DUAL MECHANISMS REGULATING ALPHA SUBUNIT-SPECIFIC ACTIVITY IN HYPOXIA-INDUCIBLE FACTOR SIGNALING

APPROVED BY SUPERVISORY COMMITTEE

Joseph Garcia, M.D., Ph.D

Lance Terada, M.D.

Zhi-Ping Liu, Ph.D.

Nikhil Munshi, M.D., Ph.D.

DEDICATION

Dedicated to my parents, Donna and Mohammed Nagati, for their constant emotional support and care through the times of hardship and happiness, success and failure

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JASON SHARIF NAGATI

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DUAL MECHANISMS REGULATING ALPHA SUBUNIT-SPECIFIC ACTIVITY IN HYPOXIA-INDUCIBLE FACTOR SIGNALING

Jason Sharif Nagati, Ph.D.

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Supervising Professor: Joseph A. Garcia, M.D., Ph.D.

Abstract: The ability to adapt to and protect from environmental stresses is essential to survival and has played a major role in fitness selection during evolution. As oxygen is essential to most life, many organisms have developed a response to conditions of low oxygen availability. Throughout the animal kingdom, hypoxia-inducible factor has emerged as a master regulator of this response. These bHLH transcription factors enhance transcription of a variety of genes that work to maintain oxygen homeostasis and allow adaptation to decreased oxygen availability. Two homologues, HIF-1α and HIF-2α, have been extensively studied in this field. Though they have similar domain structures and amino acid sequences, display overlap in some gene targets, and share regulatory mechanisms, they

also perform distinct roles. They differ in tissue expression patterns, both temporally during development and spatially, hypoxia-driven expression kinetics, target genes, and fold induction. To elucidate mechanisms of this differential behavior, I investigated two aspects of HIF-2α-specific regulation. Firstly, I explored the contribution of early growth response transcription factors, EGRs, to HIF-2α-directed erythropoietin expression. Through reporter assays and chromatin immunoprecipitation, these factors were determined to occupy the erythropoietin enhancer adjacent to the HIF-2α binding site. Overexpression analysis showed they could amplify HIF-2α transactivation of erythropoietin, while knockdown experiments showed they were necessary for full, endogenous expression. And co-immunoprecipitation studies revealed a physical interaction between EGRs and HIF-2α that was necessary for cooperative activity. Secondly, I investigated the mechanism by which modulation of HIF-2α activity by CBP/SIRT1-dependent acetylation was signaled. Our studies revealed ACSS2, an acetyl CoA synthetase, as the source of acetyl CoA required for HIF-2α complex formation with the acetyltransferase CBP, subsequent HIF-2α acetylation, and target gene activation. The ACSS2 substrate acetate is produced during hypoxia, and exogenous acetate supplementation to cell culture media induced this pathway independent of hypoxia. Acetate administration in mice also augmented the HIF-2α-influenced pathways of red blood cell production and tumor growth in an ACSS2-dependent manner. Thus, EGRs represent novel HIF-2α cofactors in erythropoietin induction, while acetate, through ACSS2, regulates HIF-2α acetylation-dependent activity.

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PRIOR PUBLICATIONS

Xu, M., et al., *An acetate switch regulates stress erythropoiesis.* Nat Med, 2014. **20**(9): p. 1018-26.

Chen, R., et al., *The acetate/ACSS2 switch regulates HIF-2 stress signaling in the tumor cell microenvironment.* PLoS One, 2015. **10**(2): p. e0116515.

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ACLY – ATP citrate lyase

ACSS1/2 – acetyl CoA synthetase short-chain family member 1/2

ADM – adrenomedullin

ADP – adenosine diphosphate

AHR – aryl hydrocarbon receptor

ANG1/2 – angiopoietin 1/2

ARNT – AHR nuclear translocator

ATP – adenosine triphosphate

BCL2 – B-cell lymphoma 2

bHLH – basic helix-loop-helix

BNIP3L – BCL2/adenovirus E1B 19kDa interacting protein 3-like

Brg-1 –Brahma/SWI2-related gene

Brm – Brahma

CA – constitutively active

CBP – CREB-binding protein

ChIP – chromatin immunoprecipitation

CITED2 – CBP/p300-interacting transactivator 2

c-MYC – v-myc avian myelocytomatosis viral oncogene homolog

CoIP – co-immunoprecipitation

CTAD – C-terminal activation domain

DcytB – duodenal cytochrome B

DMT1 – divalent metal transporter 1

DN – dominant negative

EGL-9 – egg-laying defect 9

EGR – early growth response

EMSA – electrophoretic mobility shift assay

EPAS1 – endothelial PAS-1, HIF-2α

EPO – erythropoietin

ERK1/2 – extracellular signal-regulated kinase 1/2

ETS – E26 transformation specific

FIH-1 – factor inhibiting HIF

FLK1 – fetal liver kinase 1

FLT1 – FMS-like tyrosine kinase 1

GCN5 – general control of amino acid synthesis 5

GLUT1 – glucose transporter 1

GTA – glyceryl triacetate (triacetin)

HDM2 – mouse double minute 2, human homolog

HIF – hypoxia-inducible factor

HNF-4α – hepatic nuclear factor 4 alpha

HRE – hypoxia response element

HOX – homeobox

IB – immunoblot

IP – immunoprecipitate

KAT – lysine acetyltransferase

KO – knockout

MEK1/2 – mitogen/extracellular signal-regulated kinase 1/2

MMP9 – matrix metalloproteinase 9

NAB2 – NGFI-A binding protein

NAD – nicotinamide adenine dinucleotide

NADH – nicotinamide adenine dinucleotide (reduced)

NIX – NIP3-like protein X

NTAD – N-terminal activation domain

ODD – oxygen-dependent degradation

P1P2N – oxygen-stable HIF-α, 2 prolines (P) and 1 asparagine (N) mutated

PAI1 – plasminogen activator inhibitor-1

PAS – per-arnt-sim domain

PCAF – p300/CBP-associated factor

Per – period (drosophila)

PDH – pyruvate dehydrogenase

PDK1 – PDH kinase 1

PGK1 – phosphoglycerate kinase 1

PHD – prolyl hydroxylase

PHZ – phenylhydrazine

redox – reduction/oxydation

ROS – reactive oxygen species

SLC2A1 – solute carrier family 2 (GLUT1)

SIRT – sirtuin

Sim – single-minded

SOD2 – superoxide dismutase

SWI/SNF – switch/sucrose non-fermentable

TAD – transactivation domain

TBI – traumatic brain injury

TCA – tricarboxylic acid

TIE2 – Tunica interna endothelial cell kinase

UR – unique region

USF2 – upstream stimulatory factor 2

UTR – untranslated region

VEGF – vascular endothelial growth factor

VHL – von Hippel-Lindau

WT – wild-type

WT1 – Wilm's tumor suppressor

CHAPTER ONE
INTRODUCTION

HYPOXIA-INDUCIBLE FACTORS

Hypoxia-inducible factor is a key regulator of the hypoxia response

Efficient conversion of energy for basic cellular function is essential to most life. Highly reduced bonds from carbohydrates are oxidized to a lower energy state through multiple processes. The energy released from these reactions is captured predominantly through conversion of ADP to ATP, either directly or indirectly through reduction of NAD+ to NADH, which is then used as an energy source throughout the cell. In glycolysis, glucose is converted into pyruvate, which is then converted into acetyl CoA by pyruvate dehydrogenase for entry into the tricarboxylic acid (TCA) cycle. NADH formed during glycolysis and the TCA cycle enters oxidative phosphorylation, where it is re-oxidized to NAD+ to produce several ATP molecules, a step which requires molecular oxygen as the oxidant. When oxygen is unavailable, such as during hypoxia, oxidative phosphorylation cannot proceed. Thus, cells rely predominantly on glycolysis for energy production. Ultimately, glycolysis produces a net gain of 2 ATP compared to 30 or more ATP produced when combined with the TCA cycle and oxidative phosphorylation. With the exception of anaerobes, organisms must adapt to conditions of low oxygen to survive. Hypoxia-inducible factor (HIF) has emerged as a key regulator of the hypoxia response.

HIFs, first cloned in 1995 (Wang, Jiang et al. 1995), are a family of bHLH transcription factors that direct transcription of genes involved in multiple aspects of the

hypoxia response. In order to facilitate energy production in the absence of oxidative phosphorylation, glycolysis is upregulated through multiple mechanisms. The capacity for glucose uptake is increased through increased expression of glucose transporters, GLUT1 (Ouiddir, Planes et al. 1999) and GLUT3 (Baumann, Zamudio et al. 2007), predominantly by HIF-1α, though HIF-2α may regulate expression in certain cell types (Raval, Lau et al. 2005). HIF-1α regulates expression of several enzymes involved in the glycolytic pathway, such as hexokinase II (Riddle, Ahmad et al. 2000), phosphofructokinase (Obach, Navarro-Sabate et al. 2004), phosphoglycerate kinase 1 (PGK1) (Semenza, Roth et al. 1994), aldolase, and enolase (Semenza, Jiang et al. 1996). During normal energy metabolism, the end product of glycolysis, pyruvate, is converted by pyruvate dehydrogenase (PDH) into acetyl CoA, which enters the TCA cycle. Under hypoxia, HIF-1α activates expression of pyruvate dehydrogenase kinase 1, PDK1, (Kim, Tchernyshyov et al. 2006), which phosphorylates and deactivates PDH, thus suppressing further metabolism by the TCA cycle and oxidative phosphorylation, and in turn protecting the cell by reducing production of reactive oxygen species (ROS) in the mitochondria (Kim, Tchernyshyov et al. 2006). NAD+ is converted to NADH during a step of glycolysis. Another HIF-1α target, lactate dehydrogenase (Semenza, Jiang et al. 1996), converts pyruvate to lactate and, in the process, oxidizes NADH to NAD+, replenishing it for further glycolysis.

Oxygen homeostasis is regulated by HIFs. Angiogenesis, the growth of new blood vessels from pre-existing vasculature, is triggered under hypoxia to increase the flow of blood bringing oxygen and nutrients to deficient regions of the organism. This new blood vessel growth is triggered by the secreted signaling protein vascular endothelial growth factor

(VEGF), which is transactivated by both HIF-1α (Forsythe, Jiang et al. 1996) and HIF-2α (Ema, Taya et al. 1997). HIF-2α is also known to control transcription of the VEGF receptors FLT1 (Takeda, Maemura et al. 2004) and FLK1 (Kappel, Ronicke et al. 1999). Vascular endothelium is normally maintained through angiopoietin-1 (ANG1) signaling through its receptor TIE2. During angiogenesis, ANG2 antagonizes this signaling, disrupting the endothelium to allow efficient vessel remodeling (Scharpfenecker, Fiedler et al. 2005). Both TIE2 (Tian, McKnight et al. 1997) and ANG2 (Simon, Tournaire et al. 2008) are HIF targets. The oxygen-carrying capacity of the blood is increased during hypoxia or anemia by an increase in red blood cell mass, a process regulated by HIF-2α target gene (Scortegagna, Ding et al. 2005) erythropoietin (EPO). EPO is a cytokine which regulates hematopoiesis, promoting increased erythrocyte maturation through inhibition of progenitor cell apoptosis. As hemoglobin and other proteins require iron for oxygen transport, iron metabolism is also important in oxygen homeostasis. HIF-2α regulates expression of genes involved in iron uptake, transport, and oxidation state (Mastrogiannaki, Matak et al. 2009, Shah, Matsubara et al. 2009).

