α protein levels were comparable in Hep3B cells and were not affected by Sirt1 over-expression (**Figure 3-7A**). Similar results for all transfection studies performed in Hep3B cells were observed in HEK293 cells (data not shown). Endogenous SIRT1 activity can be altered genetically by shRNA knockdown or pharmacologically by Sirtinol treatment. Neither pharmacological nor genetic inhibition of Sirt1 affected endogenous HIF-2α protein steady-state levels (**Figure 3-7B C**). Sirt1 does not affect endogenous HIF-2α half-life, as determined in hypoxia-treated Hep3B cells after cycloheximide treatment, by Sirt1 pharmacological inhibition with Sirtinol or by HIF-2α increased acetylation status with TSA-Sirtinol treatment (**Figure 3-7C**).

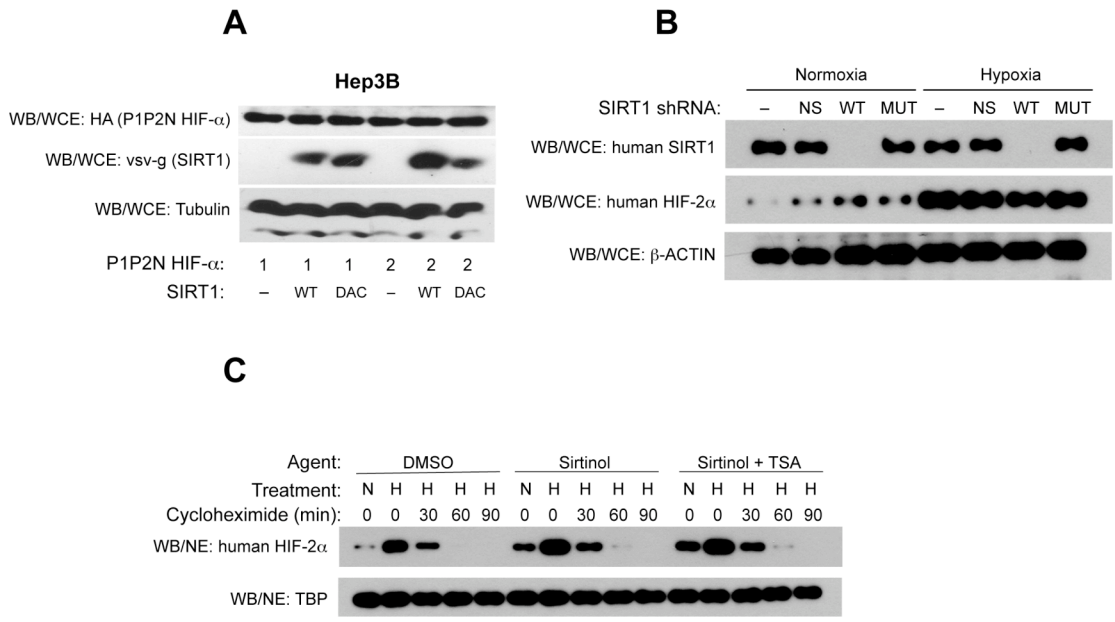


Figure 3-7. SIRT1 does not affect HIF-2α protein level. **(A)** Sirt1 over-expression does not affect exogenous P1P2N HIF-1α or P1P2N HIF-2α protein levels. Whole cell extracts (WCE) from Hep3B cells expressing P1P2N HIF-1α:HA or HIF-2α:HA along with wild-type (WT) or deacetylase mutant (DAC) vsv-g:Sirt1 were immunoblotted with antibodies recognizing the HA epitope tag, vsv-g epitope tag, or α-tubulin. **(B)** Sirt1 knockdown does not affect endogenous HIF-2α protein levels. Whole cell extracts (WCE) from normoxia- or hypoxia-exposed Hep3B cells transfected with control, Sirt1 wild-type shRNA, or Sirt1 mutant shRNA expression plasmids were immunoblotted with antibodies recognizing human Sirt1, HIF-2α or β-actin. **(C)** Pharmacological inhibition of Sirt1 activity does not affect endogenous HIF-2α protein stability. Whole cell extracts (WCE) from hypoxia-exposed Hep3B cells treated with DMSO, TSA, or sirtinol plus TSA followed by cycloheximide-mediated protein inhibition were prepared at the indicated time-points and immunoblotted with antibodies recognizing human HIF-2α or TBP.

Sirt1 gene expression increases under hypoxia

In Hep3B cells, endogenous HIF-2α protein and Sirt1 protein levels rose with continuous hypoxia (**Figure 3-8A**). Although Sirt1 protein levels may increase passively during hypoxia as an indirect consequence of HIF-2α stabilization, Sirt1 is itself likely subject to hypoxia-induced regulation. Sirt1 mRNA levels modestly increased during hypoxia exposure with a peak at 4 hours post-hypoxia exposure (**Figure 3-8B**). Examination of the human and mouse *Sirt1* proximal promoter region reveals multiple conserved HREs (**Figure 3-8C**). Transient transfection assays using an isolated human *Sirt1* proximal promoter reporter revealed efficient activation by P1P2N HIF-2α, but not by P1P2N HIF-1α (**Figure 3-9A).** Site-directed mutation of an HRE in the isolated human *Sirt1* promoter (HRE 5) eliminated preferential activation by P1P2N HIF-2α (**Figure 3-9B**).

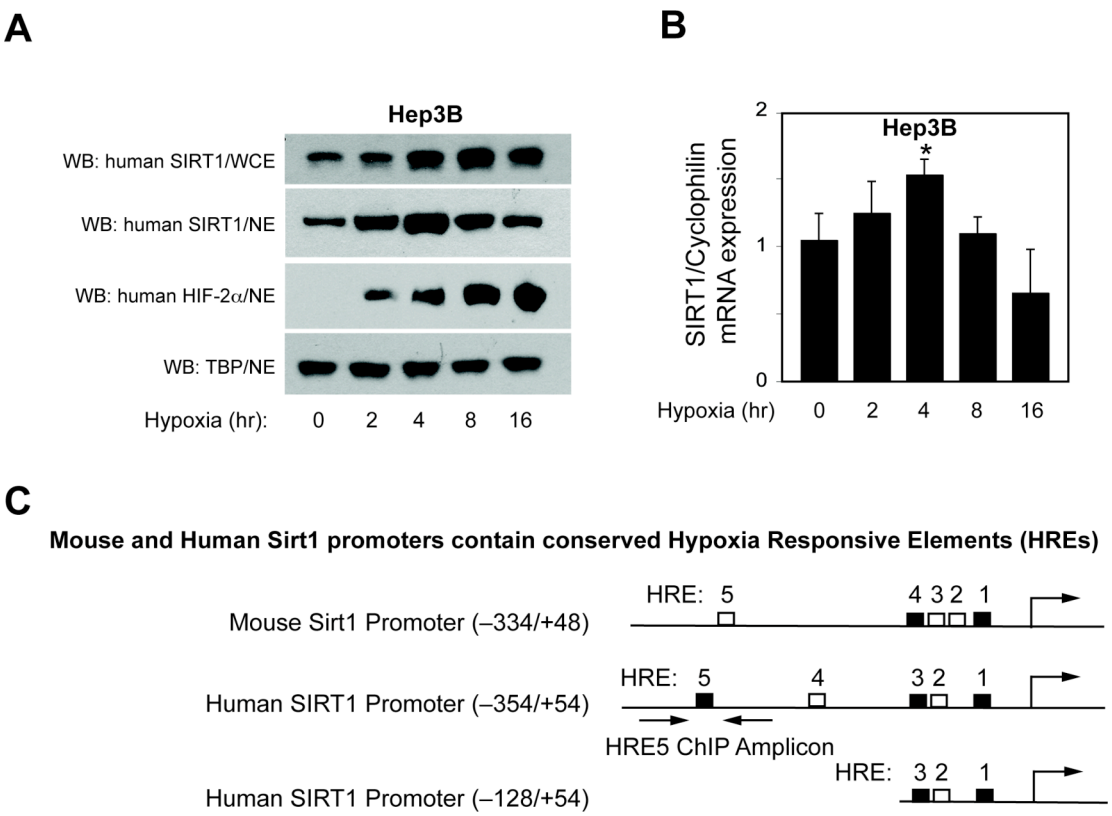


Figure 3-8: Sirt1 mRNA levels are regulated by HIF-2α during hypoxia.

(A) Sirt1 and HIF-2α protein levels increase during hypoxia. Hep3B cells maintained under normoxia or hypoxia were used to generate whole cell (WCE) or nuclear extracts (NE). Western blot analyses were performed to identify endogenous human HIF-2α, SIRT1, and TFIID. **(B)** Sirt1 steady-state mRNA levels increase during hypoxia. Real-time RTPCR analyses were performed in parallel to (A) for *epo* and *cyclophilin B* mRNA at the indicated time-points. Sirt1 mRNA levels modestly increased by 4 hr after hypoxia exposure (*$p<0.05$, one-tailed Student's t-Test). The data represent the mean ± SEM of three independent samples harvested from 6-well plates. **(C)** Mouse and human *Sirt1* promoters contain evolutionary conserved hypoxia-responsive elements (HRE). Sequence comparisons of the mouse and human proximal *Sirt1* promoter regions were performed to identify and localize HREs conforming to the consensus sequence ACGTG (black box) or GCGTG (white box).

Consistent with participation of HIF-2α in the regulation of *Sirt1* gene expression during hypoxia, endogenous HIF-2α was recruited to the HRE 5 in the human *Sirt1* promoter during hypoxia (**Figure 3-9C**).

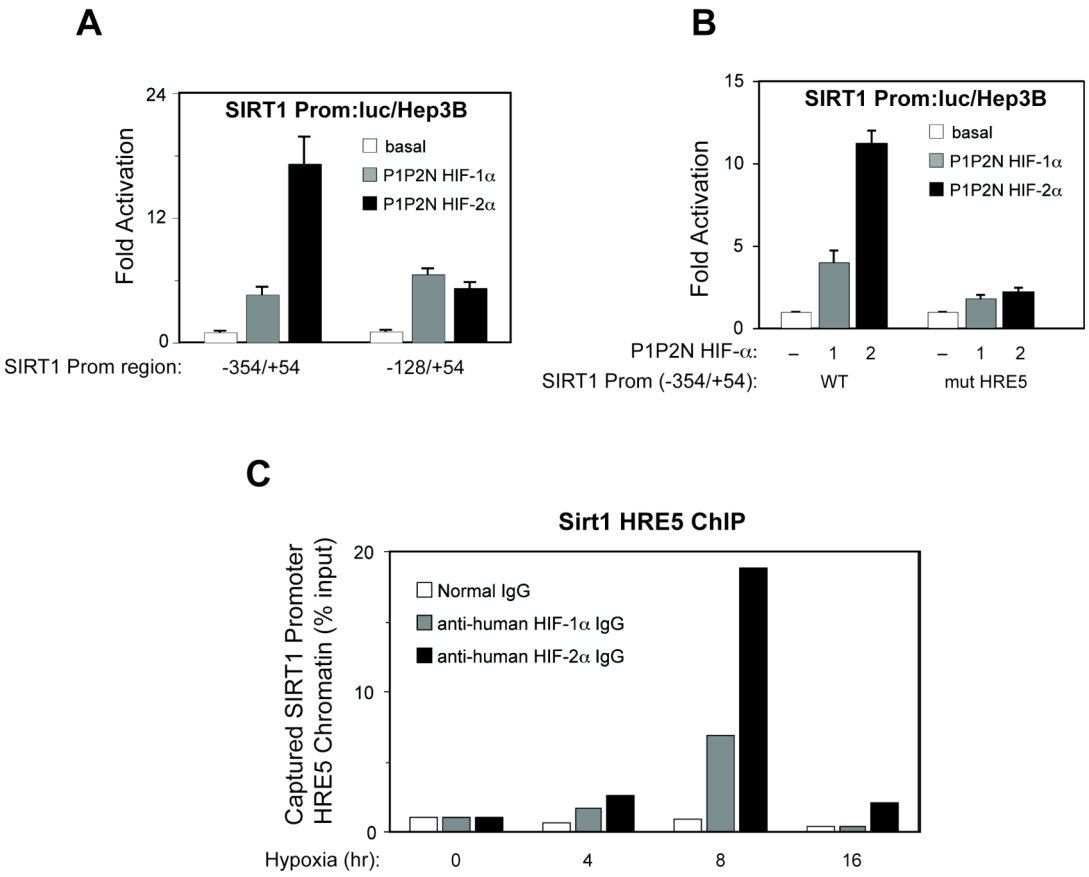


Figure 3-9: HIF-2α selectively activates the Sirt1 promoter. **(A)** The *Sirt1* proximal promoter region is preferentially activated by HIF-2α. Transfection assays reveal that P1P2N HIF-2α, more so than P1P2N HIF-1α, efficiently activates the isolated human Sirt1 promoter region ($p<0.001$). **(B)** A hypoxia-responsive element (HRE) in the Sirt1 promoter confers preferential HIF-2α activation. Site-directed mutations of the human Sirt1 proximal promoter indicate HRE5 is a primary determinant of HIF-2α selective trans-activation. The data represent the mean ± SEM of three independent transfections with each transfection performed in triplicate. **(C)** HIF protein binding to HRE5 in the endogenous *Sirt1* promoter increases during hypoxia. Hep3B cells maintained under normoxia or exposed to hypoxia for the indicated time-points were used in chromatin immunoprecipitation assays with antibodies recognizing human HIF-1α or HIF-2α. Quantitative PCR analyses were performed with primers encompassing HRE5 in the proximal human *Sirt1* promoter.

HIF-2α acetylation status is regulated by Sirt1

Sirt1 synergistic activation of HIF-2 signaling requires Sirt1 deacetylase function. We asked if HIF-2α exhibits stress-dependent acetylation. Exogenous P1P2N HIF-2α prepared using TSA plus sirtinol was progressively acetylated during continuous hypoxia (**Figure 3-10A**). Exogenous P1P2N HIF-1α was not acetylated with extracts prepared using TSA plus sirtinol despite comparable protein expression levels. HIF-2α acetylation was not observed with extracts prepared using the class I/II histone deacetylase inhibitor TSA alone (**Figure 3-10B)**, an otherwise effective inhibitor of histone H4 acetylation (**Figure 3-10C**). A similar pattern of increased acetylation during hypoxia was observed with endogenous HIF-2α with the exception that a reduced level of acetylation was evident during normoxia (**Figure 3-10D**). Similarly, deacetylation of hypoxia-induced acetylation of endogenous HIF-2α also requires Sirt1 as evident by Sirt1 knockdown (**Figure 3-10E**). Acetylated HIF-2α was efficiently deacetylated, as assessed *in vitro*, by wild type (WT) Sirt1, but not by deacetylase inactive (DAC) Sirt1 ((**Figure 3-10F**).

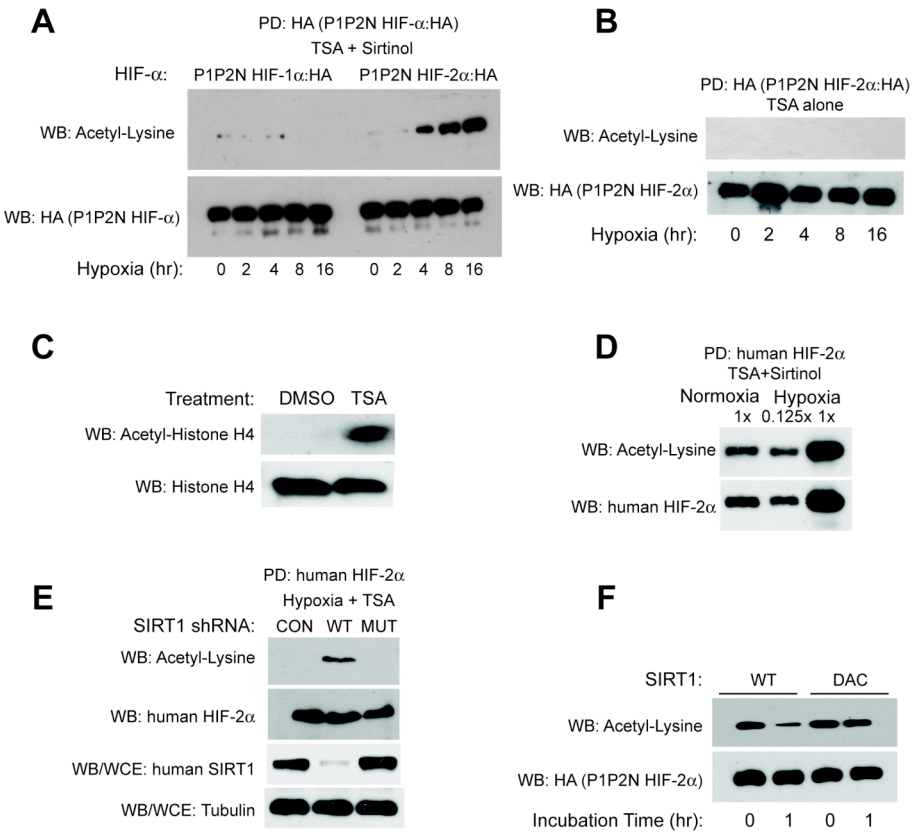


Figure 3-10: HIF-2α acetylation is regulated by Sirt1.
(A) Exogenous HIF-2α acetylation increases during hypoxia. SP:P1P2N HIF-1α:HA or SP: P1P2N HIF-2α:HA were expressed in Hep3B cells treated with trichostatin A (TSA) plus sirtinol and harvested at the indicated time-points following hypoxia exposure. After pull-down (PD), acetylated and total HIF-2α was assessed. **(B)** Exogenous P1P2N HIF-2α acetylation during hypoxia is not observed with HDAC I/II inhibition alone. SP:P1P2N HIF-2α:HA expressed in hypoxia-maintained Hep3B cells in the presence of TSA alone was purified and acetylated as well as total HIF-2α assessed. **(C)** Histone H4 acetylation during hypoxia is observed with HDAC I/II inhibition. Acid-extracted protein extracts prepared from control (DMSO) or TSA-treated Hep3B cells were assessed for acetylated or total Histone H4 protein. (D) Endogenous HIF-2α protein was immunoprecipitated from normoxia or hypoxia maintained Hep3B cells. Acetylation and total HIF-2α levels were determined. The hypoxia sample was loaded at one-eighth or an equivalent amount of the normoxia sample. **(E)** Endogenous HIF-2α deacetylation requires Sirt1. Acetylation of endogenous HIF-2α from hypoxia-exposed Hep3B cells treated with TSA was evident with wild type, but not with control or mutant, Sirt1 shRNA treatments. **(F)** Acetylated HIF-2α is a substrate for Sirt1. Acetylated P1P2N HIF-2α:HA was incubated with wild-type (WT) or deacetylase mutant (DAC) Flag:Sirt1. After one hour, acetylated and total HIF-2α was assessed by immunoblotting.

The capacity of HIF-2α to be dynamically acetylated during hypoxia suggests a potential regulatory role by this epigenetic modification. We first sought to identify the lysine residues acetylated during hypoxia. Mass spectrometry revealed significant acetylation of exogenous P1P2N HIF-2α at multiple sites including three lysine residues within the HIF-2α carboxy terminus (**Figure 3-11A**). Substitution of the three lysines that are acetylated during hypoxia (K3) with alanine (A3) or arginine (R3) residues results in a HIF-2α form that retained the ability to bind Sirt1 (**Figure 3-11B**) in co-immunoprecipitation assays and synergistically activated HIF-2α signaling (**Figure 3-11D**) in epo reporter assays. The carboxy terminus A3 and R3 lysine substitutions steady-state level of acetylation was determined in transfected HEK293 cells treated with TSA and Sirtinol. Acetylation of C terminal lysine mutants (A3. R3) is substantially lower compared to the parental wild type P1P2N HIF-2α (R3) (**Figure 3-11C**). However, the mutant HIF-2α forms (A3 or R3), unlike the parental construct (K3), were not subject to pharmacological regulation by Sirtinol, inhibitors of Sirt1 deacetylase activity (**Figure 3-11D**).

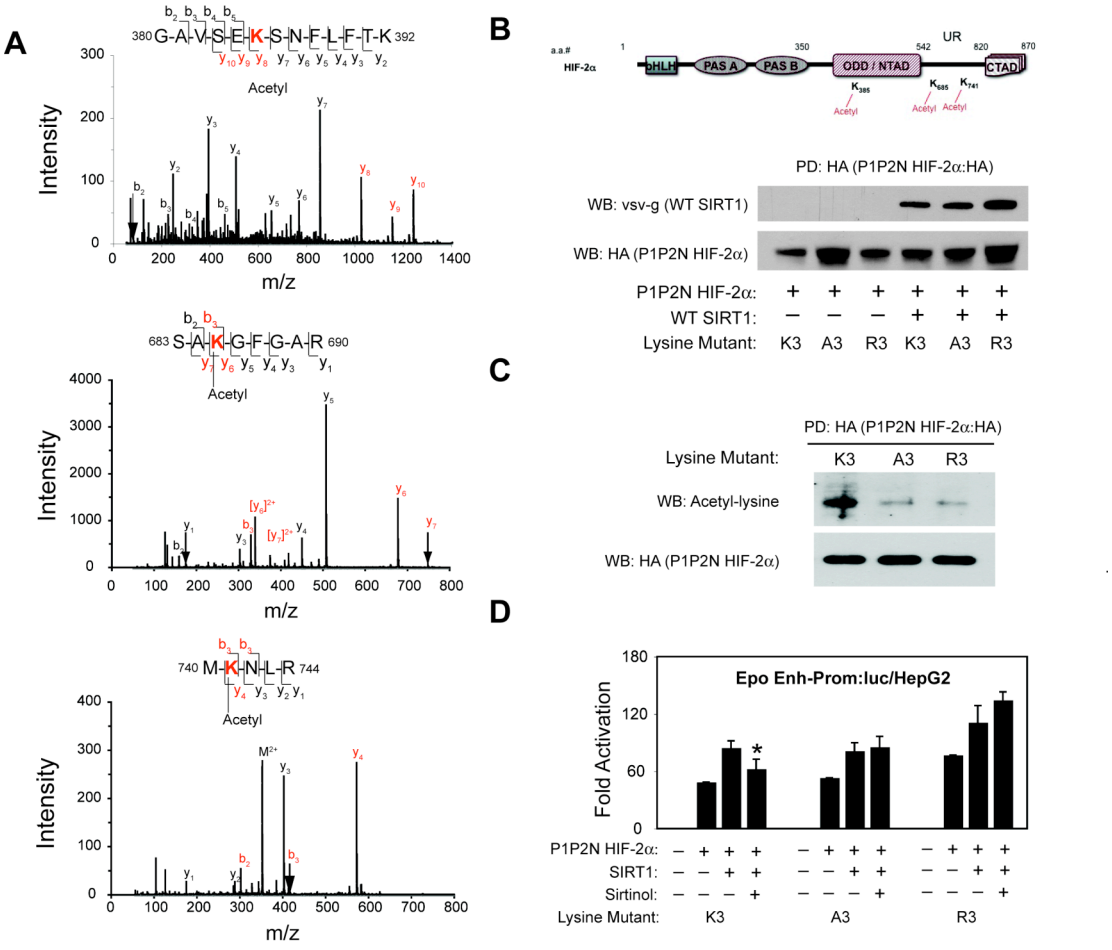


Figure 3-11: The HIF-2α carboxy terminus is acetylated during hypoxia.
(A) Specific lysine residues in the HIF-2α carboxy terminus are acetylated during hypoxia. Mass spectrometry performed on SP:HIF-2α:HA purified from hypoxia-exposed Hep3B cells treated with TSA plus sirtinol identifies three acetylated residues in the carboxy terminus. **(B)** Schematic of the acetylated lysines in the HIF-2α C terminus. Mutant HIF-2α forms with substitutions in carboxy terminal acetylated lysine residues retain Sirt1 binding capacity. Alanine (A3) or arginine (R3) substitutions of the three acetylated lysine residues (K3) in P1P2N HIF-2α results in HIF-2α forms that retain the ability to complex with Sirt1. **(C)** Reduced acetylation status of WT P1P2N HIF-2α:HA (K3) or alanine (A3), arginine (R) substitution of the three highly acetylated lysine residues in the HIF-2α carboxy terminus under hypoxia **(D)** HIF-2α carboxy terminal acetylated lysine residues confer regulated Sirt1 activation. A3 or R3 unlike K3 results in a sirtinol-insensitive P1P2N HIF-2α form as assessed by trans-activation of the Epo Enh/luc reporter by wild-type Sirt1. The data represent the mean ± SEM of three independent transfections with each transfection performed in triplicate.

Sirt1 modulates HIF-2α dependent epo gene expression *in vivo*

Since Sirt1 augments HIF-2α dependent trans-activation of isolated promoter reporters, we next determined if Sirt1 participates in regulation of endogenous HIF-2α target genes. We focused on evaluation of *epo* as this gene is preferentially regulated by HIF-2α in both Hep3B cells as well as in mice. Knockdown of Sirt1 levels by short hairpin RNA (shRNA) resulted in reduced induction of *epo* mRNA in Hep3B cells treated with hypoxia compared to control non silencing shRNA or a mutant shRNA (**Figure 3-12A**). Pharmacological manipulations of Sirt1 activity resulted in increased *epo* mRNA levels with agents that stimulate Sirt1 activity (resveratrol), and reduced *epo* mRNA levels with agents that inhibit Sirt1 activity (Sirtinol) (**Figure 3-12B).** Chromatin immunoprecipitation (ChIP) experiments revealed increased recruitment of Sirt1 and HIF-2α to the endogenous *epo* enhancer region in Hep3B cells during hypoxia (**Figure 3-12C**).

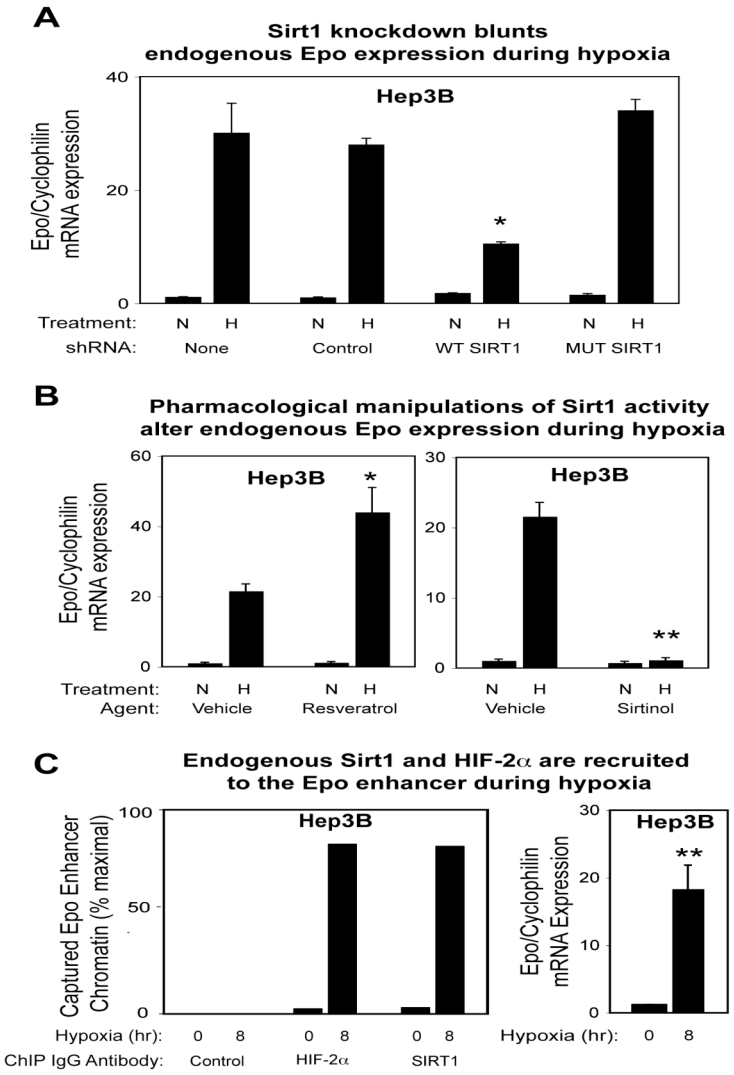


Figure 3-12: Sirt1 modulates epo gene expression in cell culture. (A) Sirt1 knockdown blunts endogenous *epo* expression during hypoxia. *Epo* gene expression was assessed by RT-PCR in Hep3B cells transfected with control, wild-type Sirt1 shRNA, or mutant Sirt1 shRNA expression vectors. After hypoxia exposure, control and mutant Sirt1 shRNA, but not wild-type Sirt1 shRNA, treated cells exhibited significant *epo* induction. **(B)** Pharmacological manipulations of Sirt1 activity alter endogenous *epo* expression during hypoxia. After hypoxia exposure, *epo* expression increased in vehicle (DMSO+PBS) and Resveratrol-treated cells, but not in sirtinol-treated cells. Resveratrol further augmented *epo* expression after hypoxia exposure relative to control cells. The data represent the mean ± SEM of triplicate wells treated as indicated. **(C)** Endogenous Sirt1 and HIF-2α are recruited to the *epo* enhancer during hypoxia. Chromatin immunoprecipitation (ChIP) assays were performed at the indicated time-points following hypoxia exposure after IP with anti-Sirt1, or anti-HIF-2α antibodies and using primers encompassing the human *epo* enhancer.(*$p<0.05$,**$p<0.01$)

If Sirt1 signaling is physiologically relevant to HIF-2α signaling, reduced Sirt1 gene dosage should affect *in vivo* regulation of the HIF-2α target gene *epo*. *Sirt1*$^{-/-}$ mice exhibit significant pre-/perinatal lethality and postnatal pathology (Cheng et al., 2003). *Sirt1*$^{+/-}$ mice, in comparison, lack gross abnormalities or gestational lethality. To assess if Sir1 deficiency affects *in vivo* regulation of *HIF-2α* target genes in adult mice during hypoxia, renal *epo* gene expression was determined in *Sirt1*$^{+/-}$ and *Sirt1*$^{+/+}$ mice subjected to systemic short-term continuous hypoxia (STCH). *Sirt1*$^{+/-}$ mice had blunted induction of renal *epo* gene expression after STCH exposure (**Figure 3-13A**). This was not due to a general reduction in renal *epo* gene expression as both groups of mice had similar levels of *epo* gene expression under ambient room air (RA) oxygen conditions. During mouse development, the liver is a major source of epo at the mid- to late-gestational stages (Koury et al., 1988). At embryonic day 14.5 (E14.5), when HIF-2α deficiency results in lower *epo* gene expression in the fetal liver, *Sirt1*$^{-/-}$ embryos had significantly lower levels of *epo* gene expression compared to *Sirt1*$^{+/+}$ mice (**Figure 3-13B**). In contrast, at E12.5 when HIF-2α deficiency has no effect upon *epo* gene expression in the fetal liver, *Sirt1*$^{-/-}$ embryos had similar levels of *epo* gene expression as *Sirt1*$^{+/+}$ mice (**Figure 3-13C**).

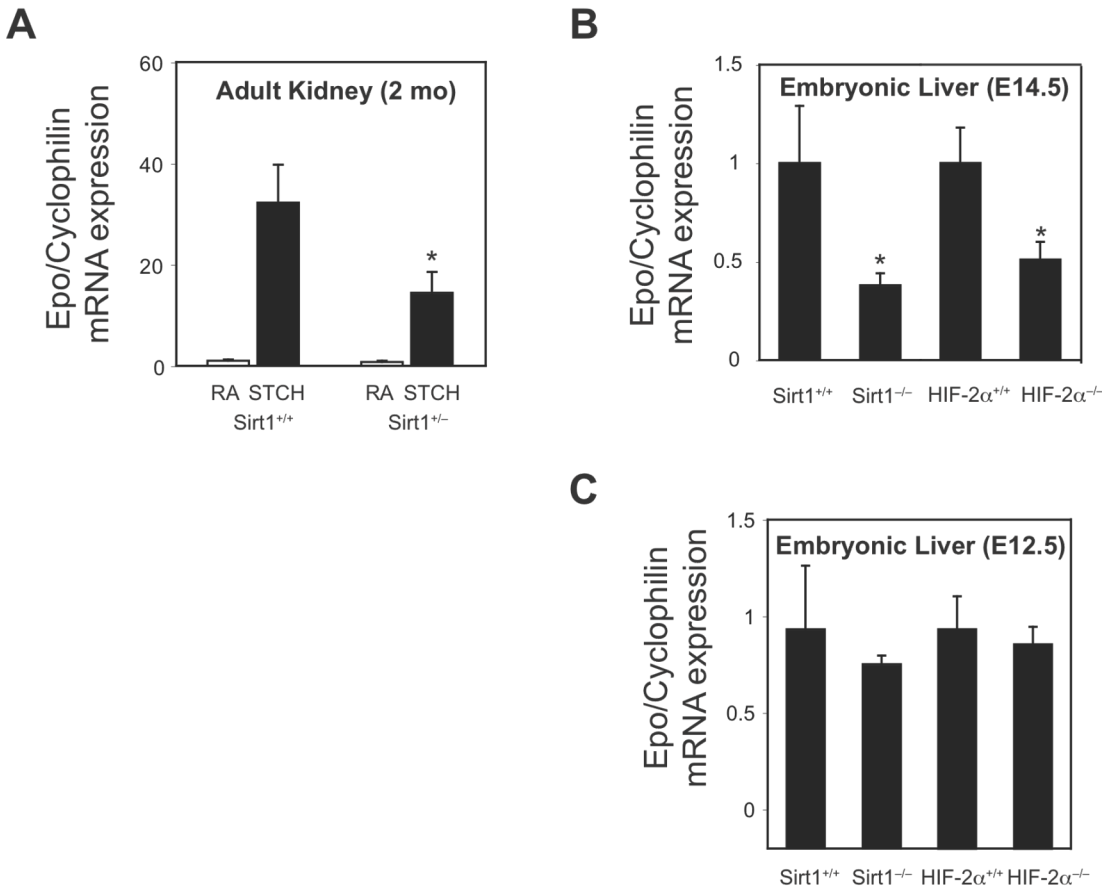


Figure 3-13 Sirt1 modulates epo gene expression in mice.
(A) Sirt1 haploinsufficiency results in blunted renal *epo* expression with hypoxia. Renal *Epo* mRNA levels were blunted in *Sirt1*$^{+/-}$ compared to *Sirt1*$^{+/+}$ adult mice after short-term hypoxia exposure ($p<0.05$). The bars represent the mean ± SD of six mice of each genotype. **(B)** Sirt1 deficiency results in reduced hepatic *epo* expression during development (E14.5). *Epo* levels in *Sirt1*$^{-/-}$, but not in *Sirt1*$^{+/+}$, embryonic day 14.5 livers were reduced ($p<0.05$). In a similar manner, *Epo* levels in *HIF-2*$\alpha^{-/-}$, but not in *HIF-2*$\alpha^{+/+}$, embryonic day 14.5 livers were reduced ($p<0.05$). The bars represent the mean ± SD of five embryos of each genotype. **(C)** Sirt1 and HIF-2α deficiency do not affect hepatic *epo* gene expression during early development (E12.5). *Epo* levels were similar in *HIF-2*$\alpha^{+/+}$ and *HIF-2*$\alpha^{-/-}$ as well as *Sirt1*$^{+/+}$ and *Sirt1*$^{-/-}$ embryonic day 12.5 livers. The bars represent the mean ± SD of seven mice per treatment group.

The adult liver is normally an ancillary site of epo expression. However, under conditions of sever chronic hypoxia, epo liver production rises to more than a third of total epo production (Eckardt and Kurtz, 2005). This indicates the liver is permissive for epo induction. We next asked if acute elevations in HIF-2α and Sirt1 levels modulated *epo* gene expression in adult mice liver. Adenoviruses encoding P1P2N HIF-1α, P1P2N HIF-2α, and/or WT or DAC Sirt1 were injected into adult mice; hepatic *epo* levels and hematocrits were measured one week later. Ectopic P1P2N HIF-2α, but not P1P2N HIF-1α, expression markedly increased hepatic *epo* levels (**Figure 3-14A**) and resulted in increased spleen weights, indicative of a dramatic increase in circulating red blood cell mass (**Figure 3-15C**). Combined over-expression of P1P2N HIF-2α and WT, but not DAC, Sirt1 further augmented *epo* levels (**Figure 3-14A**). These changes in *epo* gene expression resulted in increased hematocrits values that were highest with combined P1P2N HIF-2α and WT Sirt1 over-expression (**Figure 3-15A**).

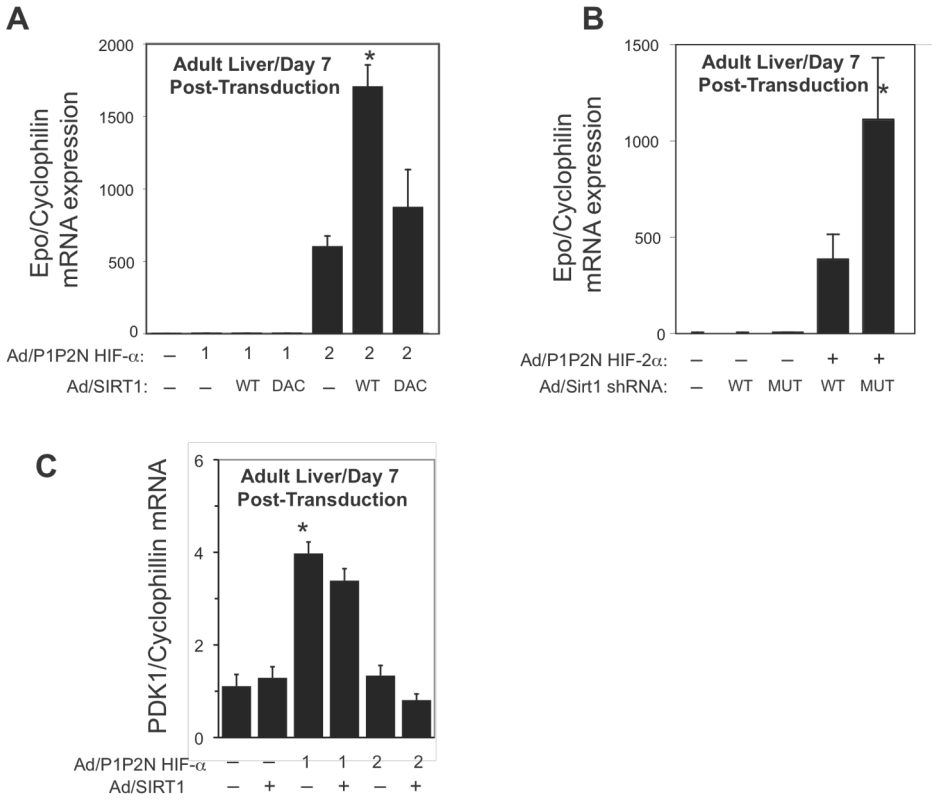


Figure 3-14: Sirt1 affects HIF-2α dependent regulation of hepatic *epo* expression in mice. (A) Ectopic Sirt1 and HIF-2α augment endogenous hepatic *epo* expression. Adenovirus encoding mock, P1P2N HIF-1α, P1P2N HIF-2α, P1P2N HIF-1α + WT Sirt1, P1P2N HIF-1α + DAC Sirt1, P1P2N HIF-1α + WT Sirt1, or P1P2N HIF-2α + DAC Sirt1 were delivered into mice. Hepatic *epo* mRNA levels at day 7 following injection increased for Ad/P1P2N HIF-2α ($p<0.01$) and were further augmented with Ad/WT Sirt1 ($p<0.05$). The bars represent the mean ± SD of five mice per treatment group. **(B)** Sirt1 knockdown decreases ectopic HIF-2α mediated induction of endogenous hepatic *epo* expression. Adenovirus encoding mock, Sirt1 wild-type shRNA, Sirt1 mutant shRNA, P1P2N HIF-2α, P1P2N HIF-2α + Sirt1 wild-type shRNA, or P1P2N HIF-2α + Sirt1 mutant shRNA were delivered into mice. The increased hepatic *epo* mRNA levels at day 7 for Ad/P1P2N HIF-2α ($p<0.01$) was blunted with Sirt1 wild-type shRNA relative to Sirt1 mutant shRNA ($p<0.05$). The bars represent the mean ± SD of five mice per treatment group. **(C)** Ectopic Sirt1 over-expression does not affect HIF-1α target gene PDK1. Adenovirus encoding mock, P1P2N HIF-1α, P1P2N HIF-2α, P1P2N HIF-1α + WT Sirt1, P1P2N HIF-1α + DAC Sirt1, P1P2N HIF-1α + WT Sirt1, or P1P2N HIF-2α + DAC Sirt1 were delivered into mice. Hepatic *Pdk1* mRNA level at day 7 following injection increased for Ad/P1P2N HIF-1α ($p<0.005$) and was not further augmented with Ad/WT Sirt1 co-expression. The bars represent the mean ± SD of five mice per treatment group.

To assess if an acute reduction in Sirt1 levels affects hepatic *epo* gene expression induced by ectopic P1P2N HIF-2α expression, adenovirus encoding shRNA against Sirt1 were used to generate Sirt1 knockdown (*Sirt1*^{kd}) mice. In *Sirt1*^{kd} mice expressing ectopic P1P2N HIF-2α in the liver, hepatic *epo* gene expression was reduced (**Figure 3-14B**) and the rise in hematocrits (**Figure 3-14B**) was blunted for mice receiving wild-type (WT) Sirt1 shRNA relative to mutant (MUT) Sirt1 shRNA treated mice. To determine the specificity of the Sirt1 effect on HIF-2α activation, we measured levels of Pdk1, a HIF-1α selective target gene. PDK1 expression was not affected by over-expression of HIF-2α and was increased with concomitant over-expression with P1P2N HIF-1α. Concomitant Sirt1 over-expression did not affect induction of Pdk1 by P1P2N HIF-1α(**Figure 3-4C**).