As these and other pathways demonstrate, HIF function is an essential response to low oxygen. Hypoxia plays a role in normal processes such as development, angiogenesis, and hematopoiesis. Pathophysiologically, hypoxic response influences tumor growth, damage from stroke and other ischemic injury, and anemia. Elucidating the mechanisms of expression and activation of the key regulators, HIFs, will be of significance to understanding the science of hypoxic response and will be of clinical relevance in combating and treating hypoxia-related disease.

Identification of HIF-2α as a regulator of Epo expression

EPO is a regulator of hematopoiesis

Erythropoietin (EPO) is a hematopoietic cytokine that controls the circulating red blood cell mass. Hypoxia or anemia stimulates expression of *EPO*, leading to increased production of red blood cells and, subsequently, the oxygen-carrying capacity of the blood. Strict regulation of *EPO* is necessary, as inappropriate levels lead to pathological problems. Increased levels of EPO can cause an overproduction of red blood cells, or polycythemia, which can lead to hypertension and blood clot formation. Transgenic mice expressing exogenous *EPO* suffer from erythrocytosis, as well as cardiac hypertrophy and vascular endothelial cell degeneration (Semenza, Traystman et al. 1989). These mice have higher hematocrit levels, causing the blood to be more viscous, increasing hemodynamic stress. Conversely, EPO deficiency is a major cause of chronic anemia. To maintain appropriate expression, several factors are involved, contributing to basal expression, tissue specificity, and expression levels, and these act on multiple elements of the *EPO* enhancers and promoter. The master regulator of *EPO* expression in response to hypoxia has been identified as HIF-2α (Warnecke, Zaborowska et al. 2004, Scortegagna, Ding et al. 2005, Chavez, Baranova et al. 2006).

EPO expression is controlled by multiple regulatory elements

EPO expression is temporally and spatially regulated. Embryonic hematopoiesis is regulated by EPO produced in the liver (Dame, Fahnenstich et al. 1998). The liver also

contributes to EPO production during higher levels of anemia in the adult, where it is expressed in hepatocytes surrounding the central vein, a region of low oxygen levels, as well as nonepithelial cells in the sinusoids (Koury, Bondurant et al. 1991, Obara, Suzuki et al. 2008). During development, there is a switch to the kidney as the main source of EPO in the adult (Dame, Fahnenstich et al. 1998), where it is expressed in the peritubular interstitial cells (Lacombe, Da Silva et al. 1988, Semenza, Koury et al. 1991, Obara, Suzuki et al. 2008). EPO is also produced in the brain during hypoxia (Digicaylioglu, Bichet et al. 1995) where it is mainly restricted to astrocytes (Masuda, Okano et al. 1994, Chavez, Baranova et al. 2006). *EPO* expression is controlled by multiple elements in the gene found in the promoter and enhancers. A 4-kb section of the human *EPO* gene, including the sequence 400 bp 5' of the start site and 700 bp 3' of the stop site, was analyzed in transgenic mice (Semenza, Traystman et al. 1989, Semenza, Dureza et al. 1990), and though expressed in all tissues analyzed, this sequence was able to direct the strongest expression of the transgene to the fetal liver, kidney, and brain. Subjecting the transgenic mice to anemia resulted in an increase in *EPO* levels only in the fetal and adult livers, though expression was retained in other organs. Subsequently, the 3' end of this sequence was shown to be an enhancer necessary to drive hypoxia-inducible expression of a reporter in Hep3B cells (Beck, Ramirez et al. 1991). This 3' enhancer is the major regulator of hepatic *EPO* expression. Mice lacking this enhancer are born anemic, but recover two weeks after birth from kidney-derived EPO (Pan, Suzuki et al. 2011). When an expanded region of the locus that included an additional 6 kb of sequence 5' of the start site was analyzed, constitutive expression was lost in all tissues (Semenza, Dureza et al. 1990). Transgene expression was only detected in the livers of

anemic mice, namely hepatocytes near the central vein (Koury, Bondurant et al. 1991). Thus, the sequence flanking the 3' end of *EPO* contains a liver-specific, hypoxia-inducible element and regulatory elements capable of directing expression to normal *EPO* expressing regions, while a regulatory element in the 5' region may suppress constitutive expression.

Further analysis of the *EPO* locus revealed that the region from 6 kb to 14 kb 5' of the start site is able to control kidney expression (Semenza, Koury et al. 1991). Transgenic mice expressed human *EPO* in kidney, as well as liver, after phenylhydrazine (PHZ)-induced anemia. Additionally, these mice had a higher degree of polycythemia than those containing a shorter, 10-kb transgene, likely due to the excess *EPO* expression in the kidney, indicating an anemia-regulated enhancer in the distal 5' region. Little is known about the 5' enhancer, though recently, a putative HIF binding site was characterized in this region (Storti, Santambrogio et al. 2014). This element was shown to enhance transcription from a reporter after exposure to hypoxia. In common with the 3', liver-specific enhancer, a highly conserved HIF binding site and nearby CACA region was detected. Additionally, HIF-2α was detected by chromatin immunoprecipitation at the 5' enhancer after hypoxia treatment. Thus, analysis of the regions both 5' and 3' of the *EPO* gene reveal distinct enhancer elements controlling hypoxia-directed expression in different organs.

Further analysis of the 3' enhancer, a 256-nucleotide span, indicated that there may be multiple sites of regulation (Semenza, Nejfelt et al. 1991, Blanchard, Acquaviva et al. 1992). DNase footprinting of the 3' flanking region of *EPO* incubated with liver nuclear extracts revealed at least four protected sites. Gel shift assays further showed nuclear factor binding on these regions from both liver and kidney extracts, with binding being further

enhanced in most cases with extracts from anemic animals. Experiments showed hypoxic induction is conferred by an element of 120 bp or less (Beck, Ramirez et al. 1991, Pugh, Tan et al. 1991), which was further narrowed down to a minimal hypoxia-induced enhancer of 43 to 50 nucleotides (Blanchard, Acquaviva et al. 1992, Semenza and Wang 1992). A hypoxia responsive element (HRE) was delineated at the 5' end of this minimal enhancer (Semenza and Wang 1992). Mutation of any portion of a short sequence in the 5' end, CTACGTGCT, resulted in a complete loss of hypoxia induced activity. The authors discovered through gel shift assays that this element could be bound by a nuclear factor present in extracts from hypoxia-treated cells (Semenza and Wang 1992, Wang and Semenza 1993). This factor which binds to the HRE was designated hypoxia-inducible factor (HIF-1).

EPO expression is controlled by multiple factors

In addition to HIF-1, other factors regulate *EPO* expression. When nucleotides 34-50 of the minimal enhancer were lost, activity was greatly reduced, indicating a control element exists in those nucleotides. This was further confirmed by the presence of a protected area in this region of a DNase footprint assay (Blanchard, Acquaviva et al. 1992, Semenza and Wang 1992). Interestingly, this region appeared bound during normoxia as well as hypoxia. The authors of both studies noted that direct repeats found in this region have the same sequence as binding sites for hormone receptor family members, though reporter assay activity was unaffected by the addition of multiple steroid hormones and vitamins (Blanchard, Acquaviva et al. 1992). This indicated a possible orphan nuclear receptor was involved in *EPO* expression, which was later determined to be hepatic nuclear factor 4 alpha,

HNF-4α (Galson, Tsuchiya et al. 1995). HNF-4α at the time was an orphan nuclear receptor, though lineolic acid has been identified as a possible ligand (Yuan, Ta et al. 2009), inhibiting transcriptional activity when bound. It is expressed in both the kidney and liver, major sites of EPO production, and thus likely contributing to tissue-specific expression. Binding sites for this factor are present in both the promoter and 3' enhancer (Galson, Tsuchiya et al. 1995). Though the putative binding sites in the promoter may contribute to endogenous expression, the 3' enhancer elements located 3' of the HRE are essential to hypoxic induction.

Another level of tissue specific regulation of *EPO* expression may be through repression by GATA factors, transcription factors that bind the sequence GATA. The *EPO* gene contains a GATA box in the promoter rather than a TATA box and was shown to be a binding site for GATA factors 1, 2, and 3 using EMSA (Imagawa, Yamamoto et al. 1997) or ChIP (Obara, Suzuki et al. 2008). Overexpression of these factors was shown to repress *EPO* expression, and knockdown of GATA-2 led to increased expression of *EPO*. Mutation of the GATA box led to constitutive reporter expression in epithelial cells of multiple tissues, cells that do not normally express *EPO*, and this expression was unaffected by anemia (Obara, Suzuki et al. 2008). Yet, reporter activity in normal, *EPO*-expressing cells in the kidney and liver were unaffected by the mutation, expressing only during anemia. This indicates that the GATA box may be important for tissue-specific restriction of *EPO* expression. Contradictively, GATA-2 is essential for hematopoiesis since GATA-2 -/- mice die by e11 from severe anemia (Tsai, Keller et al. 1994), though this may be from defects in development of the hematopoietic system rather than from its effect on *EPO* expression.

The *EPO* promoter is also important in regulation, conferring six-fold hypoxic induction alone (Blanchard, Acquaviva et al. 1992). When used to drive reporter expression, the 3' enhancer typically confers a 6-10 fold induction. When used together, the *EPO* enhancer and promoter are able to enhance reporter transcription 50 fold or more, similar to the 50-100 fold induction of endogenous *EPO* under hypoxia, indicating cooperation in normal transcriptional activation. Analysis of the promoter also reveals putative steroid hormone receptor response elements, similar to those found in the enhancer.

EPO is expressed at a steady state to maintain normal, physiological red blood cell levels. Angiotensin II (ANG II) plays a role in this steady state maintenance (Kim, Mungunsukh et al. 2014). ANG II is a hormone involved in multiple aspects of cardiovascular function, such as regulating blood pressure and blood volume (Rodgers, Xiong et al. 2000). It activates *EPO* by binding the Ang II type 1 receptor and inducing a signaling cascade through the MEK1/2-ERK1/2 pathway (Kim, Mungunsukh et al. 2014). Interestingly, ANG II treatment was shown to induce nuclear translocation of EGR1. Additionally, while exogenous *EGR1* expression alone did not affect *EPO* reporter levels, it enhanced ANG II-dependent activation, while dominant negative EGR1 completely negated any stimulatory effect. This indicates that EGR1 may play an accessory role in normal *EPO* expression as a cofactor, enabling other transcription factors to more efficiently enhance transcription, a role I have explored further in my first aim.