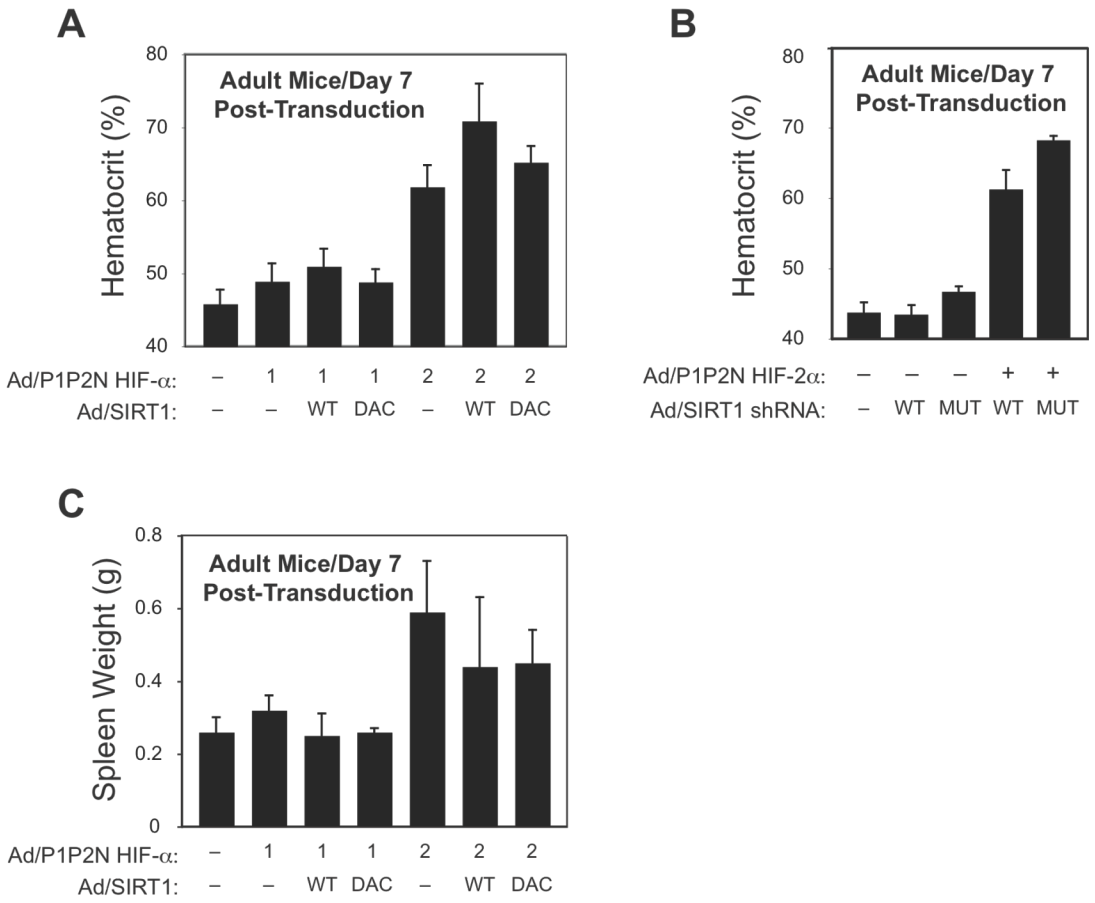


Figure 3-15: Ectopic alteration of Sirt1 affects HIF-2α dependent increase of hematocrits in mice. (A) The rise in hematocrits of mice expressing ectopic P1P2N HIF-2α in adult mouse livers is augmented by Sirt1. Hematocrits were significantly increased for all mice receiving HIF-2α. Hematocrits were further increased in mice receiving WT, but not DAC, Sirt1 adenovirus. **(B)** Knockdown of Sirt1 in adult mouse livers blunts ectopic P1P2N HIF-2α induced increases in hematocrits. Mice receiving P1P2N HIF-2α:HA adenovirus along with Sirt1 wild-type (WT) shRNA expressing adenovirus had significantly blunted rises in hematocrits relative to mice receiving P1P2N HIF-2α:HA adenovirus along with Sirt1 mutant (MUT) adenovirus ($p<0.05$). The bars represent the mean ± SD of five mice per treatment group. **(C)** Spleen weights of mice expressing ectopic HIF-2α in adult mouse livers are increased. Two month old wild-type mice were injected via the tail vein with adenovirus encoding mock, P1P2N HIF-1α:HA, P1P2N HIF-2α:HA, vsv-g:WT Sirt1 or vsv-g:DAC Sirt1 as indicated. Spleen weights were increased significantly for all mice receiving HIF-2α indicating marked increases in erythropoiesis.

Acetylation of HIF-2α by p300

HIF-1α has been described as being acetylated by Ard1 under normoxia (Jeong et al., 2002). To determine whether the mouse-specific ARD1 isoforms 225 ($ARD1^{225}$) implicated in the acetylation processes with HIF-1α or the more widely expressed mammalian ARD1 235 isoform ($ARD1^{1235}$) (Kim et al., 2006) is implicated in the acetylation of HIF-2α, I over-expressed P1P2N HIF-1α or P1P2N HIF-2α together with ARD1 isoforms in HEK293 (data not shown) or Hep3B cells with epo enhancer-epo promoter or Sod2 reporter constructs. $ARD1^{225}$ or $ARD1^{235}$ over-expression has no affect on HIF-1α or HIF-2α trans-activation of Sod2 or epo enhancer-epo promoter reporters **(Figure 3-16 A)** In acetylation experiments in HEK293 cells, ARD1 failed to acetylate HIF-2α (**Figure 3-16C**).

The protein p300, a histone acetyl transferase (HAT) enzyme is a classical HIF co-activator (Ema et al., 1999). In transient transfection assays, p300 over-expression can effectively increase both HIF-1α and HIF-2α trans-activation of the epo enhancer-epo promoter or Sod2 promoter reporters (**Figure 3-16B**). However, Sirt1 selectively augments p300 induced increase of P1P2N HIF-2α activation, but not of P1P2N HIF-1α activation. In acetylation experiments, p300 over-expression under normoxia can effectively induce acetylation of HIF-2α in HEK293 cells treated with TSA and Sirtinol (**Figure 3-16C)**. In follow-up experiments, we will determine the nature of the modified lysines compared to those acetylated during hypoxia.

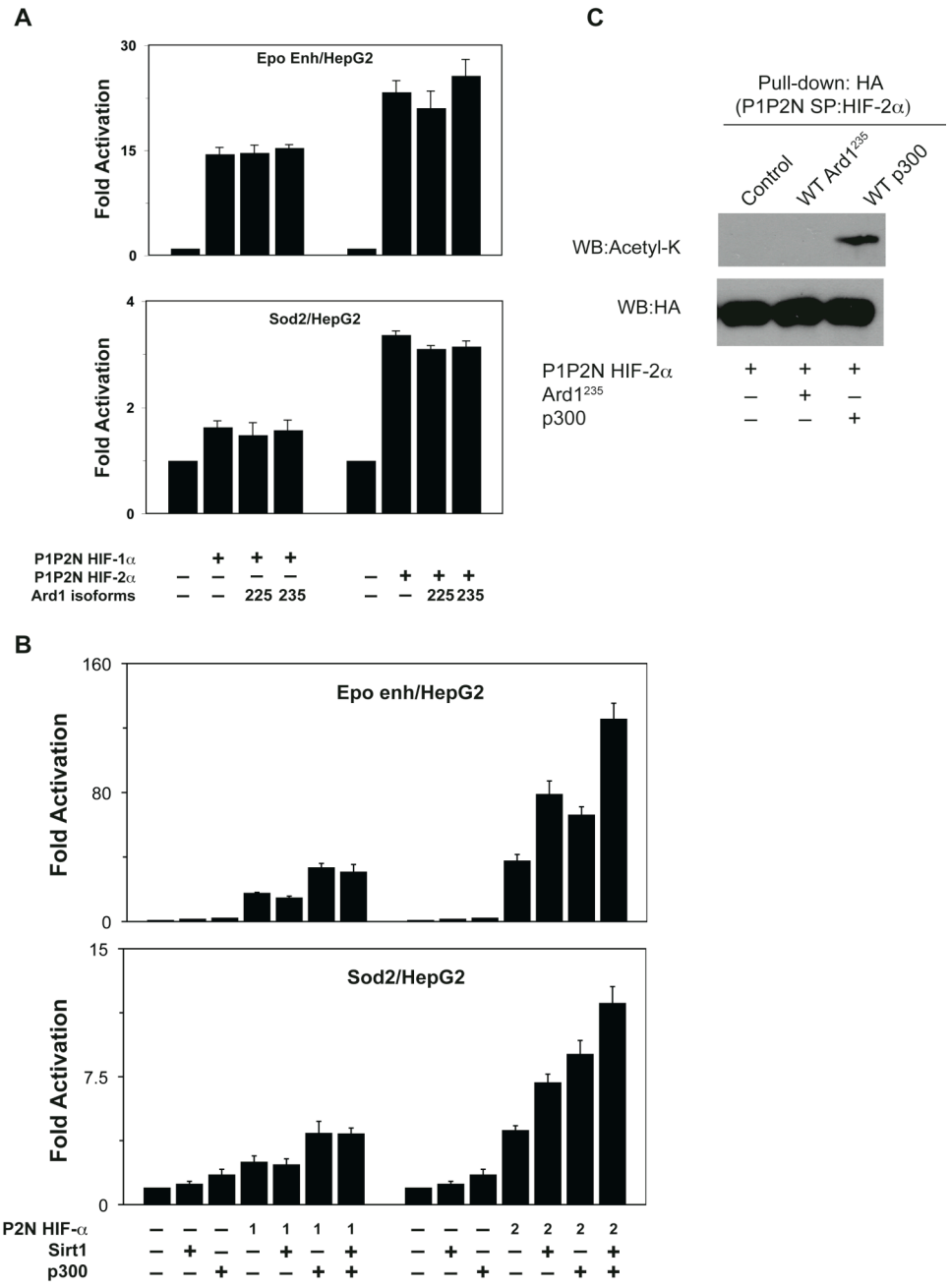


Figure 3-16. p300 Acetylates HIF-2α, and trans-activation by p300 is further increased by Sirt1. (A) ARD1 isoforms do not affect HIF transactivation. Hep3B transfected with expression plasmid encoding P1P2N HIF-1α or P1P2N HIF-1α with epo enhancer or Sod2 promoter reporters together with ARD1 isoforms 225 (ARD1225) or 235 (ARD1235), or (B) with expression plasmid encoding p300 and/or Sirt1. (C) *In vivo* acetylation of P1P2N HIF-2α:HA. HEK293 cells over-expressing ARD1 or p300 together with P1P2N HIF-2α were treated with TSA + Sirtinol. HA pull-down (PD) of P1P2N HIF-2α was followed by SDS-PAGE and western blotting (WB) against total protein (HA) or acetylated P1P2N HIF-2α (Acetyl lysine).

DISCUSSION

HIF members have important and unique biological roles in stress response. Adult HIF-2$\alpha^{-/-}$ mice exhibit mitochondrial dysfunction and associated metabolic abnormalities (Oktay et al., 2007; Scortegagna et al., 2003a). HIF-2α target genes identified from *in vivo* studies with HIF-2α null and knockdown mice (Ding et al., 2005; Kojima et al., 2007; Scortegagna et al., 2003a; Scortegagna et al., 2005) include *Cas1*, *Sod2*, and *Fxn*. In other studies of HIF-2$\alpha^{-/-}$ mice, we also found that HIF-2α is an essential regulator of *epo* in the kidney (Scortegagna et al., 2005) and eye (Ding et al., 2005). Selective ablation of HIF-2α in the liver of VHL$^{-/-}$ mice, which have constitutively activated HIF-1α and HIF-2α signaling, largely eliminates hepatic *epo* gene expression (Rankin et al., 2007). These studies led us to propose that HIF-2α is an essential sensor and responder to *in vivo* stresses with *epo* and *Sod2* being HIF-2α target genes induced during hypoxia and/or oxidative stress states (Scortegagna et al., 2003a; Scortegagna et al., 2005). These genes will in turn allow cells to survive the inciting stresses.

Sirt1 involvement in the aging process in mammals is still being debated. Presently, it is widely accepted that Sirt1 is involved in the maintenance of cellular homeostasis in response to a variety of stresses including genotoxic, metabolic, and oxidative stresses, by facilitating biological process that promote cytoprotection, cell survival and inhibit apoptosis (Anastasiou and Krek, 2006). Here we describe for the first time the implication of Sirt1 in the regulation of gene expression in response to hypoxic stress.

Sirt1 is activated by altered redox states. Since hypoxia affects cellular redox state, we reasoned that Sirt1 might also participate in HIF signaling during hypoxia. Several molecular and biochemical findings herein support this hypothesis. HIF-2α, but not HIF-1α, trans-activation is synergistically activated by Sirt1. Endogenous HIF-2α selectively complexes with Sirt1, and the endogenous Sirt1/HIF-2α complex increases with hypoxia exposure. Stable Sirt1/HIF-2α complex formation requires the HIF-2α carboxy terminal NTAD/ODD and the UR regions (**Figure 3-11)**. During hypoxia, several lysine residues are acetylated in the presence of Sirt1 inhibitors, as determined by mass spectrometry (**Supplemental Figure 5**), or when Sirt1 is genetically depleted by shRNA knock-down. Furthermore, the carboxy terminus of HIF-2α is sufficient for Sirt1 interaction and synergistic activation using the Gal4/VP16 system (**Figure 3-9 B, C**). The acetylated lysine residues in the HIF-2α carboxy terminal, distinct from sites of poly-ubiquitination or ARD1 acetylation (Paltoglou and Roberts, 2007), are relevant for the observed HIF2α/Sirt1 synergy. The data confirm that the acetylated lysines in the HIF-2α carboxy terminus are required for repression of Sirt1 by the small molecule inhibitor sirtinol **(Figure 3-11**). Finally, acetylated HIF-2α is a direct substrate for the class III HDAC Sirt1 deacetylase (**Figure 3-10 E, F**).

Our cell culture (**Figure 3-12**) and mouse model experiments (**Figure 3-13, Figure 3-14**) support a functional role for Sirt1 in HIF-2α dependent regulation of *Epo* gene expression. In HepG2 cells exposed to hypoxia, Sirt1 and

HIF-2α are recruited to the *Epo* enhancer. Pharmacological as well as genetic modulation of Sirt1 activity affects *epo* gene expression accordingly. Our data support a major role for Sirt1 in control of *epo* gene expression in Hep3B cells, a model cell line for HIF and epo biologists. Epo, generally considered an erythrogenic growth factor, is also a potent prosurvival factor that protects developing stem cell and progenitor cells in a variety of organs (Fliser et al., 2006; Noguchi et al., 2007). The participation of Sirt1 in *Epo* regulation may facilitate epo production in cellular niches characterized by nutrient, hypoxic, and possibly other environmental stresses.

In developing mice, hepatic *Epo* gene expression is normally induced by physiological hypoxia induced by growth. Sirt1 deficiency affects embryonic hepatic *Epo* gene expression at the same developmental time-point when HIF-2α deficiency has its consequence. Reflecting a need for exquisite and coordinate of Epo regulation by both Sirt1 and HIF-2α, renal *epo* gene expression is reduced with either Sirt1 or HIF-2α haploinsufficiency in adult mice treated with hypoxia (**Figure 3-13**). As further evidence of Sirt1 regulating HIF-2 signaling and *epo* gene expression *in vivo*, acute Sirt1 knockdown in adult liver blunts ectopic HIF-2α induced increases in hepatic *Epo* gene expression, whereas concomitant Sirt1 and P1P2N HIF-2α over-expression further augments hepatic *epo* gene expression in normoxic mice compared to P1P2N HIF-2α over-expression alone.

Our data reveal Sirt1/HIF-2 signaling may be regulated by at least two mechanisms. The first involves Sirt1 recruitment by the HIF-2α carboxy terminus

where it can serve as a conventional co-activator. Amino acid substitutions of the hypoxia-induced acetylated lysine residues in the HIF-2α carboxy terminus do not affect Sirt1/HIF-2α complex formation. Also, Sirt1 still retains the ability to stimulate these HIF-2α lysine mutants signaling indicating that acetylation is not required for Sirt1 recruitment and binding.

A second mechanism regulating Sirt1/HIF-2 signaling involves HIF-2α regulation by post-translational modification. Transcriptional activation of HIF-2α lysine substitution mutants by Sirt1 is not repressed by sirtinol. These findings are similar to those observed with the loss of NAM-induced repression for lysine substitution mutants of PGC-1α, also a target for Sirt1-mediated deacetylation and augmentation (Rodgers et al., 2005).

Mitochondrial complexes I and II oxidize the energy rich molecules NADH and FADH, and with the electron transport chain, they generate ROS (Klimova and Chandel, 2008; Turrens, 2003). ROS and the increased NAD^+ levels during hypoxia positively regulate Sirt1 activity, suggesting that hypoxia increases Sirt1 enzymatic activity. Hypoxia increases Sirt1 enzymatic activity and HIF-2α protein stability, and leads to synergistic activation of the *epo* gene. This synergy may be a result of direct interaction and dynamic deacetylation of HIF-2α by Sirt1. Thus, Sirt1/HIF-2α signaling may be controlled through regulation of Sirt1 deacetylation activity by NAD^+ levels or NAD^+/NADH ratios, changing during hypoxia and other environmental stresses. Linking Sirt1/ HIF-2α signaling with pyridine nucleotide homeostasis raises additional scenarios in which Sirt1 may alter HIF-2 signal

transduction, including settings in which HIF-2α is activated through hypoxia-independent mechanisms. Future studies will be needed to address these possibilities as well as to identify the precise molecular mechanism by which HIF-2α acetylation/deacetylation regulates HIF-2 signaling.

Our model (**Supplemental Figure 6)** suggests that HIF-2α can be acetylated both under normoxia and hypoxia by an unknown acetylase. TSA treatment alone is not sufficient to observe HIF-2α acetylation. However, TSA favors p300 interaction with and acetylation of Sp1 (Huang et al., 2005) or p53 (Stiehl et al., 2007) proteins. p300 is a HIF co-activator; it is also a candidate acetyl transferase in our model. Under normoxia, the asparaginyl hydroxylase FIH cannot efficiently hydroxylate the HIF-2α CTAD to a degree similar to HIF-1α (Bracken et al., 2006; Koivunen et al., 2004) and p300 can still interact with HIF-2α under normoxia. Thus, p300 may play a dual role, as an acetylase and as a HIF co-activator.

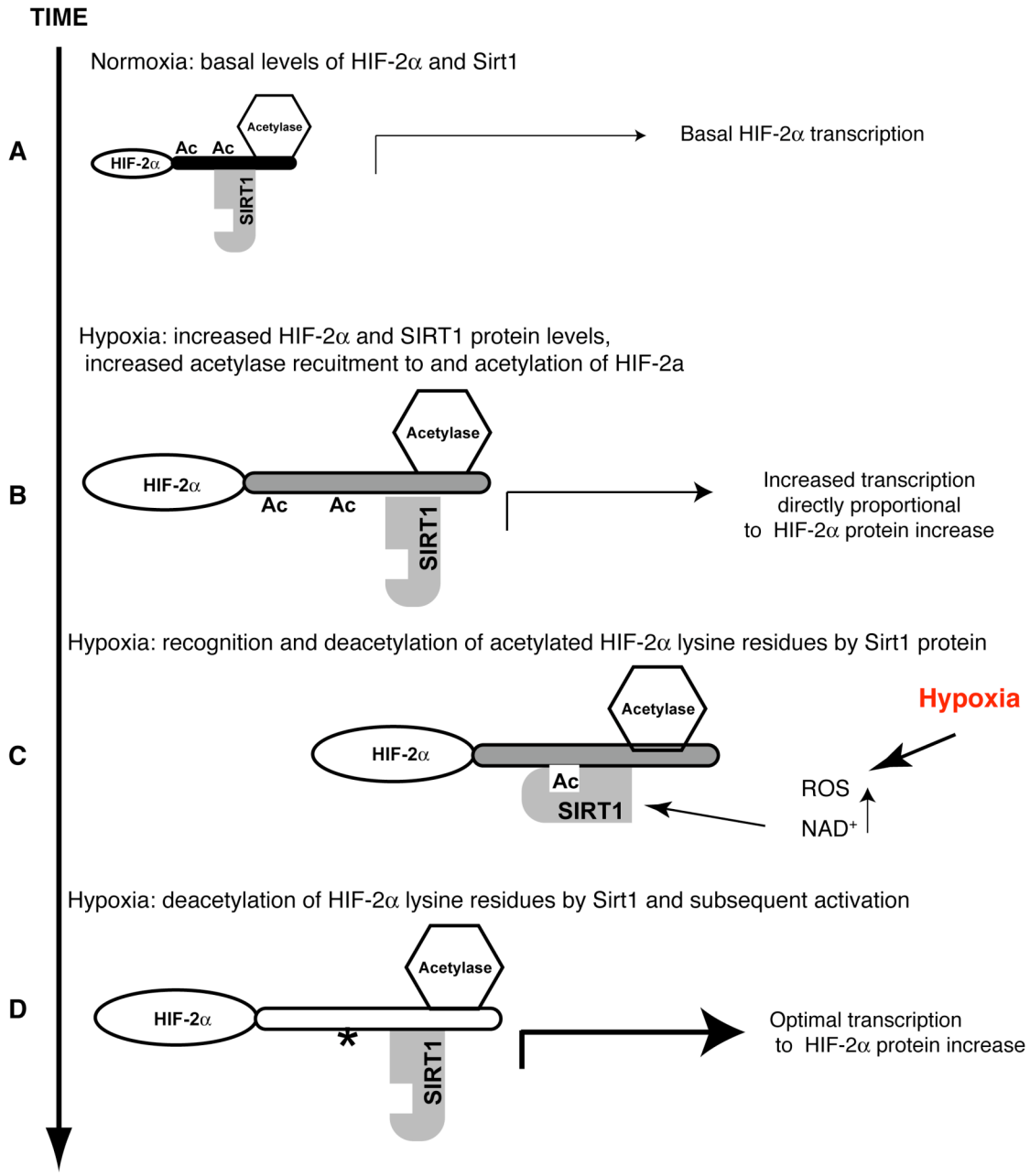
Under normoxia, HIF-2α is acetylated and can interact with Sirt1. We assume that Sirt1 enzymatic activity is not optimal. HIF-2α, but not HIF-1α, steady-state acetylation under normoxia, as well as during hypoxia, occurs in HEK293 as well as Hep2B cells only when Sirt1 enzymatic activity is inhibited pharmacologically with Sirtinol treatment or is inhibited genetically by shRNA knockdown (**Figure 3-10 E**). A point mutation that inhibits Sirt1 deacetylation activity (H363Y Sirt1, DAC Sirt1) leads to disruption of the Sirt1/HIF-2α complex.