Wilms tumor suppressor, WT1, has been shown to be a regulator of *EPO* expression in fetal liver (Dame, Kirschner et al. 2006). In *WT1* knockout mice, EPO is still produced in the fetal liver, but expression is impaired. WT1 activity appears to be distinct from that of the

HIFs. Exogenous *WT1* expression will induce *EPO* expression under normoxic conditions and in HEK293 cells, a cell line in which *EPO* expression is unresponsive to hypoxia. WT1 has no effect on *EPO* expression in Hep3B cells during hypoxic stimulation. WT1 co-localizes with EPO in cells in the fetal liver, as well as the dorsal root ganglia and Sertoli cells of the testes, but it is not detected in the renal interstitial cells where EPO is produced in response to hypoxia. Additionally, WT1 associates with the *EPO* promoter and has not been detected binding to the 3' enhancer. These characteristics indicate a pathway of activation distinct from that of HIF-2α.

Identification of Hypoxia Inducible Factor as a regulator of EPO expression

Analysis of the 3' *EPO* enhancer led to the detection of a DNA binding activity, labeled as HIF-1, at the HRE in Hep3B nuclear extract (Semenza and Wang 1992). This binding activity was also detected in the nuclear extracts of several other cell lines that had been hypoxia-treated (Wang and Semenza 1993). Putative HREs were detected in the enhancer regions of several glycolytic enzymes, proteins upregulated during hypoxia (Semenza, Roth et al. 1994). These HREs displayed the same hypoxia-regulated binding activity, as well as the ability to drive reporter expression under hypoxia. Through large-scale affinity purification of $CoCl_3$-treated HeLa cells, the HRE-binding activity was purified several thousand fold, revealing two subunits of HIF-1 (Wang and Semenza 1995). The components of this heterodimer, known as HIF-1α and HIF-1β, 120 and 92 kDa respectively, bind to the HIF binding site in the HRE in a sequence-dependent manner. Through peptide sequencing, followed by cDNA amplification through use of degenerate primers, both genes

were cloned (Wang, Jiang et al. 1995). HIF-1α, 826 amino acids in length, contains an N-terminal basic helix-loop-helix (bHLH) and two PAS domains. The PAS (Per-Arnt-Sim) domain is shared by Drosophila period (Per) and single-minded (Sim), and the dioxin receptor subunits aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1β was previously identified as the mammalian ARNT homolog, which heterodimerizes with AHR to form a nuclear receptor responsive to environmental, aromatic hydrocarbons (Reyes, Reisz-Porszasz et al. 1992).

Two homologs to HIF-1α have been identified, HIF-2α (Ema, Taya et al. 1997, Flamme, Frohlich et al. 1997, Tian, McKnight et al. 1997) and HIF-3α (Gu, Moran et al. 1998). HIF-2α is more closely related, sharing 45% sequence identity with HIF-1α, including 84% and 66% identity between the bHLH and PAS domains, respectively. HIF-3α has not been extensively studied. Though it has been shown to negatively regulate HIF target genes through a dominant negative splice variant (Makino, Kanopka et al. 2002), HIF-3α is a hypoxia-responsive transcription factor with distinct target genes (Zhang, Yao et al. 2014).

The generation of *Hif-2α* knockout mice gave evidence that it plays a significant role in the regulation of red blood cell mass (Scortegagna, Morris et al. 2003). These mice had a lower, basal hematocrit level than wild type mice and lower levels of other blood cell types overall. Irradiated mice receiving *Hif-2α*-null bone marrow transplants had normal hematocrit levels, indicating it was unlikely the global *Hif-2α* knockout effect was due to a perturbation of red blood cell production in the bone marrow. Transplantation of wild-type bone marrow into an irradiated *Hif-2α*-null mouse, though, displayed the pancytopenia of the global knockout, pointing to an endocrine factor produced elsewhere. It was subsequently

discovered that HIF-2α is the major regulator of *EPO* expression in Hep3B and Kelly cells (Warnecke, Zaborowska et al. 2004), as well as *in vivo* (Scortegagna, Ding et al. 2005, Chavez, Baranova et al. 2006). *HIF-2α* was determined to be expressed in renal interstitial cells, the major site of *EPO* expression. While HIF-2α is enriched in these *EPO* producing cells (Scortegagna, Ding et al. 2005, Pan, Suzuki et al. 2011), HIF-1α is not (Pan, Suzuki et al. 2011). When *HIF-2α* is knocked out, mice are anemic, and *EPO* production in the kidneys remains depressed and unresponsive to this anemia, even under hypoxic conditions. *EPO* expression is even diminished in *HIF-2α* heterozygous mice.

Regulation of HIF activity

HIF-α subunits share multiple domains, including the bHLH and PAS domains in the N-terminus, and activation domains in the C-terminus. The function of the bHLH-PAS domain and C-terminus of HIF-1α was analyzed through deletion mutants (Jiang, Rue et al. 1996). DNA binding was found to be mediated through the basic region of the bHLH, and required dimerization with HIF-1β. Dimerization was achieved through the bHLH and both (Yang, Zhang et al. 2005) PAS domains. Activation of transcription of a reporter required an intact C-terminus, likely to contain an activation domain. DNA binding of the heterodimer is necessary for activation, but not sufficient. Though HIF-1α levels increased with administration of hypoxia mimic $CoCl_2$, HIF-1β levels remain constant, indicating that hypoxia-induced activity is regulated through the α-subunit (Kallio, Pongratz et al. 1997). Additionally, HIF-α protein levels increase after $CoCl_2$ treatment or hypoxia, but mRNA

levels typically remain the same, indicating a post-transcriptional mechanism for regulation of HIF activity.

The levels and activity of HIF-α subunits are tightly regulated in an oxygen-dependent manner by post-translational modifications catalyzed by two classes of oxygen-dependent enzymes, prolyl hydroxylases and asparaginyl hydroxylases. Deletion mutants revealed at least two regions that conferred hypoxia-induced transcriptional activity in HIF-1α (Jiang, Zheng et al. 1997) and the corresponding domains in HIF-2α (O'Rourke, Tian et al. 1999), repressing function under normoxic conditions. These activation domains are located in the C-terminal half of the proteins, and are labeled NTAD and CTAD (N-terminal and C-terminal activation domains, respectively).

HIF stability is regulated by a shared domain

The NTAD renders the protein oxygen labile, resulting in rapid degradation in the presence of oxygen (Huang, Gu et al. 1998, Ema, Hirota et al. 1999). The region of the NTAD that confers oxygen instability is referred to as the oxygen-dependent degradation (ODD) domain. This property is transferable to fusion proteins containing the NTAD. Degradation is directed by an ubiquitin-dependent, proteasome-mediated mechanism that is lost when the NTAD is removed (Huang, Gu et al. 1998, Kallio, Wilson et al. 1999, Tanimoto, Makino et al. 2000). Under normoxia, HIF is recognized by the von Hippel-Lindau (pVHL) E3 ubiquitin ligase complex (Tanimoto, Makino et al. 2000) by a mechanism that requires oxygen and iron (Ivan, Kondo et al. 2001, Jaakkola, Mole et al. 2001), thus targeting it for degradation by the proteasome. Through alanine substitution mutations, a

proline residue in the ODD domain was determined to be necessary for interaction with pVHL and consequent degradation. Furthermore, through mass spectrometry analysis it was determined that this proline is hydroxylated by cell lysate and that this modification is required for pVHL binding. Overall, two proline residues within the ODD domain of both HIF-1α and HIF-2α have been identified that direct pVHL recognition after hydroxylation (Masson, Willam et al. 2001). Prolyl-4-hydroxylases (PHDs), which require iron and molecular oxygen for activity, and are also dependent on 2-oxoglutarate and ascorbate, were candidate enzymes for HIF hydroxylation. The abilities of ascorbate to increase hydroxylation of HIF-1α and a 2-oxoglutarate competitive inhibitor to block proline modification (Jaakkola, Mole et al. 2001) indicated that a PHD was indeed responsible for the proline modification that leads to HIF degradation. A candidate enzyme was first discovered in *C. elegans*, egg laying defect 9 (EGL-9), which was shown to hydroxylate *C. elegans* HIF-1 (Epstein, Gleadle et al. 2001). Subsequently, three mammalian homologs of EGL-9, which regulate HIF stability, PHD-1, -2, and -3, were identified (Bruick and McKnight 2001, Epstein, Gleadle et al. 2001).

HIF activity is regulated by a shared domain

The CTAD confers hypoxia-dependent activity to the HIF-α subunit. HIF activity requires lysine acetyltransferase (KAT) cofactors. It was discovered that p300 and CREB-binding protein (CBP) interact with HIF-1α through a cysteine/histidine rich region of the KAT during hypoxia (Arany, Huang et al. 1996). During hypoxia, p300 overexpression was able to enhance activity of an *EPO* reporter, suggesting a role as a co-activator. This role is

essential as introduction of the p300/CBP-sequestering protein E1a through either adenoviral infection or transfection completely blocks hypoxic induction of HIF target genes *EPO* and *VEGF*. Both CBP and p300 are recruited by HIF through the CTAD (Ema, Hirota et al. 1999, Carrero, Okamoto et al. 2000, Gu, Milligan et al. 2001). Though HIF stability during normoxia is unaffected by the CTAD in contrast to the NTAD, HIF transcriptional activity is regulated by this domain (Ema, Hirota et al. 1999), suggesting another post-translational modification of HIF in the CTAD regulated by oxygen levels. Through mass spectrometry analysis, a conserved asparagine residue, N851 in HIF-2α, was found to be hydroxylated during normoxia, a modification that was nearly absent during hypoxia (Lando, Peet et al. 2002). Mutation of the asparagine rendered hybrid proteins constitutively active, as well as constitutively showing interaction with the p300 CH1 domain in coIP experiments, indicating the hydroxylation modification blocks KAT recruitment by HIF. An enzyme, Factor Inhibiting HIF (FIH-1), first discovered as a factor which binds to the HIF CTAD during normoxia and inhibits its activity (Mahon, Hirota et al. 2001), was shown to be the asparaginyl hydroxylase that modifies the asparagine residue in the CTAD during normoxia, blocking KAT interaction (Mahon, Hirota et al. 2001, Lando, Peet et al. 2002). Similar to the PHDs, FIH-1 requires iron and oxygen for enzymatic activity.

HIF hydroxylases are hypoxia regulated

During hypoxia, the activities of PHDs and FIH-1 are inhibited. This results in less turnover or inhibition of HIF activity. Mutation of the hydroxylation-targeted proline and asparagine residues of HIF-α renders the protein constitutively active, no longer dependent

on oxygen levels for activation. The requirement of molecular oxygen for hydroxylase activity by both enzymes provides a mechanism for hypoxia-dependent regulation; lack of oxygen substrate results in lack of hydroxylation and therefore stabilization of HIF-α subunits. Reactive oxygen species (ROS) have been proposed as another level of regulation of these enzymes (Pan, Mansfield et al. 2007, Masson, Singleton et al. 2012), though this idea is disputed. ROS, which are produced in the mitochondria during hypoxia (Chandel, McClintock et al. 2000), upregulate transcription of hypoxia-related genes (Chandel, Maltepe et al. 1998). They are thought to inhibit both FIH-1 and PHD, possibly through the oxidation of reduced iron, enzyme-required Fe(II), to Fe(III) (Kaelin and Ratcliffe 2008, Hagen 2012). Despite the lack of firm consensus on the complete mechanism, both HIF-1α and HIF-2α are regulated by these same two hydroxylation modifications to some degree. Yet, there are differences that imply alternate methods of regulation.