These data suggest that HIF-2α is a direct substrate for Sirt1 deacetylation. A direct demonstration of Sirt1 deacetylating HIF-2α is seen with our *in vitro* data.

In adult mice, concomitant Sirt1 and P1P2N HIF-2α over-expression in livers of normoxic mice augments hepatic *epo* gene expression compared to P1P2N HIF-2α over-expression alone. In developing mice, hepatic *epo* gene expression is normally induced by physiological hypoxia experienced with growth. As further evidence of Sirt1 regulating HIF-2α signaling *in vivo*, Sirt1 knockdown in adult liver also affects HIF-2α dependent *epo* gene expression.

Sirt1/HIF-2α signaling during hypoxia involves dynamic changes in HIF-2α acetylation and Sirt1-mediated deacetylation rates (**Figure 3-10**). HIF-2α is only present in vertebrates and Sirt1 has no effect upon HIF-1α signaling, suggesting that Sirt1/HIF-2α signaling serves a more specialized role in higher metazoans. Integrating Sirt1/HIF-2α signaling, likely operant in pathophysiological conditions associated with chronic or repetitive environmental stresses, with input from other stress-responsive signal transducers may invoke crucial protective mechanisms. FoxO (Bakker et al., 2007) and HIF-2α (Tian et al., 1997) are both activated under hypoxic conditions. I propose signaling by Sirt1/HIF-2α, like that of Sirt1/FoxO, regulates expression of pro-survival factors under hypoxia and other adverse environmental conditions. Future studies will be needed to determine whether Sirt1/HIF-2α signaling has broader implications for stress signaling mechanisms as well as for aging in mammals.

Supplemental Figure 5. HIF-1α and HIF-2α protein sequence homology. In red are hydroxylated prolines and asparagine residues (P1P2N) implicated in the oxygen-dependent regulation of HIF-α Residues in green are acetylated lysines in HIF-2α identified by mass-spec and conservation in HIF-1α.



Supplemental Figure 6. Model of Sirt1 activation of HIF-2α. (**A**) In normoxia, HIF-2α interacts with Sirt1 (Figure 3-4) and induce basal HIF-2 target genes such as Epo. During hypoxia (**B**) increased stabilization of HIF-2α and transcription of Sirt1 (Figure 3-8) lead to increased Sirt1/HIF-2α complex (Figure 3-4D) and HIF-2α target genes transactivation. Here Sirt1 acts as a transcriptional co-activator (Figure 3-12). Oxidative stress (Brunet at al 2004) and NAD^+ (Imai et al., 2000) increase Sir1 activity. (**C**) During hypoxia, increases ROS levels and NADH oxidation (Klimova and Chandel, 2008; Turrens, 2003) stimulate Sirt1 enzymatic activity and deacetylation of HIF-2α (Figure 3-10). Deacetylated HIF-2α/Sirt1 complex (**D**) induce optimal HIF-2α target genes transcription during hypoxia.

CHAPTER FOUR

Materials And Methods

Materials and methods for chapter two

Bioinformatics Analyses.

Genomic sequences of various vertebrate erythropoietin enhancer regions were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) and UCSC (http://genome.ucsc.edu) genome browsers. Sequence analysis was performed using the default setting of the rVISTA algorithm program from the University of California in Santa Cruz (UCSC) genome browser. I also performed sequence manually using the ClustalW multiple sequence alignment program (http://www.ebi.ac.uk/cgi-bin/clustalw). Putative transcription factor binding sites (BS) in the conserved sequences were predicted using the Tfsitescan/dynamic Plus server (Ghosh, 2000)

Mammalian reporter and expression plasmids

The epo enhancer-promoter reporter constructs were amplified by PCR from mouse genomic DNA. The epo promoter was cloned as –410 to +1 relative to the start of transcription of the epo gene. The 176 bp parental epo enhancer regions, representing the conserved regions evaluated from Blast search, rVISTA and manual alignment using ClustalW, located 627 bp downstream of exon 5

from NCBI reference sequence M11319.1 were amplified by PCR from mouse genomic DNA. The epo enhancer NheI-XhoI fragment fused to the epo promoter SalI-HindIII fragment were cloned upstream of the luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI) as BS123-Box1+ Epo enhancer. Several 5' and 3' deletions were generated (boundaries are represented by solid arrows and open arrows respectively) by PCR (**Figure 2-3**), each representing delimited conserved regions. Point mutations of the binding sites BS2 or HRE were generated with QuickChange site directed mutagenesis (Stratagene).

Wildtype human HIF-1α and HIF-2α expression plasmids were constructed from carrier EST plasmids. The oxygen-independent HIF mutant forms, P1P2N HIF-1α and P1P2N HIF-2α, were previously described (Dioum et al., 2008). The hybrid HIF proteins P1P2N-HIF-1α/HIF-2α and P1P2N-HIF-2α/HIF-1α exchange the carboxy termini (CTERM) downstream of the PAS domain. The hybrid as well as amino or carboxy deletion HIF constructs were generated by zipper or deletion PCR techniques. All HIF expression constructs have a carboxy terminal HA epitope tag P1P2N HIF-1α:HA and P1P2N HIF-2α:HA deletion constructs were generated by PCR and cloned into pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA).

Full length human Sp1, Sp1, WT1, Egr family members Egr1, Egr2, Egr3 and Egr4) and NAB2 expression vectors were made by PCR cloning from cDNA library purchased from Openbiosystems Technology. The Egr family members and NAB2 were engineered with a VSV-G and FLAG epitope tag respectively.

The constitutively active forms of Egr1 (I292F Egr1) (Lee et al., 2005) and Egr2 (I268E) and the dominant negative form of NAB2 (E51K NAB2) (Svaren et al., 1996) were generated by QuickChange site directed mutagenesis. Egr2/Egr3-hybrids expression vectors were constructed by exchange of the Egr2 transactivation domain (TAD, residues 1-99), or Egr2 TAD and R1 domain (residues 1-269) with respectively the Egr3 R1 and zinc-finger domains (residues121-387) or zinc-finger domain alone (residues 254-387) and *vice-versa*. The hybrid constructs were generated by zipper pcr techniques with amino terminus VSV-G tag. All expression plasmids, unless otherwise indicated, are in pIRES-hr GFP (Stratagene, La Jolla, CA). Mutations were introduced using PCR-based site-directed mutagenesis (QuikChange II; Stratagene) and sequence-verified prior to cloning.

Cell culture and transfections

Reporter plasmids (30 ng per well), and expression plasmids (100 ng per well) were used in transient transfection analyses. HEK293 (Cat. No. CRL-1573, ATCC, Manassas, VA) and Hep3B cells (Cat. No. HB-8064, ATCC) were maintained in complete media [DMEM, 4.5 g/l glucose, 4 mM glutamate (Cat. No. SH30022, HyClone, Logan, UT), 10% fetal bovine serum (FBS; Cat. No. S10650H, Atlanta Biologicals, Lawrenceville, GA) supplemented with penicillin (100U/mL)/streptomycin (100 μg/mL) (Cat. No. 15140-148, Gibco BRL, Carlsbad, CA)] in a 5% $C0_2$, 95% air incubator. Cells at 50-60% confluency were

transfected in 48-well plates (Cat. No. 3548, CoStar, Corning Inc., Lowell, MA) with a maximum of 500 ng total DNA per well using Lipofectamine 2000 (Cat. No.11668-019, Invitrogen). Within each transfection set, DNA amount was kept constant with addition of empty expression vector. At 24 hr post-transfection, cells were harvested for luciferase and β-galactosidase assays. For hypoxia treatments, I transferred cells to a humidified environmental chamber (Coy Laboratory Products, Inc., Grass Lake, MI), replaced culture media with deoxygenated media, and maintained the cells under hypoxic (1% O_2, 5% CO_2, 94% N_2) conditions for the specified periods. Cells were harvested within the chamber to prevent any oxygen-induced, post-translational modifications.

Nuclear extract preparations

Hep3B cells (80% confluent) in 6 cm cell culture d dish were treated with hypoxia for the indicated time period (0, 1 2, 4, 8 and 16 hrs) using a Coy hypoxia chamber. After hypoxic treatment, the medium was discarded, and the Hep3B cells were lysed with 200ul of nuclear extract (NE) lysis buffer (20mM Hepes pH7.4, 10mM NaCl, 1.5mM MgCl2, 20% Glycerol, 0.1% Triton X-100, 1 mM DTT, 1× protease inhibitor cocktail (Cat. No. P8340, Sigma) and 1mM PMSF) before incubation on ice outside of the bag for 5 minutes. The cells lysate was transferred into a clean 1.5mL centrifuge tube and vortex for 10 sec to re-suspend cells and incubate for another 5 min. After centrifugation of the cell lysate 2600g at 4°C for 10 min, the supernatant representing the cytosolic

fraction was discarded and the nuclear pellet was resuspended in 175μl of lysis buffer NE by gentle vortex for 10 seconds. To nuclear membrane was lysed by adding 25μl of 5M NaCl and incubated with a gentle rotation at 4°C for 1 hr followed by vigorous vortex three times for 5 sec. The nuclear extract was centrifuges at 16000g at 4°C for 10 min to separate nuclear protein from cell debris and the nuclear protein concentration was estimated by Bradford assay. The nuclear protein was mixed with 50 μl of 4X SDS-PAGE loading buffer and heated at 95°C for 5 min followed by vigorous vortex for 10 sec.

For endogenous protein expression analysis, 40 μg of total nuclear protein was loaded on an 8% SDS-PAGE mini gel with 1.5 mm thickness and analyzed by western blot against HIF-1α (BD bioscience 610959 1:500 in 5% milk-TBS-T), HIF-2α (Novus Biological NB100-132 1:1000 in 5% milk-TBS-T), Egr1 (Santa Cruz sc-110 1:500 in 5% milk-TBS-T), or Egr2 (Covance PRB-236P 1:500 in 5% milk-TBS-T). As loading controls, I used antibodies to TBP (Santa Cruz sc-204 1:1000 in 5% milk-TBS-T), Sp1 from Santa Cruz sc-59 (1:500 in 5% milk-TBS-T), or ARNT from BD bioscience 611078 (1:500 in 5% milk-TBS-T).

Exogenous protein pulldown experiments

Co-immunoprecipotation (Co-IP) assays of the full length or deletion constructs of HIF-1α or HIF-2α together with the Egr isoforms were performed in HEk293 cells. HEK293 cells seeded at 1.5 x10^6 cells/60 mm dish were transfected with 4.5 μg of the indicated P1P2N HIF-α:HA expression constructs

with WT vsv-g:Egr1, vsv-g:Egr2, vsv-g:Egr3, or vsv-g:Egr4. The Egr1 protein is very unstable under normoxia and is barely detectable. To circumvent this problem, 8hrs after transfection, Egr1 transfected cells were treated with 10 μM MG132 (Cat. No. 474790 Calbiochem, Gibbstown, NJ stock 10 mM in DMSO) for 16 hrs. Twenty four hours after transfection, cells were harvested according μMACS^{TM} HA Tag Protein Isolation Kit (Cat. No. 130-091-122, Miltenyi Biotec, Auburn, CA) recommendations and HIF-α HA tagged protein-complexes bound to the anti-HA magnetic beads were thoroughly washed and eluted in 50 μl SDS-PAGE sample buffer heated at 95°C. Immune complexes were analyzed by immunoblotting with anti-vsv-g antibodies (1:1000 dilution; Cat. No. ab18612, Abcam, Cambridge, MA) or with anti-HA antibodies (1:1000 Dilution; Cat. No. H9658, Sigma). P1P2N HIF-1α:HA and P1P2N HIF-1α:HA deletion constructs were co transfected with vsv-g:Egr2 in HEK293 cells and immunoprecipitation is performed as described above.

shRNA knockdown studies

The Egr1 short hair RNA microRNA (shRNAmir) 5'-CGCTTCGGTTCTCCAGAATGTAtagtgaagccacagatgtaTACATTCT GGAGAACCGAAGCT-3' used to knockdown human Egr1 in Hep3B cells was purchased from OpenBiosystems technology. The Egr2 shRNA expression plasmid (5'-GATC AACATTGACATGACTGGAGAGAAGA-3') was purchased from Origen technologies (Cat no. TR313276). For cell culture studies, Hep3B

cells in 6-well plates were transfected at 60% confluency in triplicate with 2.5 μg of Egr1, Egr2 or Egr1 and Egr2 shRNA or control expression plasmids using 7μl of Lipofectamine LTX (Cat. No. 15338-100, Invitrogen, Carlsbad, CA) in DMEM plus 10% fetal bovine serum following the manufacturer's instructions. Forty-eight hours following transfection, the transfected cells were subjected to hypoxia for 16 hours and then total RNA was purified using the GenElute Total RNA Kit (Cat. No. RTN70-1KT, Sigma). Erythropoietin and cyclophilin gene expression were determined by real-time RTPCR analyses from cDNA generated by reverse transcription.

Real-time RT-PCR analyses

The expression of endogenous epo and cyclophilin were determined by reverse transcription of total RNA followed by real time PCR analysis. Total RNA from Hep3B cells was extracted using GenElute™ mammalian Total RNA kit (Cat. No. RTN70-1KT, Sigma). 1μg of total RNA was reverse transcribed by extension of oligo-dT primers using M-MLV reverse transcriptase (Cat. No. 28025-013,Invitrogen) according to the manufacturer's recommendations. Total RNA was isolated from 1/3 of the total mouse livers (right lobule) and extracted using the FastRNA Pro Green Kit (Cat. No. 6045, MP Biomedicals, Solon, OH). 1 μg of total RNA was then reverse transcribed using a M-MLV reverse transcriptase Kit (Cat. No.28025-013, Invitrogen). Mouse epo levels were normalized to a mouse loading control. Values were normalized to cyclophilin as

previously described (Dioum et al., 2008). Real time quantitative PCR was performed on an Applied Biosystems ABI Prism 7000 Thermocycler using Power SYBR Green Master Mix following the manufacturer's protocol and one-tenth of total cDNA with the following pairs of human primers:

Epo (forward)	5'-GAGGCCGAGAATATCACGACGGG-3',
Epo (reverse)	5'-TGCCCGACCTCCATCCTCTTCCAG-3',
cyclophilin (forward)	5'-ATGTGGTTTTCGGCAAAGTTCTA-3',
cyclophilin (reverse)	5'-GGCTTGTCCCGGCTGTCT-3',
VEGF (forward)	5'-CGCTGGTAGACGTCCATGA -3',
VEGF (reverse)	5'-CACGACAGAAGGAGAGCAGAA 3',
Egr1 (forward)	5'-GGGAGCCGAGCGAACA-3'
Egr1 (reverse)	5'- TCAGAGCGATGTCAGAAAAGGA-3',
Egr2 (forward)	5'-CCCTTTGACCAGATGAACG-3'
Egr2 (reverse)	5'-TAGGTGCAGAGATGGGAGC-3',

Statistical analyses

Comparisons between experimental groups were made by Student's t-Test for groups with equal sample size or by z-Test for comparisons between groups of unequal sample size. Two-tailed analyses were performed unless otherwise indicated. The statistical analyses were performed in Microsoft Excel (Microsoft Corporation, Redmond, WA).

Materials and methods for chapter three

Mammalian reporter and expression plasmids

The mouse *Sod2* (-1452/+40) (Scortegagna et al., 2003a), *VegfA* (-1106/+123) (Dioum et al., 2008), and *Epo* enhancer-promoter (Dioum et al., 2008), reporters were previously described. By PCR, I amplified from mouse genomic DNA the mouse Epo promoter representing –410 to +1 relative to the start of transcription of the epo gene and epo enhancer regions. The PCR products representing the mEpo enhancer Nhe I-Xho I and the mEpo promoter Sal I-Hind III were cloned into the luciferase reporter plasmid pGL3-Basic (Promega) as BS123-Box1+ Epo enhancer. Several 5' and 3' deletions were generated (boundaries are represented by solid arrows and open arrows respectively) by PCR (Figure 1a) each one representing delimited conserved regions. Point mutations of the binding sites BS23 were generated with Quick-Change site directed mutagenesis (Stratagene).

The human *SIRT1* promoters (-354/+54 and 128/+54) were isolated by PCR amplification of human genomic DNA and inserted into the firefly luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI). The 8xFBE-tk reporter was generated by PCR isolation of the 8xFBE element from a previously described FoxO reporter (Biggs et al., 1999) (generously provided by Dr. William Biggs) and replacement of the 3xHRE element upstream of the minimal tk region

in 3xHRE-tk/luc (Tian et al., 1997). Full length human Sp1, Sp1, WT1, Egr family members Egr1, Egr2, Egr3 and Egr4) and NAB2 expression vectors were made by PCR cloning from cDNA library purchased from OpenBiosystems Technology and subcloned into pIRES-hrGFP expression vector. The deacetylase (DAC) Sirt1 mutant (H363Y) was generated by site-directed mutagenesis (Vaziri et al., 2001). Constitutively active (nuclear) FoxO expression plasmids contained triple alanine substitutions (A3) for serine/threonine phosphorylation sites that regulate nuclear export (Furuyama et al., 2000) (generously provided by Dr. Tatsuo Furuyama). Wild type human HIF-1α and HIF-2α expression plasmids were constructed from carrier EST plasmids.

The oxygen-independent HIF mutant forms, P1P2N HIF-1α and P1P2N HIF-2α, were previously described (Dioum et al., 2008). The hybrid HIF proteins P1P2N-HIF-1α/HIF-2α and P1P2N-HIF-2α/HIF-1α exchange the carboxy termini (CTERM) downstream of the PAS domain. The hybrid as well as amino or carboxy deletion HIF constructs were generated by zipper or deletion PCR techniques. All HIF expression constructs have a carboxy terminal HA epitope tag. The HIF expression constructs used in the co-immunoprecipitation and pull-down experiments have an S protein (SP) tag at the amino terminus and/or a hemagglutinin A (HA) tag at the carboxy terminus. The SP:P1P2N HIF-α:HA and P1P2N HIF-α:HA forms have intact transactivation properties compared to the untagged parental forms as judged by transient transfection assays (data not

shown). SP:P1P2N HIF-2α:HA was used for mass spectroscopy determinations of hypoxia-induced acetylated lysine residues.

For mammalian two-hybrid experiments, Gal4:Sirt1 (aa 194-557), VP16:HIF-1α:HA (aa 351-826), or VP16:HIF-2α:HA (aa 353-870) fusion constructs were generated by pcr, cloned into CMX/Gal4DBD (Sirt1) and CMX/VP16 (HIFs) (CMX/Gal4 DBD and CMX/VP16 generously provided by Dr. Ron Evans), and confirmed by sequencing. The wild-type (WT) Sirt1 insert for the Gal4 fusion contains the core region of Sirt1 with homology to yeast Sir2. The mutant (MUT) Sirt1 insert also contains mutations that inactivate the deacetylase (H363Y) and ADP-ribosyl transferase (G261A) activities in Sirt1 (positions relative to full-length Sirt1). The Gal4-responsive reporter was generated by replacement of the 3xHRE element in 3xHRE-tk/luc with a synthetic linker consisting of four Gal4 upstream activating sequences (4xUAS). All expression plasmids, unless otherwise indicated, are in pIRES. Mutations were introduced using PCR-based site-directed mutagenesis (QuikChange II; Stratagene) and sequence-verified prior to cloning.

Cell culture and transfections

Reporter plasmids (30 ng per well), and expression plasmids (100 ng per well) were used in transient transfection analyses. HEK293 (Cat. No. CRL-1573, ATCC, Manassas, VA) and Hep3B cells (Cat. No. HB-8064, ATCC) were maintained in complete media [DMEM, 4.5 g/l glucose, 4 mM glutamate (Cat. No.

SH30022, HyClone, Logan, UT), 10% fetal bovine serum (FBS; Cat. No. S10650H, Atlanta Biologicals, Lawrenceville, GA) supplemented with penicillin (100U/mL)/streptomycin (100 μg/mL) (Cat. No. 15140-148, Gibco BRL, Carlsbad, CA)] in a 5% $C0_2$, 95% air incubator. Cells at 50-60% confluency were transfected in 48-well plates (Cat. No. 3548, CoStar, Corning Inc., Lowell, MA) with a maximum of 500 ng total DNA per well using Lipofectamine 2000 (Cat. No.11668-019, Invitrogen). Within each transfection set, DNA amount was kept constant with addition of empty expression vector (Novak et al.). At 24 hr post-transfection, cells were harvested for luciferase and β-galactosidase assays. For pharmacological treatments, cells were transfected and media replaced 4 hr later with drug-containing media. Resveratrol (1 mM stock in DMSO; Cat. No. R5010, Sigma, Saint Louis, MO) sirtinol (1 mM stock in DMSO; Cat. No. 510 8474, Chembridge Corporation, San Diego, CA), or nicotinamide (1 M stock in 1x PBS; Cat. No. N0636, Sigma) were diluted at the indicated concentrations in DMEM immediately prior to use. For pharmacological treatments, DMSO (Cat. No. D-8779, Sigma) and 1x PBS were added to a final concentration 0.5% for all samples including controls. Cells were harvested 20 hr after drug treatment was initiated. For hypoxia treatments, I transferred cells to a humidified environmental chamber (Coy Laboratory Products, Inc., Grass Lake, MI), replaced culture media with deoxygenated media, and maintained the cells under hypoxic (1% O_2, 5% CO_2, 94% N_2) conditions for the specified periods. Cells were harvested in the chamber to prevent any oxygen-induced, post-translational modifications.

Mammalian two-hybrid studies were performed in Hep3B cells using 48-well plates with Lipofectamine 2000 as described earlier.

Cycloheximide assays (half-life determinations)

Hep3B cells were seeded at 0.5×10^6 cells onto 6 cm diameter plates and transfected with the indicated expression constructs. The next day, the transfected cells were treated for an additional 24 hours with 5 μM sirtinol plus 0.2 μM trichostatin A (TSA) or 0.2 μM TSA alone. The treated cells were exposed to hypoxia for the last 0 or 16 hours of drug treatment. The following day, cells were treated with 50 μg/ml cycloheximide (50 mg/ml stock in DMSO; Cat. No. C4859, Sigma) for 0, 30, 60, or 90 min before lysis. Western blotting analyses were performed as indicated.

Nuclear extract preparations

Nuclear protein was isolated from mouse livers and extracted using a commercially available nuclear extract kit (Cat. No. 40010, Active Motif, Carlsbad, CA). In brief, fresh mouse liver (300 mg) was diced using a clean razor blade, placed in a pre-chilled dounce homogenizer, and homogenized in 1ml of ice cold 1x hypotonic buffer supplemented with 1mM DTT and o.1% detergent mixture (Active motif cat. No. 40010). After 15 minutes incubation on ice, the homogenate was centrifuged for 10 minutes, 850g 4°C, and the supernatant was discarded. The pellet was gently resuspended in 500 μl of 1x hypotonic buffer by

up-an-down pipetting several times. After 15 minutes incubation on ice, 25 μl of detergent solution was added and then vortexed vigorously for 10 seconds. The suspension was centrifuged for 30 seconds, 14,000g, 4°C. The nuclear pellet was resuspended in 50 μl complete lysis buffer and then vigorously vortexed for 10 seconds. The protein suspension was centrifuged was incubated for 30 minutes on ice on a rocking plate-form set at 150 rpm, and then vortexed for 30 seconds. After centrifugation, the supernatant containing the nuclear protein suspension was transferred into a pre-chilled microfuge and the protein concentration was measured by using Bradford assay.

Exogenous protein pull-down experiments

For pull-down assays with full-length HIF proteins, HEK293 cells were seeded at 1.5 x10^6 cells/60 mm dish and were transfected with 4.5 μg of the indicated expression plasmid (pIRES, P1P2N SP:HIF-1α:HA, P1P2N SP:HIF-2α:HA, WT vsv-g:Sirt1, DAC vsv-g:Sirt1). One day after transfection, cells were maintained under normoxia or exposed to 4 hr, 8 hr, or 16 hr of hypoxia. Cell extracts were prepared with lysis buffer (Cyto Buster™ Protein Extraction Reagent, Cat. No. 71009, Novagen, Gibbstown, NJ) containing 1× phosphatase inhibitor Cocktail 1 (Cat. No. P2850, Sigma), 1× phosphatase inhibitor Cocktail 2 (Cat. No. P5726, Sigma), 1× protease inhibitor cocktail (Cat. No. P8340, Sigma), 5 μM trichostatin A (1 mM stock in DMSO; Cat. No. T8552, Sigma), and 10 mM nicotinamide (1 M stock in 1x PBS; Cat. No. N0636, Sigma). SP-tagged proteins

were purified as described with the following modifications (Hackbarth et al., 2004). Extracts were incubated with S-protein agarose (Cat. No. 69704, Novagen, Madison, WI, USA) for 2 hr at 4°C with end-over-end mixing. Following binding, samples were three times with 1 × IP buffer (Cat. No. 54001, Active Motif, Carlsbad, CA) containing 1× phosphatase inhibitor Cocktail 1, 1× phosphatase inhibitor Cocktail 2, 1× protease inhibitor cocktail, 5μM TSA, 10 mM nicotinamide. Bound complexes were released by heating in 50 μl SDS-PAGE sample buffer at 95°C for 5 minutes. For pull-down assays with HIF-2∝ deletion constructs, HEK293 cells were seeded at 1.5 x10^6 cells/60 mm dish and were transfected with 4.5 μg of the indicated P1P2N HIF-2α:HA deletion expression constructs and/or with WT vsv-g:Sirt1. One day after transfection, cells were harvested with μMACS™ HA Tag Protein Isolation Kit (Cat. No. 130-091-122, Miltenyi Biotec, Auburn, CA). Bound complexes were released by heating in 50 μl SDS-PAGE sample buffer at 95°C for 5 minutes. Immune complexes were analyzed by immunoblotting with anti-vsv-g antibodies (1:1000 dilution; Cat. No. ab18612, Abcam, Cambridge, MA) or with anti-HA antibodies (1:1000 Dilution; Cat. No. H9658, Sigma). For mammalian two-hybrid proteins, Gal4:WT Sirt1, Gal4:MUT Sirt1, VP16:HIF-1α:HA and/or VP16:HIF-2α:HA pull-down experiments were performed in HEK293 cells. Expression plasmids (5 μg each) were co-transfected using Lipofectamine 2000 in 10 cm plates seeded at 2 x 10^6 cells/10 cm plate. Twenty-four hours after transfection, cell extracts were prepared with lysis buffer (Cyto Buster™ Protein Extraction Reagent; Cat. No.

71009, Novagen, Gibbstown, NJ) containing 1× phosphatase inhibitor Cocktail 1 (Cat. No. P2850, Sigma), 1× phosphatase inhibitor Cocktail 2 (Cat. No. P5726, Sigma), 1× protease inhibitor cocktail (Cat. No. P8340, Sigma), and 1 mM PMSF (Cat. No. P762, Sigma). The HA complex was immunoprecipitated using the μMACS™ HA Tag Protein Isolation Kit (Cat. No. 130-091-122, Miltenyi Biotec, Auburn, CA). Bound complexes were released by heating in 50 μl SDS-PAGE sample buffer at 95°C for 5 minutes. Immune complexes were analyzed by immunoblotting with anti-vsv-g (1:1000 dilution; Cat. No. ab18612, Abcam, Cambridge, MA) or anti-VP16 (1:1000 Dilution; Cat. No. ab4808 Abcam, Cambridge, MA) antibodies.

Endogenous protein pull-down experiments

Immunoprecipitation of endogenous proteins was accomplished with a Nuclear Complex Co-IP kit (Cat. No. 53009, Active Motif) according to the manufacturer's protocol. Hep3B nuclear extract (500 μg) were first incubated with 20 μl protein A agarose beads (Cat. No. 07-131, Upstate Biotechnology, Inc., Lake Placid, NY) for 2 hours with gentle rotation at 4°C. The cleared supernatants were incubated with 5 μg Sirt1 specific antibody (Cat. No. 07131, Upstate Biotechnology, Inc., Temecula, CA) or normal rabbit IgG (Cat. No. 2027, Santa Cruz Biotechnology, Santa Cruz, CA) overnight before addition of 20 μl of protein A agarose beads for 4 hours. After three washes with 1x low IP buffer plus 1mg/mL BSA followed by three washes with 1x low IP buffer without BSA,

the immunoprecipitated materials were eluted with addition of 20 μl 2x SDS sample buffer followed by boiling, resolved by SDS-PAGE, and detected with human anti-Sirt1 (1:1000 Dilution; Cat. No. 07131, Upstate Biotechnology, Inc.), anti-HIF-1α (1:1000 Dilution; Cat. No. 610959, BD Biosciences, Franklin Lakes, NJ), or anti-HIF-2α (1:1000 Dilution; Cat. No. NB100-132, Novus Biologicals, Littleton, CO) antibodies.

In vitro ^{35}S-Methionine SIRT1/GST-HIF pull-down experiments

Full-length wild-type Flag-SIRT1 (WT SIRT1) or H363Y Flag-SIRT1 (DAC SIRT1) subcloned in pcDNA 3.1 (Invitrogen) were *in vitro* transcribed and translated using the TNT-Quick™ rabbit reticulocyte lysate system reaction following the manufacturer's recommendations (Cat. No. L4610, Promega). The SIRT1 proteins were labeled by incorporation of ^{35}S-Methionine (Cat. No. NEG709A500UC, Perkin Elmer, Boston, MA). For pull-down assays, 25 μL of either WT or DAC ^{35}S-Labeled SIRT1 protein extract was incubated overnight at 4°C with 0.5 ml of purified GST-HIF1α CTERM:HA or GST-HIF-2α CTERM:HA. The proteins were pulled down using anti-HA agarose beads and the beads were washed three times with 500 μl IP low buffer (Cat. No. 54001, Active Motif) before elution with 30 μl 2x SDS-PAGE sample buffer. The eluted proteins were resolved by SDS-PAGE, the HIF-α proteins were detected by coomassie staining, and ^{35}S-labeled SIRT1 was detected by autoradiography.

Bacterial expression of HIF-α carboxy terminus (CTERM) GST fusions

The carboxy terminal portion (CTERM) of human HIF-1α (amino acids 352-826) or HIF-2α (amino acids 350-870) was cloned as a GST fusion protein in the bacterial expression plasmid pGEX-4T3 (Pharmacia, Peapack, NJ). The E. coli strain Origami B (DE3) (Cat. No. 71136-3, Novagen, Madison, WI) and Rosetta-gami B (DE3) (Cat. No. 71137-3, Novagen) were transformed respectively with parental pGEX-4T3 or with pGEX-4T3 encoding GST-HIF-1α CTERM:HA and GST-HIF-2α CTERM:HA. Bacteria were grown in a 37°C shaker incubator in the presence of 34 μg/mL chloramphenicol, 15 μg/mL kanamycin, 12.5 μg/mL tetracycline and 100 μg/mL ampicillin in 25 mL Luria-Bertani medium (LB broth) overnight. The cultures were then transferred to a 500 mL LB broth with 34 μg/mL chloramphenicol, 15 μg/mL kanamycin, 12.5 μg/mL tetracycline and 100 μg/mL ampicillin at 37°C and grown to an absorbance of 0.7 at 600 nm before induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Cat. No. BP.1755-1, Fisher Scientific, Pittsburgh, PA). Before induction, a 1 ml aliquot was withdrawn and saved for a non-induced control. Cells were grown for 16 hrs at 25°C after IPTG induction. The IPTG-induced bacteria were pelleted by centrifugation (4000 rpm, 30 minute), washed 1x with ice cold PBS, and resuspended in 25 ml of B-PER II bacterial protein extraction reagent (Cat. No. 78260, Pierce, Rockford, IL) supplemented with 5 ml bacterial protease inhibitor cocktail (Cat. No. P8465, Sigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Cat. No. P762, Sigma) at room temperature for 15 minutes with gentle shaking.

The lysate were further sonicated 3x using a cell sonicator on ice for 10 sec each time using the microtip probe set at amplitude 5 with continuous burst (Cat. No. F550, Fisher Scientific). The bacterial cell lysate was cleared by centrifugation at 15000 rpm for 30 minute at 4°C. The purification of GST-HIFs by glutathione-sepharose affinity chromatography was performed according to the manufacturer's suggestions. Briefly, the bacterial lysate was incubated with 0.4 ml of a 1:1 (vol/vol) slurry of 1x PBS:glutathione-sepharose beads (Cat. No. 17-0756-01, GE Healthcare, Piscataway, NJ) at 4°C overnight on a rotator. The resin was spun down and the supernatant discarded, and the pelleted resin was washed 5x with 1 ml 1x PBS containing 1:10 dilution of B-PER II binding solution (Cat. No. 78260, Pierce). GST fusion proteins were eluted by incubation with 1 ml wash buffer containing 1 mM reduced glutathione (Cat. No. G4251-10G, Sigma) for 4 hr at 4°C.

Exogenous HIF-2α acetylation experiments

I transfected HEK293 cells seeded in 10 cm culture dishes with expression vectors encoding SP:P1P2N HIF-1α:HA or SP:P1P2N HIF-2α:HA using Lipofectamine 2000 (Cat. No. 11668-019, Invitrogen). Eight hours after transfection, I treated cells with 5 μM sirtinol plus 0.2 μM trichostatin A (TSA) or 0.2 μM TSA alone for an additional 24 hours. To assess the contribution of hypoxia on HIF acetylation, the transfected cells were exposed to hypoxia for the last 0, 2, 4, 8 or 16 hours following drug treatment. SP:P1P2N HIF-1α:HA or

SP:P1P2N HIF-2α:HA were purified from the cell lysates using pull-down with SP-agarose and subjected to immunoblot analyses using antibodies recognizing acetylated lysine residues (Cat. No. KAP-TF120, Stressgen, Ann Arbor, MI). The same membranes were re-probed with antibodies recognizing the HA epitope to assess SP:P1P2N HIF-1α:HA or SP:P1P2N HIF-2α:HA loading levels.

To verify that TSA treatment inhibited class I/II HDACs activities, acid-extracted histone preparations were prepared from Hep3B cells grown in a 60 mm dish treated with either DMSO or .2 μM TSA for 16 hrs. Cells were harvested in 500 μL of 1x hypotonic buffer (cat no. 100505 Active motif) supplemented with 1× protease inhibitor cocktail (Cat. No. P8340, Sigma), 0.2 μM TSA, 25μl of detergent solution (cat 100512 Active motif) and 1 mM DTT (cat no. D9779-5G Sigma) vortex for 10 seconds. The cell lysate was centrifuged at 10,000 g for 5 minutes and the supernatant (cytoplasmic fraction) discarded. The nuclear fraction pellet was re-suspended very well in 200 μl of 0.4N HCl and incubated on rotator for 1 hour. Nuclear debris were cleared by centrifugation at 4°C, 16,000 g for 10 minutes and the supernatant containing histones was transferred into a clean 1.5 ml eppendorf tube. Total histones were precipitated by adding trichloro-acetic acid (TCA 100% w/v solution cat. no. 9169) with gentle mixing to a final concentration of 10%. The solution (appear milky) was incubated on ice for 30 minutes and proteins were centrifuged at 4°C, 16,000 g for 10 minutes. Pelleted proteins were gently washed with 500 μl of ice-cold acetone and air dried for 20 minutes at room temperature. Histones were dissolved in 100 μl of PBS by

vigorous mixing and quantitated by Bradford protein assay. Twenty five μg of protein was loaded on 15% SDS-PAGE gel and immunoblotting was performed using antibodies recognizing acetyl-histone H4 (1:2000 cat. no. 06-866 Millipore) or total histone H4 (dilution 1:3000 cat. no. 05-858 Millipore).

Endogenous HIF-2α acetylation experiments

Hep3B cells were grown in 15 cm plates in complete medium (DMEM, 10% FBS, 100 μg/ml Penicillin, 100 U/ml Streptomycin) to 90% confluency. Cells were treated with either normoxia (3 plates) or 12 hrs of hypoxia (3 plates) in complete medium supplemented with 2 μM TSA and 5 μM sirtinol. Cells were fractionated and the nuclear protein extracted using a high-salt fractionation protocol (Miltenyi Biotech, Auburn, CA) supplemented with 1× protease inhibitor cocktail (Cat. No. P8340, Sigma), 0.2 μM TSA, and 5 μM sirtinol. The nuclear protein concentration was diluted to 10 μg/μl and endogenous HIF-2α was immunoprecipitated overnight at 4°C with 30 μg of a monoclonal human HIF-2α antibody (Cat. No. NB100-132, Novus Biologicals, Littleton, CO) using gentle rotation. The bound HIF-2α antibody was precipitated with 40 μl of Protein A/G agarose beads (Cat. No. sc-2003, Santa Cruz Biotechnology, Santa Cruz, CA) for 4 hrs. The bound protein was washed three times using high stringency wash buffer (Cat. No. 130-091-122, Miltenyi Biotec), and then eluted with 30 μl of preheated (95°C) 2x SDS-PAGE sample buffer. Aliquots of the normoxia (80%) and hypoxia (20%) samples were separated on an 8% SDS-PAGE gel and

immunoblotted overnight for endogenous HIF-2α (1:500 dilution; Cat. No. NB100-132, Novus Biologicals) or for acetyl lysine (Cat. No. KAP-TF120, Stressgen, Ann Arbor, MI). Normalization was performed using antibodies recognizing TATA-binding protein (TBP; 1:1000, cat no. sc-204 Santa Cruz Biotech), α-tubulin (1:3000 cat. no. T 5168 Sigma), or β-actin (1:1000 cat. no. A 5316, Sigma).

***In vitro* deacetylation experiments**

To prepare acetylated HIF-2α substrate, HEK293 cells (1.5 x 10^6 cells, one 15 cm plate) were transfected with an expression plasmid encoding SP:P1P2N HIF-2α:HA (25 μg) in 60 μl of Lipofectamine 2000 in Opti-MEM 1 reduced serum medium (Cat. No. 31985, Invitrogen). Four hours after transfection, Opti-MEM 1 was replaced with complete media (1x DMEM, 10% FBS, Penicillin/Streptomycin) and allowed to recover for four hours. Next, the transfected cells were treated with sirtinol (5 μM) plus TSA (0.2 μM) for 24 hrs with hypoxia exposure for the last 16 hrs. Acetylated HIF-2α was purified using SP-agarose chromatography. For Sirt1 protein, WT Flag-SIRT1 or DAC Flag-SIRT1 expression plasmid DNA (15 μg) was transfected in HEK293 cells (10 cm plates) and purified twenty four hours later using anti-Flag antibody agarose (Cat. No. F-2426, Sigma). Cells were lysed in 1mL Cyto Buster™ Protein Extraction Reagent (Cat. No. 71009, Novagen) as described earlier. The whole cell lysate was spun down (14000 rpm, Eppendorf 5415 micro-centrifuge) at 4°C for 30 minute and the supernatant incubated with 40 μl of EZ view Flag affinity gel (50%

slurry) previously equilibrated 2x with the lysis buffer (500 μl) of Cyto Buster™ buffer. Binding was performed by gently agitation of the mixture in the rotator kept at 4°C in the cold room for two hours to overnight. The Flag beads were pelleted down by centrifugation (8000 rpm, Eppendorf 5415 micro-centrifuge) for 1 minute at 4°C and washed 3x with ice-cold Tris-buffered saline (TBS; 20 mM Tris-pH 8.0, 150 mM NaCl) at 4°C. After the last wash, the flag beads were resuspended in 100 μl of TBS plus 1 mM PMSF. To verify purification of Sirt1, an aliquot of the resuspended beads (10 μl) was separated by electrophoresis on an 8% SDS-PAGE and immunoblotted with anti-Flag antibody. Before the deacetylation reaction, the SP-agarose bound P1P2N HIF-2α:HA and the Flag-bead bound SIRT1 were each spun down, equilibrated with 500 μl of deacetylation reaction buffer (50 mM Tris pH 9.0, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, and 10 mM NAD), and resuspended in a final volume of 250 μl of deacetylase reaction buffer for SP:P1P2N HIF-2α:HA and 100 μl for Flag:SIRT1. I performed the *in vitro* deacetylation reaction for zero or one hour at 30°C by mixing 25 μl of acetylated SP:P1P2N HIF-2α:HA (25% of over-expressed SP:P1P2N HIF-2α:HA from one 15 cm plate) with 20 μl of either WT or DAC SIRT1 in a total volume of 50 μl deacetylase reaction buffer. The reaction was stopped with addition of 14 μl of 4x SDS-PAGE sample buffer and heat-inactivation at 95°C for 5 minutes. The reaction (30 μl) was separated using SDS-PAGE chromatography and immunoblotted using antibody recognizing acetylated-lysine or the HA epitope.