CHAPTER TWO

HIF-2 ALPHA ACTIVITY IS INFLUENCED BY UNIQUE MECHANISMS

HIF-1α and HIF-2α have distinct functions

Though not exclusively, HIF-1α typically functions as a more immediate response to hypoxia. As a regulator of glycolysis, for example, it allows cells to continue energy production without oxygen. Additionally, HIF-1α can initiate apoptosis in response to severe hypoxia (Greijer and van der Wall 2004). These functions may indicate the necessity for ubiquitous expression of HIF-1α, as they may benefit any cell experiencing hypoxia, or benefit the whole organism by being ubiquitously available, such as through apoptosis of damaged cells. HIF-2α, on the other hand, normally regulates functions that provide long term, global adaptation to hypoxia, such as hematopoiesis and angiogenesis, obviating the need for ubiquitous expression. As many targets are secreted proteins which require precise control and conditions, or act only in specific organs, expression of HIF-2α is localized to distinct regions that provide the proper conditions to signal its transcriptional response. For example, renal interstitial cells, which produce HIF-2α target EPO, are less susceptible to the transient hypoxic conditions common in active muscle cells, and would thus respond only to prolonged exposure to low oxygen. And EPO, as a secreted hormone that acts in the bone marrow, needs only remote access to marrow by way of the blood stream.

The differential regulation of HIF-1α and HIF-2α pathways is evident at multiple levels. Their expression is regulated spatially, with HIF-2α displaying a more restricted

expression pattern. Though there is overlap, they activate unique sets of target genes, and those targets may depend on cell type and developmental stage. HIF-1α and HIF-2α homozygous null mice have related but unique abnormalities. And, they frequently have opposing effects on cancer. Thus, though HIF-1α and HIF-2α are related, key regulators of the hypoxic response, they have distinct functions.

HIF-2α is expressed in distinct domains

The expression pattern of HIF-2α differs from that of HIF-1α. While expression of HIF-1α is widespread (Wenger, Rolfs et al. 1996), HIF-2α expression is more restricted to a subset of tissues, first described localized to endothelial cells, as evidenced by the alternate name endothelial PAS-1 (EPAS1) (Tian, McKnight et al. 1997). Grossly, it was observed by Northern blot in the brain, kidney, liver, lung and heart (Flamme, Frohlich et al. 1997). In the umbilical cord where HIF-1α is expressed in vasculature smooth muscle, HIF-2α is found in the endothelial cells of the blood vessels (Tian, McKnight et al. 1997). It is also expressed in blood vessel endothelium within the embryo, including the aorta and capillaries in the brain where it regulates angiogenesis, as well as in the placenta, contributing to placental development, processes requiring invasion (Jain, Maltepe et al. 1998). In the kidney, HIF-2α is expressed in the peritubular interstitial cells, while HIF-1α is restricted to tubular cells (Rosenberger, Mandriota et al. 2002). HIF-2α is expressed in the duodenum in epithelial cells lining the intestinal lumen (Wiesener, Jurgensen et al. 2003), where it regulates iron uptake and metabolism from the gut (Shah, Matsubara et al. 2009), in cortical astrocytes in the brain (Masuda, Okano et al. 1994, Chavez, Baranova et al. 2006), and in the retina (Morita,

Ohneda et al. 2003, Ding, Scortegagna et al. 2005). Additionally, stabilization kinetics during hypoxia differ. HIF-1α accumulation is more transient, with protein levels increasing early during hypoxia and then falling off, while HIF-2α accumulates at later time points and persists (Wiesener, Jurgensen et al. 2003, Holmquist-Mengelbier, Fredlund et al. 2006).

HIFs activate distinct pathways

HIF-1α and HIF-2α have distinct sets of target genes, though they share certain targets. Both regulate angiogenic factors, such as VEGFa, ANG3, and ADM (Pawlus, Wang et al. 2012), but many of these genes appear to be more responsive to HIF-2α (Dioum, Clarke et al. 2008). HIF-1α is the main regulator of hypoxia-induced glycolysis. It activates expression of numerous genes in the glycolytic pathway, including glucose transporters, metabolic enzymes, and PDK1, which maintains anaerobic glycolysis by inhibiting the TCA cycle (Goda and Kanai 2012). HIF-1α regulates apoptosis during severe hypoxia, though it may also inhibit it in some circumstances (Greijer and van der Wall 2004). HIF-1α targets include pro-apoptotic genes *BNIP3L*, *NIX* (Sowter, Ratcliffe et al. 2001), and *BCL2* (Pawlus, Wang et al. 2012).

HIF-2α is the primary *in vivo* regulator of hematopoiesis through *EPO* induction (Scortegagna, Ding et al. 2005). Thus, it responds to conditions of low oxygen delivery, such as anemia. HIF-2α regulates iron metabolism. DcytB, which reduces iron from Fe(III) to Fe(II), and DMT1, a divalent metal transporter, are both HIF-2α targets (Shah, Matsubara et al. 2009), as is ferroportin (Taylor, Qu et al. 2011), which exports iron outside of the cell. HIF-2α regulates the response to oxidative stress. Antioxidant genes catalase, glutathione

peroxidase type 1, and superoxide dismutase 2 (SOD2) (Scortegagna, Ding et al. 2003), which catalyzes breakdown of superoxide, a damaging ROS produced in the mitochondria, are regulated by HIF-2α. HIF-2α also regulates frataxin (Oktay, Dioum et al. 2007), an aconitase chaperone that prevents mitochondrial dysfunction. In addition to regulating proangiogenic factors, HIF-2α enhances expression of matrix metalloproteinase 9 (MMP9) (Petrella, Lohi et al. 2005) and plasminogen activator inhibitor-1 (PAI1) (Ahn, Chua et al. 2010), which regulate tissue invasion during angiogenesis or tumor metastasis.

HIF knockout mice have differing phenotypes

HIF-1α and *HIF-2α* knockout mice display different phenotypes, reflecting the divergent roles. *HIF-1α* homozygous null embryos have disorganized and reduced vasculature of the yolk sac at E9.5, and absent neural vasculature by E8.5 (Iyer, Kotch et al. 1998, Ryan, Lo et al. 1998). This reflects the contribution of HIF-1α to angiogenesis and neovascularization, though some vasculature remains, possibly from compensation by HIF-2α. There is cranial malformation due to incompletely closed neural folds, possibly from reduced glycolysis due to loss of HIF-1α target PGK1. The embryos are reduced in size and have fewer somites. Embryos were dead by E11. The *HIF-2α* null genotype is also lethal. Embryos were dead by E13.5 (Peng, Zhang et al. 2000). As HIF-2α also regulates angiogenesis, the embryos and yolk sacs showed severe vascular defects, as well as hemorrhaging in a subset of animals. *HIF-2α* null mice are deficient in catecholamine neurotransmitters, which causes embryonic lethality due to heart failure from bradycardia (Tian, Hammer et al. 1998). HIF-2α is required for expression of proangiogenic factors in the

retina. *HIF-2α* null retinas have deficient vascularization (Morita, Ohneda et al. 2003), while *HIF-2α* heterozygous retinas had impaired neovascularization in response to an oxygen-induced retinopathy procedure (Dioum, Clarke et al. 2008). The generation of viable *HIF-2α* null mice allowed phenotype analysis in post-natal animals (Scortegagna, Ding et al. 2003). *HIF-2α* null mice were blind by one month and had structural defects in the retina and retinal vasculature (Ding, Scortegagna et al. 2005). HIF-2α is required for the normal function of multiple organs (Scortegagna, Ding et al. 2003). Null mice have defects in antioxidant response, resulting in greater oxidative stress and impaired mitochondrial function. The mice develop enlarged hearts and fatty livers, and retinal development is impaired. Thus, *HIF-α*-deficient mice have phenotypes analogous to their specific functions.

HIF-1α and HIF-2α can have opposing roles in cancer

Cancer cells are distinguished from normal cells by several characteristics that promote their pathogenic behavior. These include resisting apoptosis/cell death, promoting angiogenesis, unchecked proliferation, invasion and metastasis, and evading growth suppressors (Hanahan and Weinberg 2011). Because several HIF-α pathways regulate cancer hallmarks, HIF signaling can promote tumor growth and metastasis. The high metabolic load present in tumors creates environmental stresses by quickly depleting available oxygen and food, conditions which result in HIF-α stabilization and activity. HIF-α subunits are frequently expressed at high levels within tumors (Poon, Harris et al. 2009). Mutations in pVHL, which targets HIF-α for degradation, cause Von Hippel Lindau syndrome, an inherited disorder characterized by high incidence of certain types of cancer. Yet, as cancer is

a set of individual, unique diseases caused by different mutations or gene dysregulation in different cell types, rather than an invariant, homogeneous pathology, the contributions of HIF-1α and HIF-2α can be quite divergent and vary between cancer types.

HIF-2α is typically associated with negative outcome in cancer, while HIF-1α is often, though not exclusively, negatively correlated. Knockdown of *HIF-2α*, but not *HIF-1α*, was shown to reduce proliferation and tumor growth of xenographs in mice in several tumor cell lines (Franovic, Holterman et al. 2009). *HIF-1α* expression in certain cancer types is associated with a positive outcome, while *HIF-2α* expression is negatively correlated with survival (Bertout, Patel et al. 2008). And in clear cell renal cell carcinoma lines, expression of *HIF-2α* is a more potent promoter of tumor cell growth than expression of *HIF-1α* (Kaelin 2008). Although HIF-1α target genes regulate functions essential to tumor growth, such as angiogenesis, metastasis, and energy production, other HIF-1α-specific activities may account for its tumor suppression. HIF-1α antagonizes the function of the oncogene c-Myc, while HIF-2α enhances its activity (Gordan, Bertout et al. 2007). HIF-1α has also been shown to interact with and stabilize tumor suppressor p53 (An, Kanekal et al. 1998). Conversely, HIF-2α suppresses p53 indirectly, mediated through HDM2 (Roberts, Watson et al. 2009).

As HIF-1α and HIF-2α share regulatory mechanisms, inhibiting or promoting those mechanisms may affect both proteins, such as with prolyl hydroxylase inhibitors. Promoting HIF-2α activity would aid in recovery from anemia through enhanced hematopoiesis. Inhibiting it may reduce tumor progression. Selectively controlling HIF-2α activity without affecting HIF-1α activity would reduce off-target effects from non-related pathways.

Elucidating the different control mechanisms of HIF-α subunit activity would have significant clinical relevance and would facilitate research into targeting of subunit-specific regulation.

HIF-2α Preferential Activity

Though HIF-1α and HIF-2α bind to the same enhancer element and have a high degree of amino acid sequence identity in important activation domains, they display different preference for target gene activation, even when expressed in the same cell. While HIF-1α protein levels may increase 50-fold during hypoxia, there is only a three- to four-fold increase in HIF-2α in Hep3B cells, indicating a difference in degradation efficiency. Yet, the mRNA levels of HIF-2α target gene *EPO* can increase 40-fold or more in hypoxia-treated Hep3B cell lines (Goldberg, Dunning et al. 1988) and several hundred-fold in anemic mice (Bondurant and Koury 1986), indicating that HIF-2α activity during hypoxia is, in part, a function of increased transactivation efficiency rather than purely due to abundance or stabilization. Thus, there may be unique mechanisms that control HIF-2α-specific activity.