Large-scale acetylated HIF-2α preparation

HEK293 cells at 80% confluency plated in twenty-two 15 cm dishes were transfected with 20 μg of pSP:P1P2N HIF-2α:HA expression plasmid using 50 μl of Lipofectamine 2000 (Cat. No. 11668-019, Invitrogen) according to the manufacturer's recommendation. The next morning, the media was changed with fresh complete DMEM medium supplemented with 2 μM TSA, 2 mM sodium butyrate and 10 μM sirtinol for an additional 24 hrs with the last 16 hrs including hypoxia treatment. Before harvest, cells in each dish were washed with 2 mL of cold PBS supplemented with phosphatase inhibitor (Cat. No. 53009-102146, Active Motif) and lysed with 1 mL of Cyto Buster™ Protein Extraction Reagent (Cat. No. 71009, Novagen) supplemented with 1x protease inhibitor (Cat. No. P3840, Sigma), 2 μM TSA, 2 mM sodium butyrate, and 5 μM sirtinol at room temperature for 15 minute or until the solution become clear. The cell lysate was centrifuged at 14,000 rpm for 15 minutes at 4°C. The recombinant SP:P1P2N HIF-2α:HA protein was purified from cell lysate by overnight pulldown with 1.5 ml S-protein agarose (Cat. No. 69704, Novagen, Madison, WI, USA) at 4°C with gentle rotation. The S-protein agarose bound proteins were washed 3x for 5 minutes each at 4°C with gentle rotation with 10 ml wash buffer (Cat. No. 130-091-122, Miltenyi Biotec) supplemented with 1x protease inhibitor cocktail (Cat. No. P3840, Sigma), 1 mM PMSF, 0.2 μM TSA, and 5 μM sirtinol. The purified HIF-2α protein was eluted in 150 μl of heated (95°C) 1x SDS-PAGE sample buffer, separated on an 8% SDS-PAGE gel, stained with colloidal coomassie blue

(LC6025, Invitrogen), isolated from the gel, and subjected to liquid chromatography followed by liquid chromatography-tandem mass spectrometry (LC/MS/MS) as described below.

Mass spectrometry acetylation analyses

To detect unbiased acetylated lysines on HIF-2α, the gel slice of human HIF-2α protein from the one-dimensional SDS-PAGE gel was digested with trypsin (Cat. No. V5113, Promega, Madison, WI). The tryptic digests were analyzed by nano-LC/MS/MS using a Dionex LC-Packings HPLC (Dionex, Sunnyvale, CA) coupled with a Q Star XL mass spectrometer (Applied Biosystems, Framingham, MA). Peptides were first desalted on a 300 μm x 5 mm PepMap C18 trap column (Dionex, Sunnyvale, CA) with 0.5% formic acid in HPLC-grade water at a flow rate of 30Î¼l/min. After desalting for 5 min, peptides were flushed onto a 75 μm x 15 cm C18 LC-Packings nano column (Dionex, Sunnyvale, CA) (3 micron, 100A) at a flow rate of 250 nl/min. Peptides were eluted with a 45 min gradient of 3-35% acetonitrile in 0.1% formic acid. Mass ranges for the MS survey scan and MS/MS were 300-2000 m/z and 50-2000 m/z, respectively. The scan time for MS and MS/MS were 1.0 s and 3.0 s, respectively. The top three multiply-charged ions with MS peak intensity greater than 15 counts/scan were chosen for MS/MS fragmentation with a precursor ion dynamic exclusion of 45 s.

Chromatin immunoprecipitation (ChIP) assays

Hep3B cells seeded at 2 x 10^6 (150 mm plates) 48 hr prior to use were exposed to normoxia or hypoxia for 4 or 8 hr, and then harvested for whole cell protein or RNA. *Epo* induction after hypoxia exposure was confirmed by real-time RT-PCR. Chromatin immunoprecipitation (ChIP) assays were carried out using the ChIP-IT™ Express Magnetic assay kit (Cat. # 17-295, Upstate Biotechnology, Inc.) according to the manufacturer's instructions. The antisera used were normal mouse IgG (1-2 μg/ml; Cat. No. 2027, Santa Cruz Biotechnology), normal rabbit IgG (1-2 mg/ml; Cat. No. NI01, EMD Chemicals, Inc., Gibbstown, NJ), anti-human HIF-2α antiserum (2 mg/ml; NB 100-132, Novus Biologicals), and rabbit anti-Sirt1 (0.5 mg/ml; Cat. No. 07131, Upstate Biotechnology, Inc.). After ChIP, the precipitated genomic DNA was analyzed by quantitative PCR with an Applied Biosystems ABI Prism 7000 thermocycler (Applied Biosystems; Foster City, CA) and Power SYBR Green Master Mix (Cat. No. 4367659, Applied Biosystems) using the following primers for a human *epo* enhancer amplicon: 5'-ACTCCTGGCAGCAGTGCAGC-3' (forward) and 5'-CCCTCTCCTTGATGACAATCTCAGC-3' (reverse). The captured genomic DNA was measured by normalizing with that of input material and compared between the normoxic and hypoxic treatment.

shRNA knockdown studies

A Sirt1 miR shRNA expression plasmid was constructed inserting oligonucleotides encoding a miR into an adenoviral expression plasmid

(pACU6loxP). The resultant expression vector generates Sirt1 shRNA recognizing mouse as well as human Sirt1 mRNA as shown previously (5'tcgacGATGAAGTTGACCTCCTCATTcaagagATGAGGAGGTCAACTTCATCtttt ttggaaat-3' and 5'ctagatttccaaaaaaGATGAAGTTGACCTCCT CATctcttgAATGAGGAGGTCAACTTCATCg-3') (Sun et al., 2007) Site-directed mutations (underlined residues) were introduced into the mouse/human Sirt1 expression vector (5'tcgacGATGTTCTTGTGGTCCAGTTTcaagagAACTGGACCACAAGAACATCtttt ttggaaat-3' and 5'-ctagatttccaaaaaaGATGTTCTTGTGGTCCAGTT ctcttgA AACTGGACCACA AGAACATCg-3') that result in production of an ineffective shRNA for Sirt1 knockdown. Additional shRNA expression plasmids that encode shRNA recognizing human Sirt1 mRNA only (Cat. No. RHS1764-9692271, Open Biosystems (Narala et al., 2008) or non-silencing control miR shRNA expression plasmid (Cat. No. RHS1707, Open Biosystems) were obtained. For cell culture studies, Hep3B cells in 6-well plates were transfected at 60% confluency in triplicate with 2.5 μg of shRNA or control expression plasmids using 7μl of Lipofectamine LTX (Cat. No. 15338-100, Invitrogen, Carlsbad, CA) in DMEM plus 10% fetal bovine serum following the manufacturer's instructions. Forty-eight hours following transfection, the transfected cells were subjected to hypoxia for 16 hours and then total RNA was generated (GenElute Total RNA Kit (Cat. No. RTN70-1KT, Sigma). Erythropoietin and cyclophilin gene expression were determined by real-time RTPCR analyses from cDNA generated by reverse

transcription. For mouse experiments, the miR shRNA regions were isolated and subcloned into adenoviral expression vectors. Transductions in mice were performed as described. I confirmed Sirt1 knockdown in Hep3B cells or in mouse livers using immunoblotting nuclear extract with antibodies recognizing Sirt1 (1:1000 Dilution; Cat. No. 07131, Upstate Biotechnology, Inc.) as well as the normalization controls α-tubulin (1:3000 cat. no. T 5168 Sigma), or β-actin (1:1000 cat. no. A 5316, Sigma).

Real-time RT-PCR analyses

The expression of endogenous epo and cyclophilin were determined by reverse transcription of total RNA followed by real time PCR analysis. Total RNA from Hep3B cells was extracted using GenElute™ mammalian Total RNA kit (Cat. No. RTN70-1KT, Sigma). 1μg of total RNA was reverse transcribed by extension of oligo-dT primers using M-MLV reverse transcriptase (Cat. No. 28025-013, Invitrogen) according to the manufacturer's protocol. Total RNA was isolated from 1/3 of the total mouse livers (right lobule) and extracted using the FastRNA Pro Green Kit (Cat. No. 6045, MP Biomedicals, Solon, OH). 1 μg of total RNA was then reverse transcribed using a M-MLV reverse transcriptase Kit (Cat. No.28025-013, Invitrogen). Mouse epo levels were normalized to a mouse loading control. Values were normalized to cyclophilin as previously described (Dioum et al., 2008). Real time quantitative PCR was performed on an Applied Biosystems ABI Prism 7000 thermocycler using Power SYBR Green Master Mix

following the manufacturer's protocol and one-tenth of total cDNA with the following pairs of human primers:

Epo (forward) 5'-GAGGCCGAGAATATCACGACGGG-3',

Epo (reverse) 5'-TGCCCGACCTCCATCCTCTTCCAG-3',

cyclophilin (forward) 5'-ATGTGGTTTTCGGCAAAGTTCTA-3',

cyclophilin (reverse) 5'-GGCTTGTCCCGGCTGTCT-3',

SIRT1 (forward) 5'-GCAGGTTGCGGGAATCCAA-3',

SIRT1 (reverse) 5'-GGCAAGATGCTGTTGCAAA-3'; or

mouse primers: Epo (forward) 5'-GAGGCAGAAAATGTCACGATG-3',

Epo (reverse) 5'-CTTCCACCTCCATTCTTTTCC-3',

cyclophilin (forward) 5'-ATGTGGTTTTCGGCAAAGTTCTA-3',

cyclophilin (reverse) 5'-GGCTTGTCCCGGCTGTCT-3'.

The results of triplicate experiments were expressed as $2^{(-(\text{EPO number of cycles- cyclophilin number of cycles}))}$.

Adult mouse adenoviral experiments

Recombinant adenoviruses encoding human P1P2N HIF-1α:HA, P1P2N HIF-2α:HA, vsv-g:Sirt1 (WT or MUT), or GFP under control of the CMV promoter were generated and titered prior to use. Adult CD1 wildtype female mice (Charles River Laboratories; Wilmington, MA) approximately 7-9 weeks old (25 g) were injected through tail veins with a total of 2×10^{11} PFU (particle forming units) adenovirus diluted in 1x PBS and normalized with equivalent amounts of CMV-

GFP control adenovirus. Hematocrits were measured at day 0 and day 7 following infection using heparinized microhematocrit capillary tubes. Mice were euthanized at day 7 following injection. Fresh liver samples were obtained and snapped-frozen in liquid nitrogen for RNA or protein preparations. Hepatic epo and cyclophilin mRNA levels were determined by real-time RTPCR analyses. Ectopic Sirt1, P1P2N HIF-1α:HA, or P1P2N HIF-2α:HA protein were confirmed by western blot analyses.

Adult mouse hypoxia experiments

Heterozygous Sirt1 knockout mice lacking exons 4 through 7 were housed under standard 12 hr:12 hr light:dark conditions, fed *ad lib* with standard chow, and established as mating pairs(Cheng, 2003 #103). For hypoxic experiments, age- and gender-matched Sirt1 heterozygous knockout or wildtype littermate mice were placed in a hypoxia chamber with flow by air supply and subjected to room air (RA, 21% oxygen) or short-term continuous hypoxia (STCH, 6% oxygen) treatment for 2 hours. Mice were then euthanized, the tissues collected, and snap-frozen in liquid nitrogen. Kidneys were used for extraction of total RNA. Real-time RTPCR determinations were performed as described above.

Embryonic mouse experiments

Heterozygous Sirt1 knockout (Cheng et al., 2003) lacking exons 4 through 7 or heterozygous *HIF-2α* knockout (Scortegagna, 2003 #303) (lacking the bHLH exon) mating pairs were established for timed pregnancy mating. Embryos were

isolated at embryonic day 12.5 (E12.5) or 14.5 (E14.5) and livers harvested for total RNA preparation. Real-time RTPCR analyses were performed to measure hepatic *epo* and *cyclophilin* mRNA levels as described.

Institutional compliance

The animal and adenoviral experiments reported in this study were approved by the UTSWMC IACUC or Biosafety Committees.

Statistical analyses

Comparisons between experimental groups were made by Student's t-Test for groups with equal sample size or by z-Test for comparisons between groups of unequal sample size. Two-tailed analyses were performed unless otherwise indicated. The statistical analyses were performed in Microsoft Excel (Microsoft Corporation, Redmond, WA).

Bibliography

Afsar, B., Sezer, S., Ozdemir, F.N., Celik, H., Elsurer, R., and Haberal, M. (2006). Malnutrition-inflammation score is a useful tool in peritoneal dialysis patients. Perit Dial Int *26*, 705-711.

Akuzawa, N., Kurabayashi, M., Ohyama, Y., Arai, M., and Nagai, R. (2000). Zinc finger transcription factor Egr-1 activates Flt-1 gene expression in THP-1 cells on induction for macrophage differentiation. Arterioscler Thromb Vasc Biol *20*, 377-384.

Al-Sarraj, A., Day, R.M., and Thiel, G. (2005). Specificity of transcriptional regulation by the zinc finger transcription factors Sp1, Sp3, and Egr-1. J Cell Biochem *94*, 153-167.

Anagnostou, A., Lee, E.S., Kessimian, N., Levinson, R., and Steiner, M. (1990). Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. Proc Natl Acad Sci U S A *87*, 5978-5982.

Anastasiou, D., and Krek, W. (2006). SIRT1: linking adaptive cellular responses to aging-associated changes in organismal physiology. Physiology (Bethesda) *21*, 404-410.

Aprelikova, O., Wood, M., Tackett, S., Chandramouli, G.V., and Barrett, J.C. (2006). Role of ETS transcription factors in the hypoxia-inducible factor-2 target gene selection. Cancer Res *66*, 5641-5647.

Arai, A., Kanda, E., and Miura, O. (2002). Rac is activated by erythropoietin or interleukin-3 and is involved in activation of the Erk signaling pathway. Oncogene *21*, 2641-2651.

Awogu, A.U., and Abohweyere, A.E. (2006). Alpha erythropoietin in the management of anaemia of prematurity: a report of three cases in Nigeria. Niger Postgrad Med J *13*, 361-365.

Bae, M.H., Jeong, C.H., Kim, S.H., Bae, M.K., Jeong, J.W., Ahn, M.Y., Bae, S.K., Kim, N.D., Kim, C.W., Kim, K.R.*, et al.* (2002). Regulation of Egr-1 by association with the proteasome component C8. Biochim Biophys Acta *1592*, 163-167.

Bae, S.H., Jeong, J.W., Park, J.A., Kim, S.H., Bae, M.K., Choi, S.J., and Kim, K.W. (2004). Sumoylation increases HIF-1alpha stability and its transcriptional activity. Biochem Biophys Res Commun *324*, 394-400.

Bae, S.K., Bae, M.H., Ahn, M.Y., Son, M.J., Lee, Y.M., Bae, M.K., Lee, O.H., Park, B.C., and Kim, K.W. (1999). Egr-1 mediates transcriptional activation of IGF-II gene in response to hypoxia. Cancer Res *59*, 5989-5994.

Bakker, W.J., Harris, I.S., and Mak, T.W. (2007). FOXO3a is activated in response to hypoxic stress and inhibits HIF1-induced apoptosis via regulation of CITED2. Mol Cell *28*, 941-953.

Beck, I., Ramirez, S., Weinmann, R., and Caro, J. (1991). Enhancer element at the 3'-flanking region controls transcriptional response to hypoxia in the human erythropoietin gene. J Biol Chem *266*, 15563-15566.

Beckmann, A.M., and Wilce, P.A. (1997). Egr transcription factors in the nervous system. Neurochem Int *31*, 477-510; discussion 517-476.

Berta, M., Brahimi-Horn, C., and Pouyssegur, J. (2004). [Regulation of the Hypoxia-Inducible Factor-1alpha (HIF-1alpha): a breath of fresh air in hypoxia research]. J Soc Biol *198*, 113-120.

Berta, M.A., Mazure, N., Hattab, M., Pouyssegur, J., and Brahimi-Horn, M.C. (2007). SUMOylation of hypoxia-inducible factor-1alpha reduces its transcriptional activity. Biochem Biophys Res Commun *360*, 646-652.

Biggs, W.H., 3rd, Meisenhelder, J., Hunter, T., Cavenee, W.K., and Arden, K.C. (1999). Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. Proc Natl Acad Sci U S A *96*, 7421-7426.

Bitterman, K.J., Anderson, R.M., Cohen, H.Y., Latorre-Esteves, M., and Sinclair, D.A. (2002). Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. J Biol Chem *277*, 45099-45107.

Blanchard, K.L., Acquaviva, A.M., Galson, D.L., and Bunn, H.F. (1992). Hypoxic induction of the human erythropoietin gene: cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. Mol Cell Biol *12*, 5373-5385.

Bouras, T., Fu, M., Sauve, A.A., Wang, F., Quong, A.A., Perkins, N.D., Hay, R.T., Gu, W., and Pestell, R.G. (2005). SIRT1 deacetylation and repression of p300 involves lysine residues 1020/1024 within the cell cycle regulatory domain 1. J Biol Chem *280*, 10264-10276.

Bozzini, C.E., Alippi, R.M., and Martinez, M.A. (1971). The inhibitory effect of relative polycythemia on erythropoietin production in response to hypoxic hypoxia. Acta Physiol Lat Am *21*, 293-296.

Bracken, C.P., Fedele, A.O., Linke, S., Balrak, W., Lisy, K., Whitelaw, M.L., and Peet, D.J. (2006). Cell-specific regulation of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha stabilization and transactivation in a graded oxygen environment. J Biol Chem *281*, 22575-22585.

Bracken, C.P., Whitelaw, M.L., and Peet, D.J. (2005). Activity of hypoxia-inducible factor 2alpha is regulated by association with the NF-kappaB essential modulator. J Biol Chem *280*, 14240-14251.

Brahimi-Horn, C., Mazure, N., and Pouyssegur, J. (2005). Signalling via the hypoxia-inducible factor-1alpha requires multiple posttranslational modifications. Cell Signal *17*, 1-9.

Brines, M., and Cerami, A. (2005). Emerging biological roles for erythropoietin in the nervous system. Nat Rev Neurosci *6*, 484-494.

Brines, M., and Cerami, A. (2006). Discovering erythropoietin's extra-hematopoietic functions: biology and clinical promise. Kidney Int *70*, 246-250.

Bruick, R.K., and McKnight, S.L. (2001). A Conserved Family of Prolyl-4-Hydroxylases That Modify HIF. Science *294*, 1337-1340.

Bruick, R.K., and McKnight, S.L. (2002). Transcription. Oxygen sensing gets a second wind. Science *295*, 807-808.

Brunet, A., Sweeney, L.B., Sturgill, J.F., Chua, K.F., Greer, P.L., Lin, Y., Tran, H., Ross, S.E., Mostoslavsky, R., Cohen, H.Y.*, et al.* (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science *303*, 2011-2015.

Campo, L., Turley, H., Han, C., Pezzella, F., Gatter, K.C., Harris, A.L., and Fox, S.B. (2005). Angiogenin is up-regulated in the nucleus and cytoplasm in human primary breast carcinoma and is associated with markers of hypoxia but not survival. J Pathol *205*, 585-591.

Carrero, P., Okamoto, K., Coumailleau, P., O'Brien, S., Tanaka, H., and Poellinger, L. (2000). Redox-regulated recruitment of the transcriptional coactivators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1alpha. Mol Cell Biol *20*, 402-415.

Carroll, V.A., and Ashcroft, M. (2006). Role of hypoxia-inducible factor (HIF)-1alpha versus HIF-2alpha in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway. Cancer Res *66*, 6264-6270.

Cerami, A., Brines, M., Ghezzi, P., Cerami, C., and Itri, L.M. (2002). Neuroprotective properties of epoetin alfa. Nephrol Dial Transplant *17 Suppl 1*, 8-12.

Chandel, N.S., McClintock, D.S., Feliciano, C.E., Wood, T.M., Melendez, J.A., Rodriguez, A.M., and Schumacker, P.T. (2000). Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. J Biol Chem *275*, 25130-25138.

Chapman, N.R., and Perkins, N.D. (2000). Inhibition of the RelA(p65) NF-kappaB subunit by Egr-1. J Biol Chem *275*, 4719-4725.

Chavrier, P., Vesque, C., Galliot, B., Vigneron, M., Dolle, P., Duboule, D., and Charnay, P. (1990). The segment-specific gene Krox-20 encodes a transcription factor with binding sites in the promoter region of the Hox-1.4 gene. EMBO J *9*, 1209-1218.

Chen, Z.Y., Warin, R., and Noguchi, C.T. (2006). Erythropoietin and normal brain development: receptor expression determines multi-tissue response. Neurodegener Dis *3*, 68-75.