Cofactors regulate HIF-α subunit-specific activity

Both HIF-1α and HIF-2α are recruited to the *EPO* enhancer during hypoxia (Storti, Santambrogio et al. 2014) and with similar affinity (Ema, Taya et al. 1997), yet HIF-2α is the main regulator of expression in Hep3B cells and *in vivo*, indicating there are factors conferring a preference for HIF-2α transactivation. It has been shown that cofactors regulate HIF-2α activity. HNF-4α, which may direct expression to specific tissues, binds to the *EPO*

enhancer and is required for HIF-2α activity (Galson, Tsuchiya et al. 1995). Lysine acetyltransferases are cofactors recruited by HIFs (Ema, Hirota et al. 1999) which facilitate formation of euchromatin by acetylating histones (Grunstein 1997).

Cofactors have been shown to influence specific HIF-α subunit activity at specific target genes. The ETS transcription factor ELK-1 has been shown to cooperatively and selectively activate HIF-2α target genes (Aprelikova, Wood et al. 2006). In that study, putative ETS binding sites were detected near the HRE of several HIF-2α target genes. When ELK-1 was knocked down, hypoxic induction of several of these genes was reduced or lost, while HIF-1α target genes were unaffected. Additionally, ELK-1 displayed a synergistic activation effect with HIF-2α on the CITED2 gene. Another transcription factor, upstream stimulatory factor 2, (USF2) acts cooperatively with HIF-2α to activate gene expression during hypoxia (Pawlus, Wang et al. 2012). Knockdown of USF2 results in decreased hypoxia-induced expression of several HIF-2α target genes, but does not affect HIF-1α-dependent gene expression.

Given the complex expression pattern of *EPO*, it is possible that other, unknown transcription factors or coactivators may play a role in conferring HIF-2α-specific induction over HIF-1α. The *EPO* enhancer was shown to be bound by multiple factors during hypoxia through DNase footprinting (Semenza, Nejfelt et al. 1991). Though HIF-2α and HNF-4α have been reported, not all factors binding the *EPO* enhancer have been identified. Additionally, a reporter containing five tandem copies of the *EPO* HRE was unresponsive to HIF-2α siRNA (Warnecke, Zaborowska et al. 2004). Yet when the reporter contains an expanded region of the *EPO* enhancer, not only was this reporter more responsive to

hypoxia, but it became sensitive to HIF-2α knockdown, indicating that additional transcription factor binding sites adjacent to and working in conjunction with the HRE may be required for normal, hypoxic activation of *EPO* in Hep3B cells. I investigated a stress-responsive cofactor that binds to the *EPO* enhancer and confers HIF-2α-specific transactivation capacity.

HIF-2α specific activity is regulated by acetylation

Another known mechanism of regulation of protein activity is through post translational modifications. In contrast to HIF-1α, regulation by hydroxylation of asparagine 847 by FIH-1 in the HIF-2α CTAD is less pronounced. Mutation of this residue, or even deletion of the HIF-2α CTAD, has a less pronounced effect on reporter activity than a corresponding mutation in HIF-1α (Yan, Bartz et al. 2007). Thus, the recruitment of CBP or p300 through the CTAD is not required for HIF-2α activity as it is for HIF-1α. A different post-translational modification-mediated mechanism for acetyltransferase recruitment has been identified (Chen, Xu et al. 2012). HIF-2α activity is regulated by the post translational modification of lysine acetylation (Dioum, Chen et al. 2009). It was discovered that Sirtuin 1 (SIRT1), an NAD^+-dependent, class III histone deacetylase, has been shown to positively regulate HIF-2α-dependent activation of reporters containing regulatory regions of HIF-2α target genes, as well as regulating mouse endogenous *Epo* (Dioum, Chen et al. 2009). Deacetylase activity of SIRT1 was necessary for this function. Though HIF-1α also activated these reporters, SIRT1 did not augment this activity. Additionally, knockdown of SIRT1 blocked expression of *EPO*. Using CoIP, SIRT1 was shown to interact with HIF-2α, but not

HIF-1α, and this interaction is dependent on hypoxia. Thus, SIRT1 promotes HIF-2α-specific transcriptional activity. The known function of SIRT1 as a deacetylase indicates that HIF-2α activity is regulated by acetylation. Consequently, it was determined that HIF-2α is acetylated on three specific lysine residues during hypoxia, and SIRT1 deacetylates those residues (Dioum, Chen et al. 2009).

As NAD+-dependent enzymes, sirtuins are activated by higher ratios of NAD+ to the reduced form NADH (Fulco, Schiltz et al. 2003), and thus respond to the cellular redox state. Additionally, incubation in low glucose media results in higher NAD+ levels and increased SIRT1 activity (Canto, Jiang et al. 2010). Therefore, it follows that sirtuins may play a role alongside HIF in regulating the response to hypoxia, glucose deprivation, and other cellular stresses that affect the redox state. SIRT1 is, in fact up-regulated during early stages of hypoxia as a target of HIF-1α and HIF-2α (Chen, Dioum et al. 2011). Though Lim et al (Lim, Lee et al. 2010) report that SIRT1 is down-regulated in different cell lines during hypoxia, they looked at late hypoxia stages, eight hours at the earliest, when SIRT1 levels are returning to normal in Hep3B and HT1080 cells (Chen, Dioum et al. 2011).

Sirtuins have been implicated in the function of HIF-1α. Both SIRT3 and SIRT6 have been shown to negatively regulate HIF-1α indirectly. SIRT3 was shown to affect HIF-1α stability through a possible ROS-PHD mechanism (Finley, Carracedo et al. 2011). Loss of SIRT3, a mitochondrial deacetylase that regulates multiple oxidative, metabolic pathways, results in an increase in ROS and therefore an inhibition of PHDs. Thus, SIRT3 serves to destabilize HIF-1α indirectly by inhibiting ROS production through its normal activity. This mechanism, which is disrupted in many cancers, may represent a first level buffer to

oxidative stress which would temper HIF activity, yet still allow for a HIF response when overwhelmed, such as during extended hypoxia. SIRT6 has been shown to regulate glucose metabolism through HIF-1α (Zhong, D'Urso et al. 2010). During hypoxia, lack of oxygen renders oxidative phosphorylation ineffective, and cells revert to glycolysis for energy production. HIF-1α activates expression of multiple genes involved in glycolysis. SIRT6 acts as a corepressor of HIF-1α by deacetylating H3K9 at the promoters of HIF-1α target genes, thus inhibiting transcription. Modulation of HIF-1α-dependent glycolysis by SIRT6 is necessary as *Sirt6*-deficient mice die before one month of age due to hypoglycemia (Mostoslavsky, Chua et al. 2006), likely caused by increased cellular uptake of glucose by HIF-1α-activated glucose transporters (Zhong, D'Urso et al. 2010). Furthermore, SIRT1 has been shown to have a direct, negative effect on HIF-1α activity (Lim, Lee et al. 2010). During hypoxia, acetyltransferase P300/CBP-associated factor (PCAF) acetylates HIF-1α at lysine 674 during hypoxia (Xenaki, Ontikatze et al. 2008, Lim, Lee et al. 2010), increasing its activity through protein stabilization (Xenaki, Ontikatze et al. 2008) and interaction with cofactor p300 (Lim, Lee et al. 2010). SIRT1 deacetylates lysine 674, disrupting interaction with p300, and therefore reducing HIF-1α activity. Thus, sirtuins and HIFs are intricately linked as redox and oxygen sensors within cells.

Because HIF is known to recruit the acetyltransferases CBP and p300 as coactivators, one or both of these was likely to be involved in HIF-2α acetylation. It was determined that CBP, not p300, acetylates HIF-2α at three lysine residues during hypoxia (Chen, Xu et al. 2012). RNAi experiments show that knockdown of CBP, but not p300, resulted in the loss of HIF-2α acetylation. Additionally, knockdown of acetyltransferases PCAF and GCN5 did not

affect HIF-2α. Functionally, knockdown of CBP resulted in reduced expression of *EPO*. This reduction was specific to HIF-2α-mediated activation and required intact KAT activity. Mutation of the acetylated lysine residues resulted in loss of CBP coactivity. Knockdown of CBP did not affect expression of HIF-1α specific target gene PGK1, while knockdown of p300 resulted in reduced expression, indicating a HIF-α subunit preference for KAT coactivation. Knockdown of p300 did result in reduced *EPO* expression, though to a lesser degree than CBP knockdown. This contribution of p300 to *EPO* expression did not require intact acetyltransferase activity, possibly indicating a scaffolding/structural role. Additionally, the interaction between HIF-2α and p300 required an intact CTAD, while the interaction with CBP did not. The lack of CTAD requirement for CBP activity may indicate why FIH-1 hydroxylation, which controls HIF activity through the CTAD, has a smaller effect on HIF-2α than HIF-1α. Taken together, CBP-mediated acetylation and SIRT1-mediated deacetylation of HIF-2α constitute a hypoxia-driven, post-translational modulation of transcription factor activity specific to HIF-2α. These two enzymatic activities may work together in an acetylation-deacetylation cycling that would result in repeated recruitment of CBP and therefore augmented activity. In order to understand this mechanism further, I investigated the hypoxia-induced switch that signals HIF-2α-specific activation through acetylation.

CHAPTER THREE
Results

COFACTOR MODULATION OF HIF-2 ALPHA ACTIVITY

Early Growth Response proteins participate in HIF-2α induction of *EPO*

The major hematopoietic cytokine EPO is regulated by HIF-2α during hypoxia in cell culture and *in vivo*. To determine unknown cofactors that work cooperatively with HIF-2α, we took a candidate approach using bioinformatics to identify control elements in the enhancer and possible transcription factors which bind those elements. We identified early growth response (EGR) proteins as transcription factors that bind to the *EPO* enhancer during hypoxia. Though neither can induce *EPO* expression alone, EGR1 and EGR2 act synergistically with HIF-2α, but not HIF-1α, to induce high levels of expression. Knockdown of EGR1 or EGR2 by shRNA reduces *EPO* expression in Hep3B cells, demonstrating a necessity for these factors. EGR1 and EGR2 are recruited to the EPO enhancer during hypoxia, and as determined through CoIP experiments, and EGR2 physically interacts with HIF-2α, but not HIF-1α, indicating association is a mechanism of HIF selectivity.

EGRs are zinc finger transcription factors transcriptionally activated by mitogens and cellular stress. There have been four family members identified, EGR1-4. They regulate multiple pathways, including stress response pathways such as apoptosis and inflammation. EGR1 has been shown to be inducible by hypoxia, ROS, and ischemia (Yan, Zou et al. 1998, Rong, Hu et al. 2006, Bhattacharyya, Wu et al. 2011, Sun, Liu et

al. 2013). EGR1 mediates TGFβ signaling, and is known to be involved in wound healing. EGR2 is frequently found in the brain and regulates nerve myelination. Mutations in EGR2 can cause Charcot-Marie-Tooth disease, a demyelination disorder, while *Egr2*-null mice die shortly after birth due to nervous system defects. NAB2 is a corepressor which binds to EGRs and inhibits transcription (Svaren, Sevetson et al. 1996). EGR1-3 can form a negative feedback loop by activating NAB2 expression (Kumbrink, Kirsch et al. 2010). As transcription factors activated rapidly in response to cellular stress, EGRs and HIFs are well positioned to act cooperatively to restore oxygen homeostasis quickly.