Cheng, H.L., Mostoslavsky, R., Saito, S., Manis, J.P., Gu, Y., Patel, P., Bronson, R., Appella, E., Alt, F.W., and Chua, K.F. (2003). Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. Proc Natl Acad Sci U S A *100*, 10794-10799.

Cheng, J., Kang, X., Zhang, S., and Yeh, E.T. (2007). SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. Cell *131*, 584-595.

Chong, Z.Z., Kang, J.Q., and Maiese, K. (2003). Apaf-1, Bcl-xL, cytochrome c, and caspase-9 form the critical elements for cerebral vascular protection by erythropoietin. J Cereb Blood Flow Metab *23*, 320-330.

Chua, K.F., Mostoslavsky, R., Lombard, D.B., Pang, W.W., Saito, S., Franco, S., Kaushal, D., Cheng, H.L., Fischer, M.R., Stokes, N.*, et al.* (2005). Mammalian SIRT1 limits replicative life span in response to chronic genotoxic stress. Cell Metab *2*, 67-76.

Coleman, T.R., Westenfelder, C., Togel, F.E., Yang, Y., Hu, Z., Swenson, L., Leuvenink, H.G., Ploeg, R.J., d'Uscio, L.V., Katusic, Z.S.*, et al.* (2006). Cytoprotective doses of erythropoietin or carbamylated erythropoietin have markedly different procoagulant and vasoactive activities. Proc Natl Acad Sci U S A *103*, 5965-5970.

Conrad, P.W., Freeman, T.L., Beitner-Johnson, D., and Millhorn, D.E. (1999). EPAS1 trans-activation during hypoxia requires p42/p44 MAPK. J Biol Chem *274*, 33709-33713.

Crosby, S.D., Veile, R.A., Donis-Keller, H., Baraban, J.M., Bhat, R.V., Simburger, K.S., and Milbrandt, J. (1992). Neural-specific expression, genomic structure, and chromosomal localization of the gene encoding the zinc-finger transcription factor NGFI-C. Proc Natl Acad Sci U S A *89*, 4739-4743.

Daitoku, H., Hatta, M., Matsuzaki, H., Aratani, S., Ohshima, T., Miyagishi, M., Nakajima, T., and Fukamizu, A. (2004). Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. Proc Natl Acad Sci U S A *101*, 10042-10047.

Dame, C., Kirschner, K.M., Bartz, K.V., Wallach, T., Hussels, C.S., and Scholz, H. (2006). Wilms tumor suppressor, Wt1, is a transcriptional activator of the erythropoietin gene. Blood *107*, 4282-4290.

de Ruijter, A.J., van Gennip, A.H., Caron, H.N., Kemp, S., and van Kuilenburg, A.B. (2003). Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J *370*, 737-749.

Decker, E.L., Skerka, C., and Zipfel, P.F. (1998). The early growth response protein (EGR-1) regulates interleukin-2 transcription by synergistic interaction with the nuclear factor of activated T cells. J Biol Chem *273*, 26923-26930.

Diaz, Z., Assaraf, M.I., Miller, W.H., Jr., and Schipper, H.M. (2005). Astroglial cytoprotection by erythropoietin pre-conditioning: implications for ischemic and degenerative CNS disorders. J Neurochem *93*, 392-402.

Digicaylioglu, M., Bichet, S., Marti, H.H., Wenger, R.H., Rivas, L.A., Bauer, C., and Gassmann, M. (1995). Localization of specific erythropoietin binding sites in defined areas of the mouse brain. Proc Natl Acad Sci U S A *92*, 3717-3720.

Ding, K., Scortegagna, M., Seaman, R., Birch, D.G., and Garcia, J.A. (2005). Retinal disease in mice lacking hypoxia-inducible transcription factor-2alpha. Invest Ophthalmol Vis Sci *46*, 1010-1016.

Dioum, E.M., Clarke, S.L., Ding, K., Repa, J.J., and Garcia, J.A. (2008). HIF-2alpha-haploinsufficient mice have blunted retinal neovascularization due to impaired expression of a proangiogenic gene battery. Invest Ophthalmol Vis Sci *49*, 2714-2720.

Ebert, B.L., and Bunn, H.F. (1999). Regulation of the erythropoietin gene. Blood *94*, 1864-1877.

Eckardt, K.U., and Kurtz, A. (2005). Regulation of erythropoietin production. Eur J Clin Invest *35 Suppl 3*, 13-19.

Elvert, G., Kappel, A., Heidenreich, R., Englmeier, U., Lanz, S., Acker, T., Rauter, M., Plate, K., Sieweke, M., Breier, G.*, et al.* (2003). Cooperative interaction of hypoxia-inducible factor-2alpha (HIF-2alpha) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). J Biol Chem *278*, 7520-7530.

Ema, M., Hirota, K., Mimura, J., Abe, H., Yodoi, J., Sogawa, K., Poellinger, L., and Fujii-Kuriyama, Y. (1999). Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. EMBO J *18*, 1905-1914.

Ema, M., Taya, S., Yokotani, N., Sogawa, K., Matsuda, Y., and Fujii-Kuriyama, Y. (1997). A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. Proc Natl Acad Sci U S A *94*, 4273-4278.

Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A.*, et al.* (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell *107*, 43-54.

Erbel, P.J., Card, P.B., Karakuzu, O., Bruick, R.K., and Gardner, K.H. (2003). Structural basis for PAS domain heterodimerization in the basic helix--loop--helix-PAS transcription factor hypoxia-inducible factor. Proc Natl Acad Sci U S A *100*, 15504-15509.

Firth, J.D., Ebert, B.L., and Ratcliffe, P.J. (1995). Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and cAMP response elements. J Biol Chem *270*, 21021-21027.

Flamme, I., Frohlich, T., von Reutern, M., Kappel, A., Damert, A., and Risau, W. (1997). HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels. Mech Dev *63*, 51-60.

Fliser, D., Bahlmann, F.H., deGroot, K., and Haller, H. (2006). Mechanisms of disease: erythropoietin--an old hormone with a new mission? Nature clinical practice *3*, 563-572.

Freedman, S.J., Sun, Z.Y., Kung, A.L., France, D.S., Wagner, G., and Eck, M.J. (2003). Structural basis for negative regulation of hypoxia-inducible factor-1alpha by CITED2. Nat Struct Biol *10*, 504-512.

Fulco, M., Schiltz, R.L., Iezzi, S., King, M.T., Zhao, P., Kashiwaya, Y., Hoffman, E., Veech, R.L., and Sartorelli, V. (2003). Sir2 regulates skeletal muscle differentiation as a potential sensor of the redox state. Mol Cell *12*, 51-62.

Furuyama, T., Nakazawa, T., Nakano, I., and Mori, N. (2000). Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem J *349*, 629-634.

Garofalo, O., Cox, D.W., and Bachelard, H.S. (1988). Brain levels of NADH and NAD+ under hypoxic and hypoglycaemic conditions in vitro. J Neurochem *51*, 172-176.

Ghezzi, P., and Brines, M. (2004). Erythropoietin as an antiapoptotic, tissue-protective cytokine. Cell Death Differ *11 Suppl 1*, S37-44.

Ghosh, D. (2000). Object-oriented transcription factors database (ooTFD). Nucleic Acids Res *28*, 308-310.

Girdwood, D.W., Tatham, M.H., and Hay, R.T. (2004). SUMO and transcriptional regulation. Semin Cell Dev Biol *15*, 201-210.

Goyal, P., Weissmann, N., Grimminger, F., Hegel, C., Bader, L., Rose, F., Fink, L., Ghofrani, H.A., Schermuly, R.T., Schmidt, H.H.*, et al.* (2004). Upregulation of NAD(P)H oxidase 1 in hypoxia activates hypoxia-inducible factor 1 via increase in reactive oxygen species. Free Radic Biol Med *36*, 1279-1288.

Gradin, K., Takasaki, C., Fujii-Kuriyama, Y., and Sogawa, K. (2002). The transcriptional activation function of the HIF-like factor requires phosphorylation at a conserved threonine. J Biol Chem *277*, 23508-23514.

Grozinger, C.M., Chao, E.D., Blackwell, H.E., Moazed, D., and Schreiber, S.L. (2001). Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. J Biol Chem *276*, 38837-38843.

Gu, Y.Z., Moran, S.M., Hogenesch, J.B., Wartman, L., and Bradfield, C.A. (1998). Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. Gene Expr *7*, 205-213.

Hackbarth, J.S., Lee, S.H., Meng, X.W., Vroman, B.T., Kaufmann, S.H., and Karnitz, L.M. (2004). S-peptide epitope tagging for protein purification, expression monitoring, and localization in mammalian cells. Biotechniques *37*, 835-839.

Heiniger, C.D., Kostadinova, R.M., Rochat, M.K., Serra, A., Ferrari, P., Dick, B., Frey, B.M., and Frey, F.J. (2003). Hypoxia causes down-regulation of 11 beta-

hydroxysteroid dehydrogenase type 2 by induction of Egr-1. FASEB J *17*, 917-919.

Hisahara, S., Chiba, S., Matsumoto, H., and Horio, Y. (2005). Transcriptional regulation of neuronal genes and its effect on neural functions: NAD-dependent histone deacetylase SIRT1 (Sir2alpha). J Pharmacol Sci *98*, 200-204.

Hofer, T., Desbaillets, I., Hopfl, G., Gassmann, M., and Wenger, R.H. (2001). Dissecting hypoxia-dependent and hypoxia-independent steps in the HIF-1alpha activation cascade: implications for HIF-1alpha gene therapy. FASEB J *15*, 2715-2717.

Hogenesch, J.B., Chan, W.K., Jackiw, V.H., Brown, R.C., Gu, Y.Z., Pray-Grant, M., Perdew, G.H., and Bradfield, C.A. (1997). Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. J Biol Chem *272*, 8581-8593.

Holmberg, C.I., Tran, S.E., Eriksson, J.E., and Sistonen, L. (2002). Multisite phosphorylation provides sophisticated regulation of transcription factors. Trends Biochem Sci *27*, 619-627.

Houston, P., Campbell, C.J., Svaren, J., Milbrandt, J., and Braddock, M. (2001). The transcriptional corepressor NAB2 blocks Egr-1-mediated growth factor activation and angiogenesis. Biochem Biophys Res Commun *283*, 480-486.

Howitz, K.T., Bitterman, K.J., Cohen, H.Y., Lamming, D.W., Lavu, S., Wood, J.G., Zipkin, R.E., Chung, P., Kisielewski, A., Zhang, L.L.*, et al.* (2003). Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature *425*, 191-196.

Hu, C.J., Sataur, A., Wang, L., Chen, H., and Simon, M.C. (2007). The N-terminal transactivation domain confers target gene specificity of hypoxia-inducible factors HIF-1alpha and HIF-2alpha. Mol Biol Cell *18*, 4528-4542.

Huang, W., Zhao, S., Ammanamanchi, S., Brattain, M., Venkatasubbarao, K., and Freeman, J.W. (2005). Trichostatin A induces transforming growth factor beta type II receptor promoter activity and acetylation of Sp1 by recruitment of PCAF/p300 to a Sp1.NF-Y complex. J Biol Chem *280*, 10047-10054.

Hur, E., Chang, K.Y., Lee, E., Lee, S.K., and Park, H. (2001). Mitogen-activated protein kinase kinase inhibitor PD98059 blocks the trans-activation but not the stabilization or DNA binding ability of hypoxia-inducible factor-1alpha. Mol Pharmacol *59*, 1216-1224.

Hyun, D.H., Emerson, S.S., Jo, D.G., Mattson, M.P., and de Cabo, R. (2006). Calorie restriction up-regulates the plasma membrane redox system in brain cells

and suppresses oxidative stress during aging. Proc Natl Acad Sci U S A *103*, 19908-19912.

Imai, S., Johnson, F.B., Marciniak, R.A., McVey, M., Park, P.U., and Guarente, L. (2000). Sir2: an NAD-dependent histone deacetylase that connects chromatin silencing, metabolism, and aging. Cold Spring Harb Symp Quant Biol *65*, 297-302.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J*., et al.* (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science *292*, 468-472.

Jain, R.K., Safabakhsh, N., Sckell, A., Chen, Y., Jiang, P., Benjamin, L., Yuan, F., and Keshet, E. (1998). Endothelial cell death, angiogenesis, and microvascular function after castration in an androgen-dependent tumor: role of vascular endothelial growth factor. Proc Natl Acad Sci U S A *95*, 10820-10825.

Jeong, J.W., Bae, M.K., Ahn, M.Y., Kim, S.H., Sohn, T.K., Bae, M.H., Yoo, M.A., Song, E.J., Lee, K.J., and Kim, K.W. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell *111*, 709-720.

Jiang, B.H., Zheng, J.Z., Leung, S.W., Roe, R., and Semenza, G.L. (1997). Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension. J Biol Chem *272*, 19253-19260.

Jurgensen, J.S., Rosenberger, C., Wiesener, M.S., Warnecke, C., Horstrup, J.H., Grafe, M., Philipp, S., Griethe, W., Maxwell, P.H., Frei, U*., et al.* (2004). Persistent induction of HIF-1alpha and -2alpha in cardiomyocytes and stromal cells of ischemic myocardium. FASEB J *18*, 1415-1417.

Kang, J., Holland, M., Jones, H., and Kaysen, G.A. (1999). Coordinate augmentation in expression of genes encoding transcription factors and liver secretory proteins in hypo-oncotic states. Kidney Int *56*, 452-460.

Kashii, Y., Uchida, M., Kirito, K., Tanaka, M., Nishijima, K., Toshima, M., Ando, T., Koizumi, K., Endoh, T., Sawada, K*., et al.* (2000). A member of Forkhead family transcription factor, FKHRL1, is one of the downstream molecules of phosphatidylinositol 3-kinase-Akt activation pathway in erythropoietin signal transduction. Blood *96*, 941-949.

Kim, S.H., Jeong, J.W., Park, J.A., Lee, J.W., Seo, J.H., Jung, B.K., Bae, M.K., and Kim, K.W. (2007). Regulation of the HIF-1alpha stability by histone deacetylases. Oncol Rep *17*, 647-651.

Kim, S.H., Park, J.A., Kim, J.H., Lee, J.W., Seo, J.H., Jung, B.K., Chun, K.H., Jeong, J.W., Bae, M.K., and Kim, K.W. (2006). Characterization of ARD1 variants in mammalian cells. Biochem Biophys Res Commun *340*, 422-427.

Klimova, T., and Chandel, N.S. (2008). Mitochondrial complex III regulates hypoxic activation of HIF. Cell Death Differ *15*, 660-666.

Kobayashi, Y., Furukawa-Hibi, Y., Chen, C., Horio, Y., Isobe, K., Ikeda, K., and Motoyama, N. (2005). SIRT1 is critical regulator of FOXO-mediated transcription in response to oxidative stress. Int J Mol Med *16*, 237-243.

Koivunen, P., Hirsila, M., Gunzler, V., Kivirikko, K.I., and Myllyharju, J. (2004). Catalytic properties of the asparaginyl hydroxylase (FIH) in the oxygen sensing pathway are distinct from those of its prolyl 4-hydroxylases. J Biol Chem *279*, 9899-9904.

Kojima, I., Tanaka, T., Inagi, R., Kato, H., Yamashita, T., Sakiyama, A., Ohneda, O., Takeda, N., Sata, M., Miyata, T.*, et al.* (2007). Protective role of hypoxia-inducible factor-2alpha against ischemic damage and oxidative stress in the kidney. J Am Soc Nephrol *18*, 1218-1226.

Konstantinopoulos, P.A., Karamouzis, M.V., and Papavassiliou, A.G. (2007). Selective modulation of the erythropoietic and tissue-protective effects of erythropoietin: time to reach the full therapeutic potential of erythropoietin. Biochim Biophys Acta *1776*, 1-9.

Koury, M.J., and Bondurant, M.C. (1988). Maintenance by erythropoietin of viability and maturation of murine erythroid precursor cells. J Cell Physiol *137*, 65-74.

Koury, M.J., Bondurant, M.C., Graber, S.E., and Sawyer, S.T. (1988). Erythropoietin messenger RNA levels in developing mice and transfer of 125I-erythropoietin by the placenta. J Clin Invest *82*, 154-159.

Koury, S.T., Bondurant, M.C., Koury, M.J., and Semenza, G.L. (1991). Localization of cells producing erythropoietin in murine liver by in situ hybridization. Blood *77*, 2497-2503.

Krieg, M., Haas, R., Brauch, H., Acker, T., Flamme, I., and Plate, K.H. (2000). Up-regulation of hypoxia-inducible factors HIF-1alpha and HIF-2alpha under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. Oncogene *19*, 5435-5443.

Kumbrink, J., Gerlinger, M., and Johnson, J.P. (2005). Egr-1 induces the expression of its corepressor nab2 by activation of the nab2 promoter thereby establishing a negative feedback loop. J Biol Chem *280*, 42785-42793.

Lacombe, C., and Mayeux, P. (1999). Erythropoietin (Epo) receptor and Epo mimetics. Adv Nephrol Necker Hosp *29*, 177-189.

Lando, D., Peet, D.J., Gorman, J.J., Whelan, D.A., Whitelaw, M.L., and Bruick, R.K. (2002). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev *16*, 1466-1471.

Lando, D., Pongratz, I., Poellinger, L., and Whitelaw, M.L. (2000). A redox mechanism controls differential DNA binding activities of hypoxia-inducible factor (HIF) 1alpha and the HIF-like factor. Journal of Biological Chemistry *275*, 4618-4627.

Langley, E., Pearson, M., Faretta, M., Bauer, U.M., Frye, R.A., Minucci, S., Pelicci, P.G., and Kouzarides, T. (2002). Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. Embo J *21*, 2383-2396.

Lau, K.W., Tian, Y.M., Raval, R.R., Ratcliffe, P.J., and Pugh, C.W. (2007). Target gene selectivity of hypoxia-inducible factor-alpha in renal cancer cells is conveyed by post-DNA-binding mechanisms. Br J Cancer *96*, 1284-1292.

Lee, M., Bikram, M., Oh, S., Bull, D.A., and Kim, S.W. (2004). Sp1-dependent regulation of the RTP801 promoter and its application to hypoxia-inducible VEGF plasmid for ischemic disease. Pharm Res *21*, 736-741.

Lee, S.L., Sadovsky, Y., Swirnoff, A.H., Polish, J.A., Goda, P., Gavrilina, G., and Milbrandt, J. (1996). Luteinizing hormone deficiency and female infertility in mice lacking the transcription factor NGFI-A (Egr-1). Science *273*, 1219-1221.

Lee, Y.S., Jang, H.S., Kim, J.M., Lee, J.S., Lee, J.Y., Li Kim, K., Shin, I.S., Suh, W., Choi, J.H., Jeon, E.S.*, et al.* (2005). Adenoviral-mediated delivery of early growth response factor-1 gene increases tissue perfusion in a murine model of hindlimb ischemia. Mol Ther *12*, 328-336.

Lemaire, P., Revelant, O., Bravo, R., and Charnay, P. (1988). Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. Proc Natl Acad Sci U S A *85*, 4691-4695.

Lim, C.P., Jain, N., and Cao, X. (1998). Stress-induced immediate-early gene, egr-1, involves activation of p38/JNK1. Oncogene *16*, 2915-2926.

Lim, R.W., Varnum, B.C., and Herschman, H.R. (1987). Cloning of tetradecanoyl phorbol ester-induced 'primary response' sequences and their expression in density-arrested Swiss 3T3 cells and a TPA non-proliferative variant. Oncogene *1*, 263-270.

Liu, J., Grogan, L., Nau, M.M., Allegra, C.J., Chu, E., and Wright, J.J. (2001). Physical interaction between p53 and primary response gene Egr-1. Int J Oncol *18*, 863-870.

Liu, J.W., Chandra, D., Rudd, M.D., Butler, A.P., Pallotta, V., Brown, D., Coffer, P.J., and Tang, D.G. (2005). Induction of prosurvival molecules by apoptotic stimuli: involvement of FOXO3a and ROS. Oncogene *24*, 2020-2031.

Lo, L.W., Cheng, J.J., Chiu, J.J., Wung, B.S., Liu, Y.C., and Wang, D.L. (2001). Endothelial exposure to hypoxia induces Egr-1 expression involving PKCalpha-mediated Ras/Raf-1/ERK1/2 pathway. J Cell Physiol *188*, 304-312.

Loots, G.G., and Ovcharenko, I. (2005). Dcode.org anthology of comparative genomic tools. Nucleic Acids Res *33*, W56-64.

Lui, W.Y., Sze, K.L., and Lee, W.M. (2006). Nectin-2 expression in testicular cells is controlled via the functional cooperation between transcription factors of the Sp1, CREB, and AP-1 families. J Cell Physiol *207*, 144-157.

Luo, J., Nikolaev, A.Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001). Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell *107*, 137-148.

Mager, G.M., Ward, R.M., Srinivasan, R., Jang, S.W., Wrabetz, L., and Svaren, J. (2008). Active gene repression by the Egr2.NAB complex during peripheral nerve myelination. J Biol Chem *283*, 18187-18197.