Results

A conserved element in the EPO enhancer confers HIF-2α selective activation

Putative, previously unrecognized cis-acting elements in the *EPO* enhancer region were identified in an unbiased evolutionary comparison of the *EPO* enhancer from several mammalian organisms using a bioinformatics (Loots and Ovcharenko 2004) and visual inspection approach. The results of this survey revealed the presence of novel, conserved sequence elements located 5' and 3' of the human minimal *EPO* enhancer (Fig. 1A). These included three conserved regions identified by rVista designated Boxes 1-3. Box 1 encompasses the previously defined *Epo* enhancer restriction fragment, whereas Boxes 2 and 3 are additional conserved regions located 3' to box 1. In addition to Boxes 1-3, there are three conserved sequence elements located 5' to Box 1, designated binding sites 1-3 (BS1-3) identified by visual inspection followed by software evaluation for potential transcription factor binding sites.

To define the contribution of the conserved elements to *EPO* regulation and HIF activation, the deletion construct of the parental mouse *Epo* enhancer region that retains all the conserved elements was analyzed (BS123-Box123), followed by 3' deletion constructs that lack either Box 3 (BS123-Box12) or Box 2 and 3 (BS123-Box1). In addition, 5' deletion constructs of BS123-Box1 that lack either BS1 (BS23-Box1) or BS1 and 2 (BS3-Box1) were analyzed (Fig. 1B). To construct heterologous reporters, the mouse *Epo* enhancer deletion constructs were fused to the mouse *Epo* promoter and placed upstream of the firefly luciferase reporter gene. To assess basal expression and HIF-inducibility of these constructs, the *Epo* enhancer-*Epo* promoter reporter plasmids were co-transfected into Hep3B cells with empty expression plasmids or expression plasmids engineered to produce oxygen-independent forms (P1P2N) of HIF-1α or HIF-2α. The results of these transfection experiments (Fig. 1B) are notable for several reasons. First, Box 3 has an inhibitory effect upon HIF activation as deletion of this region (BS123-Box12) results in a significant increase in both HIF-1α and HIF-2α activation. Second, this Box 3 deletion also results in a reporter construct that is preferentially activated by HIF-2α. Third, further deletion of Box 2 (BS123-Box1) has no adverse effect upon HIF-2α activation of the *Epo* enhancer, thereby delineating a 3' boundary of the *Epo* enhancer that is maximally activated by HIF-2α. Fourth, deletion of BS1 (BS23-Box1) has no adverse effect on HIF-1α or HIF-2α activation. Fifth, subsequent elimination of BS2 from the *Epo* enhancer (BS3-Box1) results in loss of selective HIF-2α activation in Hep3B cells, resulting in a signal nearly indistinguishable from that of HIF-1α. This indicates that the BS2 element confers HIF-2α-selective activity.

The contributions of the *Epo* enhancer conserved regions to reporter activity under hypoxia were then tested. Under hypoxia, there is a 35-fold increase in activation of the BS23-Box1 reporter over normoxia (Fig. 1C). Similar to the results of HIF-2α-dependent activation in Figure 1B, deletion of BS2 (BS3-Box1) results in a significant decrease in hypoxic reporter induction. To verify the contribution of BS2 to selective HIF-2α activation, a site-specific mutant of the BS23-Box1 *Epo* enhancer reporter, designated mutBS2 BS23-Box1, with point mutations in key residues designed to disrupt potential cofactor binding, was also tested in this assay. Under hypoxia, these point mutations resulted in a loss of activity comparable to that of the reporter in which BS2 is deleted, indicating the primary sequence of BS2 is necessary for activity. The persistence of a 20-fold induction of the mutBS2 BS23-Box1 reporter during hypoxia indicates that HIF transactivation is not disrupted. To further verify the role of HIF transactivation, a point mutant of the HIF binding site based on the BS23-Box1 reporter (mutHRE BS23-Box1) was analyzed in this assay. As expected, mutation of the HRE abolished hypoxia-induced reporter activity. The lack of residual activity, despite the intact BS2 element, indicates that HIF binding is necessary to the role of BS2.

It was then determined if the sequence requirement of BS2 is specific to HIF-2α. The mutBS2 BS23-Box1 reporter was co-transfected with either P1P2N HIF-1α or HIF-2α, and activity was compared to the wild type reporter (Fig. 1D). While HIF-1α dependent activation is not affected by the mutant, HIF-2α dependent activation is reduced to a level similar to that of HIF-1α. These data indicate that the BS2 region of the *Epo* enhancer selectively confers a higher level of activity to HIF-2α over HIF-1α.

EGR family transcription factors co-activate EPO synergistically with HIF-2α

The EGR family of transcription factors was identified as candidate HIF-2α cofactors based on binding site analysis prediction of the BS2 element and known function as stress responsive transcription factors. To determine if EGR proteins affect *Epo* expression, constructs for all four EGR family members were co-transfected with the *Epo* reporter and P1P2N HIF-1α or HIF-2α. Though EGR4 did have a repressive effect on both HIF-1α and HIF-2α dependent activation, EGR1, EGR2, and EGR3 did not affect HIF-1α dependent activation (Fig. 2A). In contrast, both EGR1 and EGR2 significantly increased reporter activity when co-expressed with HIF-2α. Because none of the EGR family members transactivate the *Epo* reporter when expressed alone (see Fig. 3A), the HIF-2α-specific up-regulation of reporter activity by EGR1 or EGR2 indicates a synergistic relationship.

EGR factors bind to the Epo enhancer

EGR1 and EGR2 were then co-expressed with HIF-2α and the reporter containing a mutated BS2, mutBS2 BS23-Box1. When BS2 is mutated, EGR1 and EGR2 co-expression are no longer able to augment HIF-2α-dependent activation compared to the wild type reporter with an intact BS2 (Fig. 2B). This indicates that EGR augmentation of HIF-2α activity is mediated through the BS2 element. Because BS2 was identified as a putative EGR binding site, it is likely that EGR1 and EGR2 influence reporter expression through DNA binding rather than through an indirect effect.

To further determine whether EGR1 or EGR2 bind to the *Epo* enhancer, NAB2 was introduced into the reporter assay. NAB2 is a co-repressor of EGR1, 2, and 3, and

this co-repression requires DNA binding by the EGR factor. When NAB2 was co-expressed with HIF-2α and either EGR1 or EGR2, reporter activity was repressed, indicating that NAB2 was recruited to the enhancer (Fig. 3A). As NAB2 did not affect reporter activity without EGR coexpression, this suggests that EGR1 and EGR2 mediate NAB2 recruitment to the enhancer through direct binding. EGR factors do not enhance reporter activity alone, but require coexpression with HIF-2α, suggesting that they are not affecting the reporter indirectly.

To further verify the activity between NAB2 and EGR, constitutively active EGR1 (CA) and dominant negative NAB2 (DN) were analyzed. In both of these forms, one amino acid residue is mutated, abolishing EGR-NAB2 interaction. When introduced into the *Epo* reporter assay, DN NAB2 was not able to repress EGR1-dependent activation (Fig. 3B). When CA EGR1 is introduced, neither wild type nor DN NAB2 affects reporter activity. Thus, the repressive effect of NAB2 requires binding to EGR1, verifying a direct interaction between the reporter and NAB2.

Finally, chromatin immunoprecipitation was performed to determine if EGR1 or EGR2 occupy the endogenous *Epo* enhancer in Hep3B cells that have been subjected to hypoxia. When comparing enhancer occupancy by either EGR1 or EGR2 during hypoxia to occupancy during normoxia, both factors were enriched after four hours of hypoxia (Fig. 3C). Interestingly, EGR2 remains partially enriched after eight hours hypoxia, while EGR1 does not.

EGR factors influence endogenous Epo expression

The contribution of EGR1 and EGR2 in endogenous *Epo* expression was then analyzed through use of RNA interference. I created Lentiviral vectors that express a DsRed marker containing shRNA stem-loop sequences in the 3' UTR targeting either *EGR1* or *EGR2*, in addition to *HIF-1α*, *HIF-2α*, and a non-targeting control. Stably integrated cell lines were established in Hep3B cells with the packaged Lentivirus, and these were then tested for *EPO* induction under hypoxia. After four hours hypoxia, real-time RT-PCR reveals that *EPO* expression is reduced in both EGR1 and EGR2 knockdown cell lines (Fig. 4A). This indicates EGR1 and EGR2 are required for full, endogenous *EPO* expression. Additionally, EGR2 protein levels are nearly absent in the EGR1 knockdown cell line, likely because EGR1 activates endogenous EGR2 expression (Kumbrink, Kirsch et al. 2010). HIF-1α levels are also reduced. EGR1 protein levels are not affected by *EGR2* knockdown. *EPO* expression was lower in the *EGR1* knockdown cells than the *EGR2* knockdown cells, yet EGR2 protein was absent from both cell lines. The effect of *EGR1* knockdown on *EPO* is likely due, in part, to an indirect effect mediated through loss of EGR2 and HIF-1α.

The effect of *EGR* expression on endogenous *EPO* expression was further analyzed by over-expression of HIF and EGR factors. Hep3B cells were transfected with constitutively active EGR1 or EGR2 constructs, along with oxygen-stable P1P2N HIF-1α or HIF-2α, and real-time PCR for endogenous *EPO* was performed. Alone or when co-expressed with HIF-1α, neither EGR had an effect on *EPO* expression (Fig. 4B). When coexpressed with HIF-2α, though, CA EGR2 resulted in a nearly four-fold induction of *EPO* over that stimulated by HIF-2α expression alone. CA EGR1 had no effect on HIF-

2α-mediated *EPO* expression. This result indicates that EGR2 may play a more significant role in endogenous, HIF-2α-mediated *EPO* induction.

Preference for EGR2 as a coactivator

To examine this apparent preference for EGR2 further, a time course expression profile was created of the EGR and HIF transcription factors during a hypoxia time course. Immunoblot data shows that HIF-1α and EGR1 have similar expression profiles, both peaking early during hypoxia, and then fading by 16 hours (Fig. 5A). HIF-2α and EGR2 have a similar expression pattern to each other, though one that differs from Egr1 and HIF-1α. HIF-2α accumulates at a later time point in hypoxia than HIF-1α, but it persists through the 16-hour time point. EGR2 levels, though detectable during normoxia, increase and persist during hypoxia in a manner similar to HIF-2α. *EPO* expression (Fig. 5B) most closely mirrors the expression patterns of HIF-2α and EGR2, peaking after HIF-1α and EGR1 have faded.