Mahon, P.C., Hirota, K., and Semenza, G.L. (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev *15*, 2675-2686.

Makino, Y., Kanopka, A., Wilson, W.J., Tanaka, H., and Poellinger, L. (2002). Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3alpha locus. J Biol Chem *277*, 32405-32408.

Makita, T., Hernandez-Hoyos, G., Chen, T.H., Wu, H., Rothenberg, E.V., and Sucov, H.M. (2001). A developmental transition in definitive erythropoiesis: erythropoietin expression is sequentially regulated by retinoic acid receptors and HNF4. Genes Dev *15*, 889-901.

Marti, H.H., Bernaudin, M., Petit, E., and Bauer, C. (2000). Neuroprotection and Angiogenesis: Dual Role of Erythropoietin in Brain Ischemia. News Physiol Sci *15*, 225-229.

Marti, H.H., Wenger, R.H., Rivas, L.A., Straumann, U., Digicaylioglu, M., Henn, V., Yonekawa, Y., Bauer, C., and Gassmann, M. (1996). Erythropoietin gene expression in human, monkey and murine brain. Eur J Neurosci *8*, 666-676.

Masson, N., and Ratcliffe, P.J. (2003). HIF prolyl and asparaginyl hydroxylases in the biological response to intracellular O(2) levels. J Cell Sci *116*, 3041-3049.

Masson, N., Willam, C., Maxwell, P.H., Pugh, C.W., and Ratcliffe, P.J. (2001). Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. EMBO J *20*, 5197-5206.

Masuda, S., Okano, M., Yamagishi, K., Nagao, M., Ueda, M., and Sasaki, R. (1994). A novel site of erythropoietin production. Oxygen-dependent production in cultured rat astrocytes. J Biol Chem *269*, 19488-19493.

Maynard, M.A., Qi, H., Chung, J., Lee, E.H., Kondo, Y., Hara, S., Conaway, R.C., Conaway, J.W., and Ohh, M. (2003). Multiple splice variants of the human HIF-3 alpha locus are targets of the von Hippel-Lindau E3 ubiquitin ligase complex. J Biol Chem *278*, 11032-11040.

Michishita, E., Park, J.Y., Burneskis, J.M., Barrett, J.C., and Horikawa, I. (2005). Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Mol Biol Cell *16*, 4623-4635.

Milano, M., and Schneider, M. (2007). EPO in cancer anemia: benefits and potential risks. Crit Rev Oncol Hematol *62*, 119-125.

Milbrandt, J. (1987). A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science *238*, 797-799.

Minet, E., Arnould, T., Michel, G., Roland, I., Mottet, D., Raes, M., Remacle, J., and Michiels, C. (2000). ERK activation upon hypoxia: involvement in HIF-1 activation. FEBS Lett *468*, 53-58.

Mole, D.R., Maxwell, P.H., Pugh, C.W., and Ratcliffe, P.J. (2001). Regulation of HIF by the von Hippel-Lindau tumour suppressor: implications for cellular oxygen sensing. IUBMB Life *52*, 43-47.

Morris, B.J. (2005). A forkhead in the road to longevity: the molecular basis of lifespan becomes clearer. J Hypertens *23*, 1285-1309.

Mouillet, J.F., Sonnenberg-Hirche, C., Yan, X., and Sadovsky, Y. (2004). p300 regulates the synergy of steroidogenic factor-1 and early growth response-1 in activating luteinizing hormone-beta subunit gene. J Biol Chem *279*, 7832-7839.

Murray-Rust, T.A., Oldham, N.J., Hewitson, K.S., and Schofield, C.J. (2006). Purified recombinant hARD1 does not catalyse acetylation of Lys532 of HIF-1alpha fragments in vitro. FEBS Lett *580*, 1911-1918.

Narala, S.R., Allsopp, R.C., Wells, T.B., Zhang, G., Prasad, P., Coussens, M.J., Rossi, D.J., Weissman, I.L., and Vaziri, H. (2008). SIRT1 Acts as a Nutrient-sensitive Growth Suppressor and Its Loss Is Associated with Increased AMPK and Telomerase Activity. Mol Biol Cell *19*, 1210-1219.

Noguchi, C.T., Asavaritikrai, P., Teng, R., and Jia, Y. (2007). Role of erythropoietin in the brain. Critical reviews in oncology/hematology *64*, 159-171.

North, B.J., and Verdin, E. (2004). Sirtuins: Sir2-related NAD-dependent protein deacetylases. Genome Biol *5*, 224.

Novak, E.M., Metzger, M., Chammas, R., da Costa, M., Dantas, K., Manabe, C., Pires, J., de Oliveira, A.C., and Bydlowski, S.P. (2003). Downregulation of TNF-alpha and VEGF expression by Sp1 decoy oligodeoxynucleotides in mouse melanoma tumor. Gene Ther *10*, 1992-1997.

O'Donovan, K.J., Tourtellotte, W.G., Millbrandt, J., and Baraban, J.M. (1999). The EGR family of transcription-regulatory factors: progress at the interface of molecular and systems neuroscience. Trends Neurosci *22*, 167-173.

O'Rourke, J.F., Dachs, G.U., Gleadle, J.M., Maxwell, P.H., Pugh, C.W., Stratford, I.J., Wood, S.M., and Ratcliffe, P.J. (1997). Hypoxia response elements. Oncol Res *9*, 327-332.

O'Rourke, J.F., Tian, Y.M., Ratcliffe, P.J., and Pugh, C.W. (1999). Oxygen-regulated and transactivating domains in endothelial PAS protein 1: comparison with hypoxia-inducible factor-1alpha. Journal of Biological Chemistry *274*, 2060-2071.

Oktay, Y., Dioum, E., Matsuzaki, S., Ding, K., Yan, L.J., Haller, R.G., Szweda, L.I., and Garcia, J.A. (2007). Hypoxia-inducible factor 2alpha regulates expression of the mitochondrial aconitase chaperone protein frataxin. J Biol Chem *282*, 11750-11756.

Ouellette, A.J., Malt, R.A., Sukhatme, V.P., and Bonventre, J.V. (1990). Expression of two "immediate early" genes, Egr-1 and c-fos, in response to renal ischemia and during compensatory renal hypertrophy in mice. J Clin Invest *85*, 766-771.

Paltoglou, S., and Roberts, B.J. (2007). HIF-1alpha and EPAS ubiquitination mediated by the VHL tumour suppressor involves flexibility in the ubiquitination mechanism, similar to other RING E3 ligases. Oncogene *26*, 604-609.

Park, S.K., Dadak, A.M., Haase, V.H., Fontana, L., Giaccia, A.J., and Johnson, R.S. (2003). Hypoxia-induced gene expression occurs solely through the action of hypoxia-inducible factor 1alpha (HIF-1alpha): role of cytoplasmic trapping of HIF-2alpha. Mol Cell Biol *23*, 4959-4971.

Porntadavity, S., Xu, Y., Kiningham, K., Rangnekar, V.M., Prachayasittikul, V., and St Clair, D.K. (2001). TPA-activated transcription of the human MnSOD gene: role of transcription factors Sp-1 and Egr-1. DNA Cell Biol *20*, 473-481.

Pugh, C.W., O'Rourke, J.F., Nagao, M., Gleadle, J.M., and Ratcliffe, P.J. (1997). Activation of hypoxia-inducible factor-1; definition of regulatory domains within the alpha subunit. J Biol Chem *272*, 11205-11214.

Pugh, C.W., Tan, C.C., Jones, R.W., and Ratcliffe, P.J. (1991). Functional analysis of an oxygen-regulated transcriptional enhancer lying 3' to the mouse erythropoietin gene. Proc Natl Acad Sci U S A *88*, 10553-10557.

Rabilloud, T., Heller, M., Rigobello, M.P., Bindoli, A., Aebersold, R., and Lunardi, J. (2001). The mitochondrial antioxidant defence system and its response to oxidative stress. Proteomics *1*, 1105-1110.

Ralph, G.S., Parham, S., Lee, S.R., Beard, G.L., Craigon, M.H., Ward, N., White, J.R., Barber, R.D., Rayner, W., Kingsman, S.M.*, et al.* (2004). Identification of potential stroke targets by lentiviral vector mediated overexpression of HIF-1 alpha and HIF-2 alpha in a primary neuronal model of hypoxia. J Cereb Blood Flow Metab *24*, 245-258.

Rankin, E.B., Biju, M.P., Liu, Q., Unger, T.L., Rha, J., Johnson, R.S., Simon, M.C., Keith, B., and Haase, V.H. (2007). Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. J Clin Invest *117*, 1068-1077.

Richard, D.E., Berra, E., Gothie, E., Roux, D., and Pouyssegur, J. (1999). p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. J Biol Chem *274*, 32631-32637.

Rius, J., Guma, M., Schachtrup, C., Akassoglou, K., Zinkernagel, A.S., Nizet, V., Johnson, R.S., Haddad, G.G., and Karin, M. (2008). NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. Nature *453*, 807-811.

Rodgers, J.T., Lerin, C., Haas, W., Gygi, S.P., Spiegelman, B.M., and Puigserver, P. (2005). Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. Nature *434*, 113-118.

Russo, M.W., Sevetson, B.R., and Milbrandt, J. (1995). Identification of NAB1, a repressor of NGFI-A- and Krox20-mediated transcription. Proc Natl Acad Sci U S A *92*, 6873-6877.

Salceda, S., and Caro, J. (1997). Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem *272*, 22642-22647.

Sanchez-Elsner, T., Ramirez, J.R., Sanz-Rodriguez, F., Varela, E., Bernabeu, C., and Botella, L.M. (2004). A cross-talk between hypoxia and TGF-beta orchestrates erythropoietin gene regulation through SP1 and Smads. J Mol Biol *336*, 9-24.

Scortegagna, M., Ding, K., Oktay, Y., Gaur, A., Thurmond, F., Yan, L.J., Marck, B.T., Matsumoto, A.M., Shelton, J.M., Richardson, J.A.*, et al.* (2003a). Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1-/- mice. Nat Genet *35*, 331-340.

Scortegagna, M., Ding, K., Zhang, Q., Oktay, Y., Bennett, M.J., Bennett, M., Shelton, J.M., Richardson, J.A., Moe, O., and Garcia, J.A. (2005). HIF-2alpha regulates murine hematopoietic development in an erythropoietin-dependent manner. Blood *105*, 3133-3140.

Scortegagna, M., Morris, M.A., Oktay, Y., Bennett, M., and Garcia, J.A. (2003b). The HIF family member EPAS1/HIF-2alpha is required for normal hematopoiesis in mice. Blood *102*, 1634-1640.

Semenza, G.L. (1994). Regulation of erythropoietin production. New insights into molecular mechanisms of oxygen homeostasis. Hematol Oncol Clin North Am *8*, 863-884.

Semenza, G.L. (1999). Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol *15*, 551-578.

Semenza, G.L. (2000). Expression of hypoxia-inducible factor 1: mechanisms and consequences. Biochemical Pharmacology *59*, 47-53.

Semenza, G.L. (2004). O2-regulated gene expression: transcriptional control of cardiorespiratory physiology by HIF-1. J Appl Physiol *96*, 1173-1177; discussion 1170-1172.

Semenza, G.L., Dureza, R.C., Traystman, M.D., Gearhart, J.D., and Antonarakis, S.E. (1990). Human erythropoietin gene expression in transgenic mice: multiple transcription initiation sites and cis-acting regulatory elements. Mol Cell Biol *10*, 930-938.

Semenza, G.L., Nejfelt, M.K., Chi, S.M., and Antonarakis, S.E. (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. Proc Natl Acad Sci U S A *88*, 5680-5684.

Sharp, F.R., Ran, R., Lu, A., Tang, Y., Strauss, K.I., Glass, T., Ardizzone, T., and Bernaudin, M. (2004). Hypoxic preconditioning protects against ischemic brain injury. NeuroRx *1*, 26-35.

Silva, P.N., Soares, J.A., Brasil, B.S., Nogueira, S.V., Andrade, A.A., de Magalhaes, J.C., Bonjardim, M.B., Ferreira, P.C., Kroon, E.G., Bruna-Romero, O.*, et al.* (2006). Differential role played by the MEK/ERK/EGR-1 pathway in orthopoxviruses vaccinia and cowpox biology. Biochem J *398*, 83-95.

Sodhi, A., Montaner, S., Patel, V., Zohar, M., Bais, C., Mesri, E.A., and Gutkind, J.S. (2000). The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia-inducible factor 1alpha. Cancer Res *60*, 4873-4880.

Sperandio, S., Fortin, J., Sasik, R., Robitaille, L., Corbeil, J., and de Belle, I. (2008). The transcription factor Egr1 regulates the HIF-1alpha gene during hypoxia. Mol Carcinog.

Srinivasan, R., Jang, S.W., Ward, R.M., Sachdev, S., Ezashi, T., and Svaren, J. (2007). Differential regulation of NAB corepressor genes in Schwann cells. BMC Mol Biol *8*, 117.

Srinivasan, R., Mager, G.M., Ward, R.M., Mayer, J., and Svaren, J. (2006). NAB2 represses transcription by interacting with the CHD4 subunit of the nucleosome remodeling and deacetylase (NuRD) complex. J Biol Chem *281*, 15129-15137.

Stiehl, D.P., Fath, D.M., Liang, D., Jiang, Y., and Sang, N. (2007). Histone deacetylase inhibitors synergize p300 autoacetylation that regulates its transactivation activity and complex formation. Cancer Res *67*, 2256-2264.

Sun, C., Zhang, F., Ge, X., Yan, T., Chen, X., Shi, X., and Zhai, Q. (2007). SIRT1 improves insulin sensitivity under insulin-resistant conditions by repressing PTP1B. Cell Metab *6*, 307-319.

Suzuki, H., Tomida, A., and Tsuruo, T. (2001). Dephosphorylated hypoxia-inducible factor 1alpha as a mediator of p53-dependent apoptosis during hypoxia. Oncogene *20*, 5779-5788.

Svaren, J., Sevetson, B.R., Apel, E.D., Zimonjic, D.B., Popescu, N.C., and Milbrandt, J. (1996). NAB2, a corepressor of NGFI-A (Egr-1) and Krox20, is induced by proliferative and differentiative stimuli. Mol Cell Biol *16*, 3545-3553.

Swirnoff, A.H., Apel, E.D., Svaren, J., Sevetson, B.R., Zimonjic, D.B., Popescu, N.C., and Milbrandt, J. (1998). Nab1, a corepressor of NGFI-A (Egr-1), contains an active transcriptional repression domain. Mol Cell Biol *18*, 512-524.

Swirnoff, A.H., and Milbrandt, J. (1995). DNA-binding specificity of NGFI-A and related zinc finger transcription factors. Mol Cell Biol *15*, 2275-2287.

Taylor, B.L., and Zhulin, I.B. (1999). PAS domains: internal sensors of oxygen, redox potential, and light. Microbiol Mol Biol Rev *63*, 479-506.

Tian, H., McKnight, S.L., and Russell, D.W. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev *11*, 72-82.

Tissenbaum, H.A., and Guarente, L. (2001). Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. Nature *410*, 227-230.

Topilko, P., Schneider-Maunoury, S., Levi, G., Baron-Van Evercooren, A., Chennoufi, A.B., Seitanidou, T., Babinet, C., and Charnay, P. (1994). Krox-20 controls myelination in the peripheral nervous system. Nature *371*, 796-799.

Turrens, J.F. (2003). Mitochondrial formation of reactive oxygen species. J Physiol *552*, 335-344.

van den Beucken, T., Magagnin, M.G., Savelkouls, K., Lambin, P., Koritzinsky, M., and Wouters, B.G. (2007). Regulation of Cited2 expression provides a functional link between translational and transcriptional responses during hypoxia. Radiother Oncol *83*, 346-352.

van der Horst, A., Tertoolen, L.G., de Vries-Smits, L.M., Frye, R.A., Medema, R.H., and Burgering, B.M. (2004). FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). J Biol Chem *279*, 28873-28879.

Vannucci, R.C., and Brucklacher, R.M. (1994). Cerebral mitochondrial redox states during metabolic stress in the immature rat. Brain Res *653*, 141-147.

Vaziri, H., Dessain, S.K., Ng Eaton, E., Imai, S.I., Frye, R.A., Pandita, T.K., Guarente, L., and Weinberg, R.A. (2001). hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell *107*, 149-159.

Venken, K., Di Maria, E., Bellone, E., Balestra, P., Cassandrini, D., Mandich, P., De Jonghe, P., Timmerman, V., and Svaren, J. (2002). Search for mutations in the EGR2 corepressor proteins, NAB1 and NAB2, in human peripheral neuropathies. Neurogenetics *4*, 37-41.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A *92*, 5510-5514.

Wang, G.L., and Semenza, G.L. (1993a). Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. Blood *82*, 3610-3615.

Wang, G.L., and Semenza, G.L. (1993b). General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci U S A *90*, 4304-4308.

Wang, G.L., and Semenza, G.L. (1995). Purification and characterization of hypoxia-inducible factor 1. J Biol Chem *270*, 1230-1237.

Wang, V., Davis, D.A., Haque, M., Huang, L.E., and Yarchoan, R. (2005). Differential gene up-regulation by hypoxia-inducible factor-1alpha and hypoxia-inducible factor-2alpha in HEK293T cells. Cancer Res *65*, 3299-3306.

Warnecke, C., Zaborowska, Z., Kurreck, J., Erdmann, V.A., Frei, U., Wiesener, M., and Eckardt, K.U. (2004). Differentiating the functional role of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2alpha target gene in Hep3B and Kelly cells. FASEB J *18*, 1462-1464.

Wenger, R.H., Stiehl, D.P., and Camenisch, G. (2005). Integration of oxygen signaling at the consensus HRE. Sci STKE *2005*, re12.

Westenfelder, C., Biddle, D.L., and Baranowski, R.L. (1999). Human, rat, and mouse kidney cells express functional erythropoietin receptors. Kidney Int *55*, 808-820.

Wiesener, M.S., and Maxwell, P.H. (2003). HIF and oxygen sensing; as important to life as the air we breathe? Ann Med *35*, 183-190.

Wood, S.M., Wiesener, M.S., Yeates, K.M., Okada, N., Pugh, C.W., Maxwell, P.H., and Ratcliffe, P.J. (1998). Selection and analysis of a mutant cell line defective in the hypoxia-inducible factor-1 alpha-subunit (HIF-1alpha). Characterization of hif-1alpha-dependent and -independent hypoxia-inducible gene expression. J Biol Chem *273*, 8360-8368.

Yan, S.F., Lu, J., Zou, Y.S., Kisiel, W., Mackman, N., Leitges, M., Steinberg, S., Pinsky, D., and Stern, D. (2000). Protein kinase C-beta and oxygen deprivation. A novel Egr-1-dependent pathway for fibrin deposition in hypoxemic vasculature. J Biol Chem *275*, 11921-11928.

Yang, F., Agulian, T., Sudati, J.E., Rhoads, D.B., and Levitsky, L.L. (2004). Developmental regulation of galactokinase in suckling mouse liver by the Egr-1 transcription factor. Pediatr Res *55*, 822-829.

Yasinska, I.M., and Sumbayev, V.V. (2003). S-nitrosation of Cys-800 of HIF-1alpha protein activates its interaction with p300 and stimulates its transcriptional activity. FEBS Lett *549*, 105-109.

Yin, D., Kawabata, H., Tcherniamtchouk, O., Huynh, T., Black, K.L., and Koeffler, H.P. (2007). Glioblastoma multiforme cells: expression of erythropoietin receptor and response to erythropoietin. Int J Oncol *31*, 1193-1198.

Yoo, Y.G., Kong, G., and Lee, M.O. (2006). Metastasis-associated protein 1 enhances stability of hypoxia-inducible factor-1alpha protein by recruiting histone deacetylase 1. EMBO J *25*, 1231-1241.

Yoon, D., Agarwal, N., and Prchal, J.T. (2008). Does erythropoietin promote tumor growth? Clin Cancer Res *14*, 1920; author reply 1920-1921.

Yu, A.Y., Shimoda, L.A., Iyer, N.V., Huso, D.L., Sun, X., McWilliams, R., Beaty, T., Sham, J.S., Wiener, C.M., Sylvester, J.T.*, et al.* (1999). Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1alpha. J Clin Invest *103*, 691-696.

Yu, X., Shacka, J.J., Eells, J.B., Suarez-Quian, C., Przygodzki, R.M., Beleslin-Cokic, B., Lin, C.S., Nikodem, V.M., Hempstead, B., Flanders, K.C.*, et al.* (2002). Erythropoietin receptor signalling is required for normal brain development. Development *129*, 505-516.

Zagorska, A., and Dulak, J. (2004). HIF-1: the knowns and unknowns of hypoxia sensing. Acta Biochim Pol *51*, 563-585.

Zhao, F.H., Chen, Y.D., Jin, Z.N., and Lu, S.Z. (2008). Are impaired endothelial progenitor cells involved in the processes of late in-stent thrombosis and re-endothelialization of drug-eluting stents? Med Hypotheses *70*, 512-514.