Because EGR1 and EGR2 have a synergistic activity on the *Epo* enhancer reporter with HIF-2α, but are unable to activate transcription alone, there may be a physical interaction between them and HIF-2α. To test this, CoIP was performed on Hep3B cell extracts. Cells were exposed to hypoxia for two or eight hours, in addition to a normoxia control. Endogenous EGR1 and EGR2 were then immunoprecipitated, followed by immunoblot for HIF-1α and HIF-2α (Fig. 5C). Under endogenous conditions, only an interaction between HIF-2α and EGR2 was detected. No interactions were detected between HIF-1α and either EGR. As indicated by protein-protein

interaction, parallel expression patterns, and the effect on endogenous *EPO* expression, these data imply a preference for EGR2 in cooperatively enhancing HIF-2α activity.

EGR-HIF synergistic co-activation requires specific protein domains

To map the interaction domains, CoIP was performed after cotransfection of tagged versions of EGR2 and deletion constructs of HIF-1α and HIF-2α. Under these overexpression conditions, an interaction between EGR2 and HIF-1α was detected (Fig. 6A). EGR2 is co-immunoprecipitated by all HIF-1α or HIF-2α deletion fragments containing the N-terminal activation domain (NTAD). But, an additional interaction is detected between EGR2 and the HIF-2α unique region-C terminal activation domain (UR-CTAD) fragment (Fig. 6A).

The requirement of the HIF-2α CTAD was analyzed further using HIF-1α/HIF-2α hybrid constructs in our reporter assay. UR-CTAD domains were swapped between HIF proteins, and these hybrids were co-expressed with the *Epo* reporter. HIF-1α, as established previously, is not responsive to EGR co-expression. When the UR-CTAD is replaced with that of HIF-2α (P1P2N HIF-1α/HIF-2α), the hybrid shows synergistic activation with EGR1 and EGR2 (Fig. 6B). Conversely, when the UR-CTAD of HIF-2α is replaced with that from HIF-1α (P1P2N HIF-2α/HIF-1α), it loses its ability to be synergistically activated by EGR1 and EGR2. These results indicate that the HIF-2α CTAD is necessary for EGR co-activation.

Finally, it was determined which domains of EGR2 confered *Epo* enhancer activity. Because EGR3 does not coactivate the *Epo* enhancer, EGR2-EGR3 hybrids were created (Fig. 7A) and coexpressed in the reporter assay with HIF-2α. Constructs

containing the EGR2 transactivation domain (TAD) were able to induce a synergistic activation of the *Epo* reporter (Fig. 7B), while the EGR2 DNA binding domain (Zinc fingers), found in the activity-deficient constructs Egr2-TAD/Egr3-R1zf and Egr2-TAD-R1/Egr3-zf, was not sufficient. Additionally, CoIP was performed with the EGR2/EGR3 hybrids to determine which EGR2 domains were required for interaction with HIF-2α. Only hybrids containing the EGR2 TAD were coimmunoprecipitated with HIF-2α (Fig. 7C). These results indicate that the EGR2 TAD is necessary for both co-activation with HIF-2α through the EPO enhancer and physical interaction with HIF-2α.

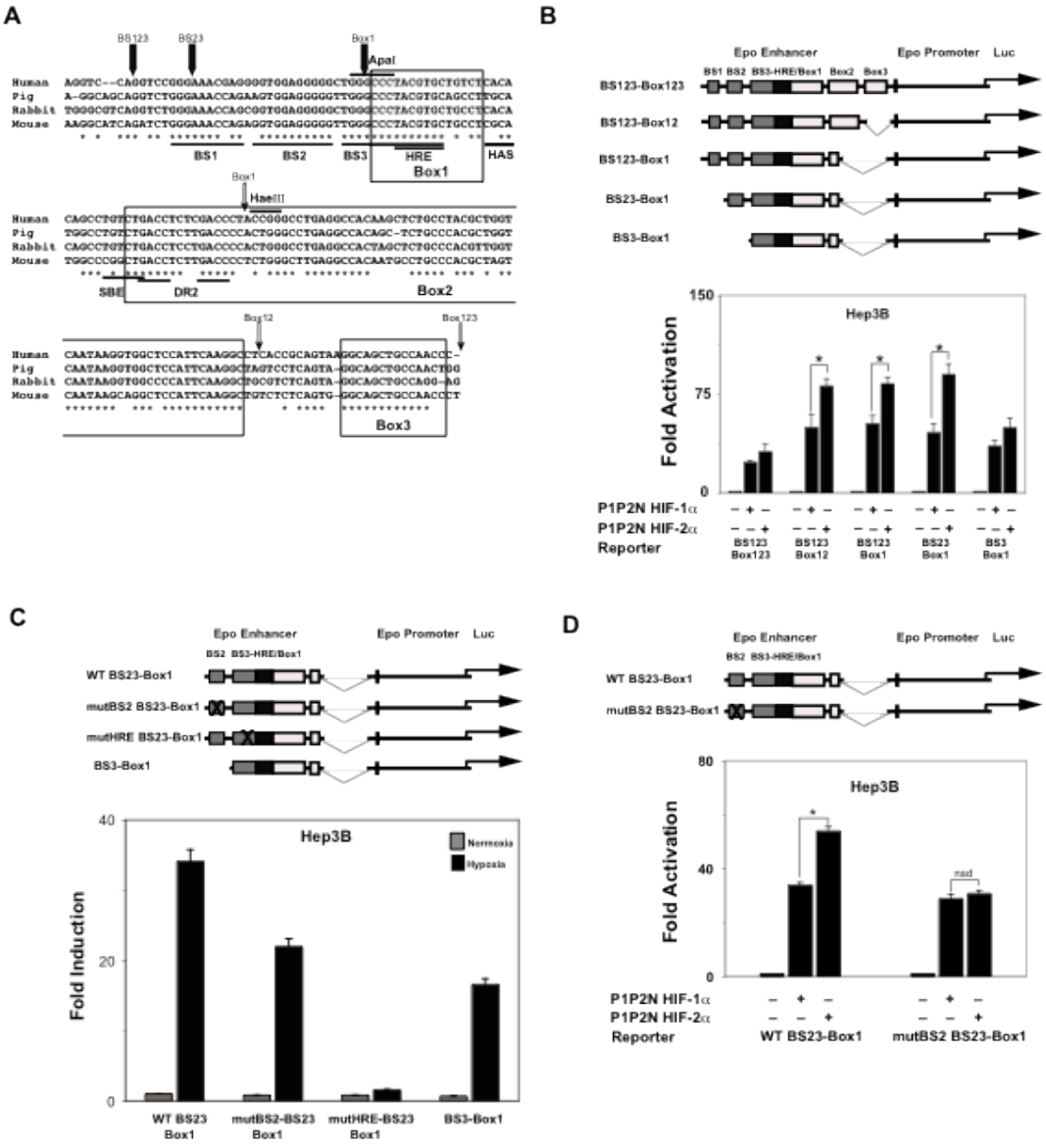


Figure 1. The BS2 element confers HIF-2α selective activity. (**A**) Multi-species alignment of *Epo* enhancer. (**B**) Analysis of activity of *Epo* enhancer reporter deletion mutants after co-expression with P1P2N HIF-1α and HIF-2α. Deletion of BS2 results in loss of HIF-2α-specific activity. Fold activation measured as luciferase activity. (**C**) Hypoxia-induced activity of *Epo* enhancer reporter containing a deletion or point mutation of the BS2 element. (**D**) HIF-α-dependent activation of the *Epo* enhancer reporter containing BS2 point mutations. BS2 contributes to HIF-2α and hypoxia-induced activity.

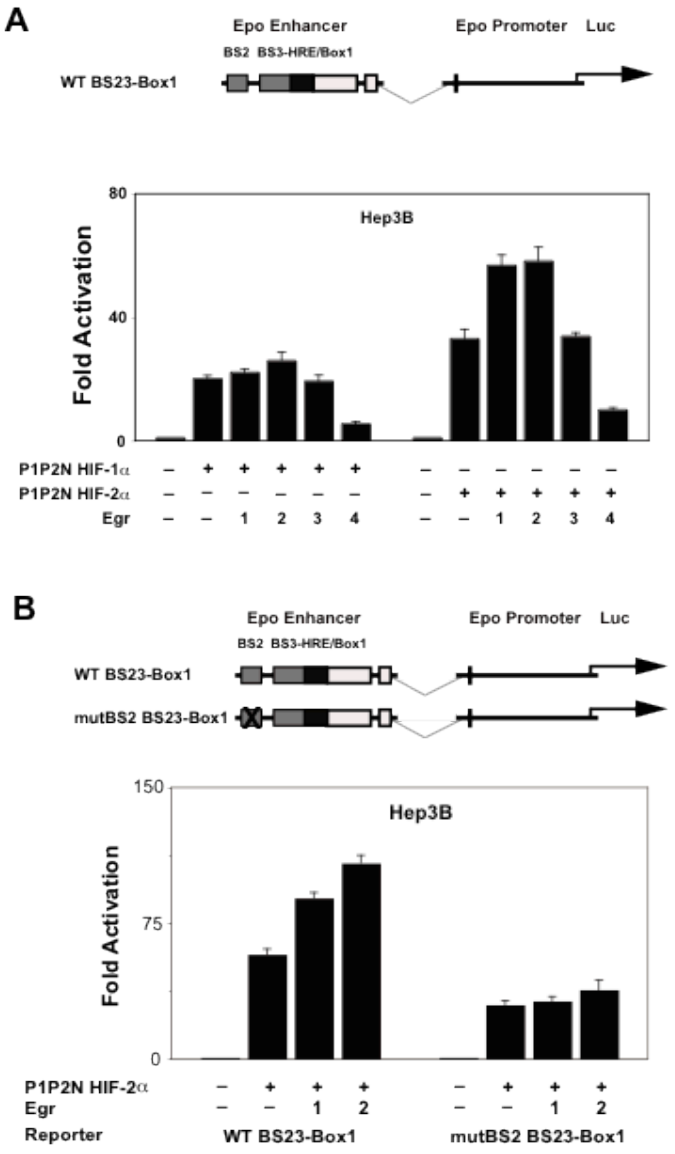


Figure 2. EGR cofactors confer HIF-2α selective activity. (**A**) *Epo* enhancer reporter activity after overexpression of EGR transcription factors with P1P2N HIF-1α and HIF-2α. EGR1 and EGR2 confer HIF-2α-selective activation. (**B**) Contribution of BS2 element to EGR-dependent reporter activity. BS2 element is required for EGR1 and EGR2 modulation of HIF-2α activity.

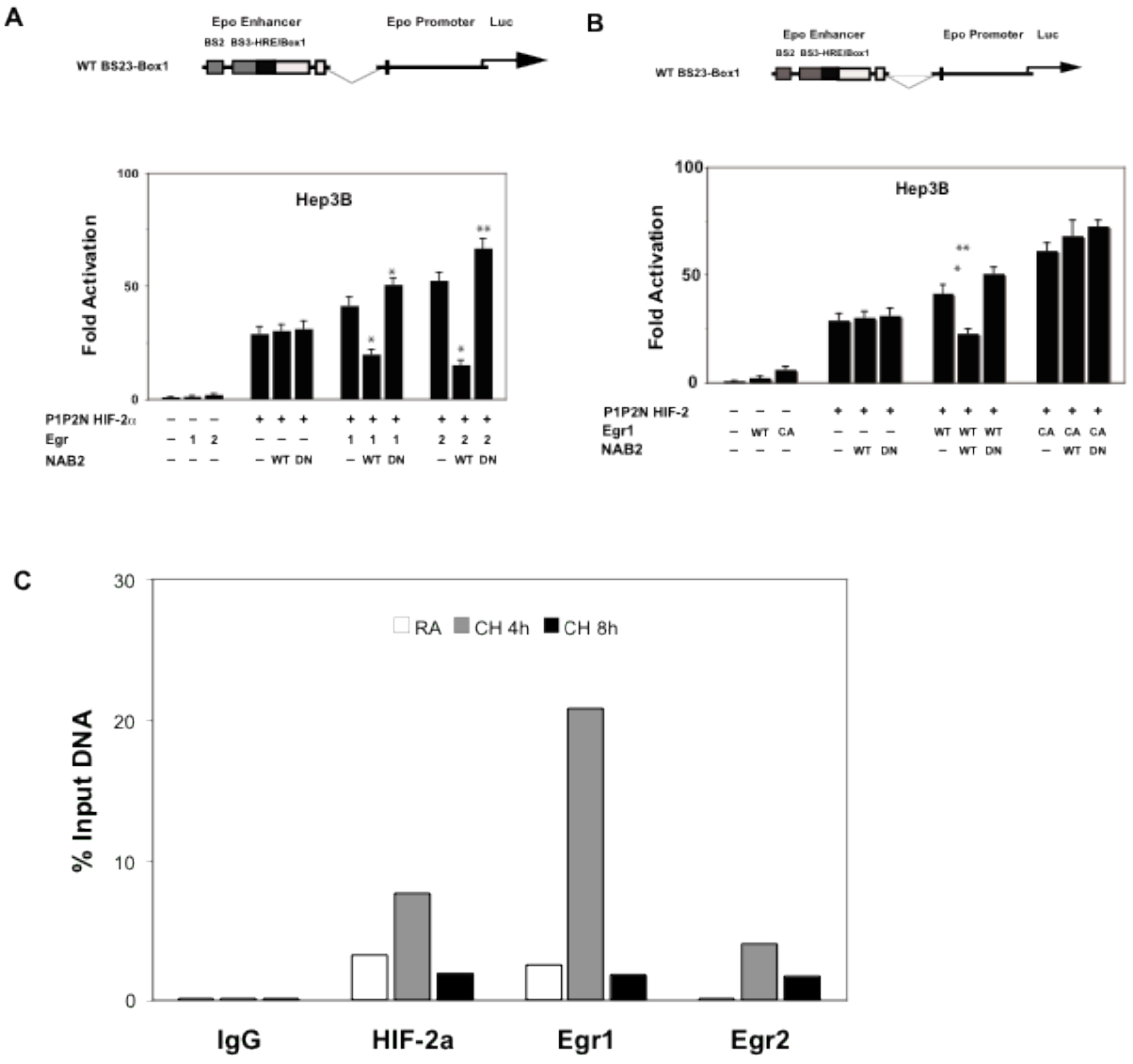


Figure 3. EGR cofactors bind to *Epo* enhancer. (**A**) *Epo* enhancer reporter activity after coexpression of EGR transcription factors, P1P2N HIF-2α, and EGR corepressor NAB2. NAB2 represses reporter activity, indicating recruitment to the reporter by EGR. (**B**) Reporter assay performed with Egr1 and NAB2 containing mutations that abolish EGR-NAB2 interactions. (**C**) Chromatin immunoprecipitation of HIF-2α, EGR1, and EGR2 after 0, 4, and 8 hours hypoxia treatment (1% O_2), followed by detection of the endogenous *Epo* enhancer.

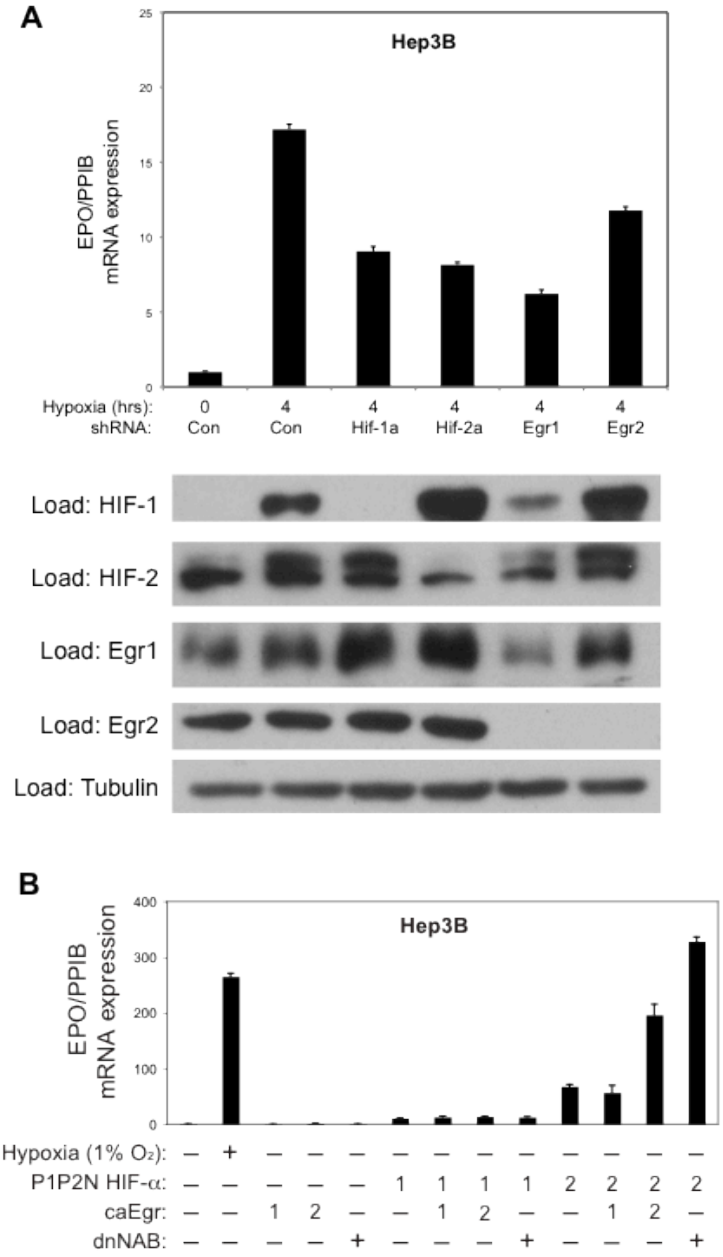


Figure 4. EGR factors influence endogenous *EPO* expression. (**A**) Real-time PCR analysis of hypoxia-induced *EPO* expression after shRNA knockdown of *HIF-1α, HIF-2α, Egr1*, and *Egr2* in Hep3B cells stably transduced with shRNA targeting constructs. *EPO* expression normalized to *cyclophilin B* (PPIB). (**B**) Effect of CA EGR1 and CA EGR2 on HIF-α-induced, endogenous *EPO* expression.

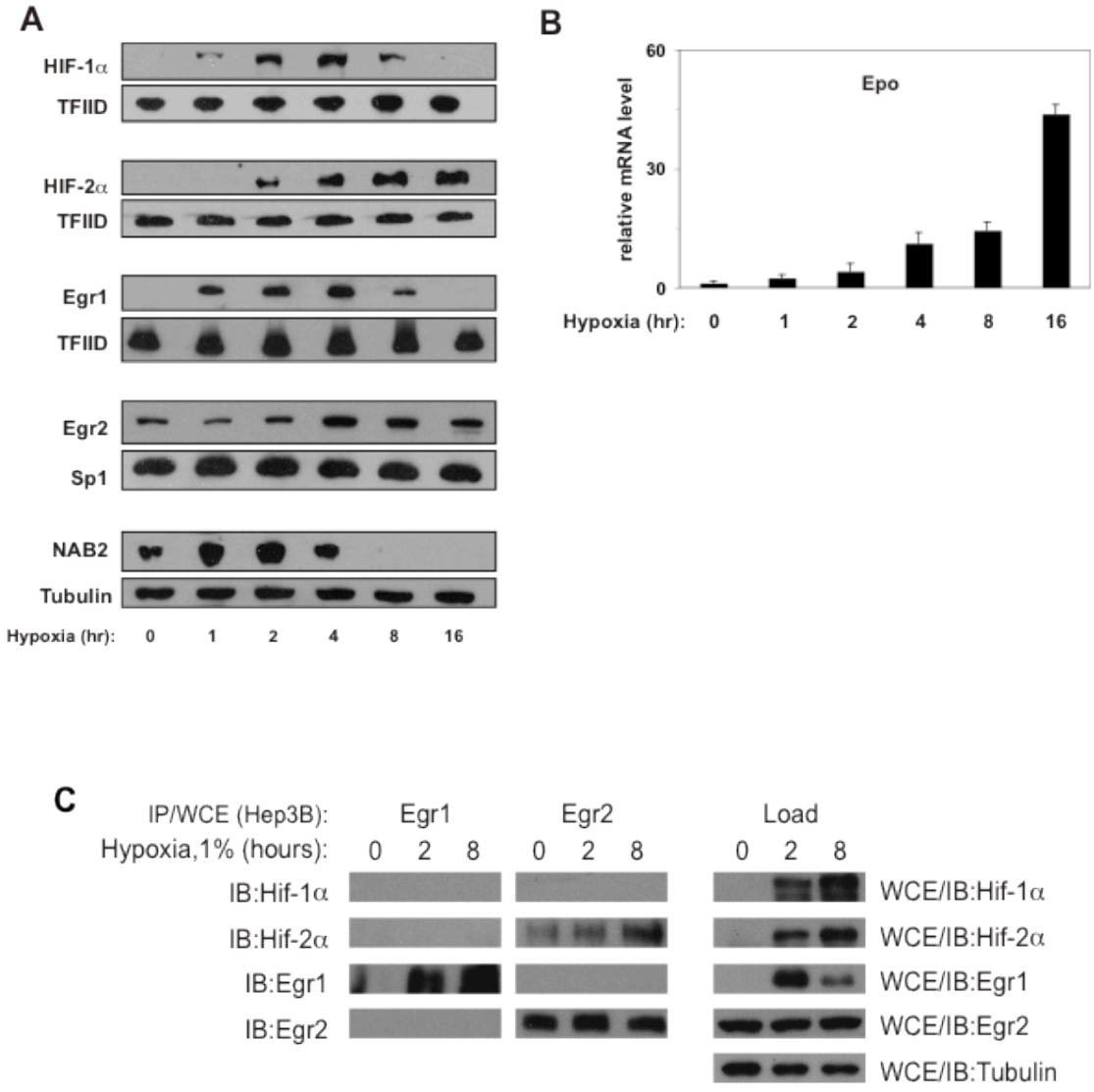


Figure 5. HIF-2α exhibits preferential interaction with EGR2 over EGR1. (**A**) Detection of HIF-α and EGR factors during a hypoxia time course by Western blot. (**B**) *EPO* expression during a hypoxia time course. *EPO* expression pattern parallels EGR2 and HIF-2α expression pattern. (**C**) Coimmunoprecipitation of endogenous EGR1 or EGR2, followed by immunoblot for HIF-1α or HIF-2α. EGR2 forms a complex with HIF-2α.

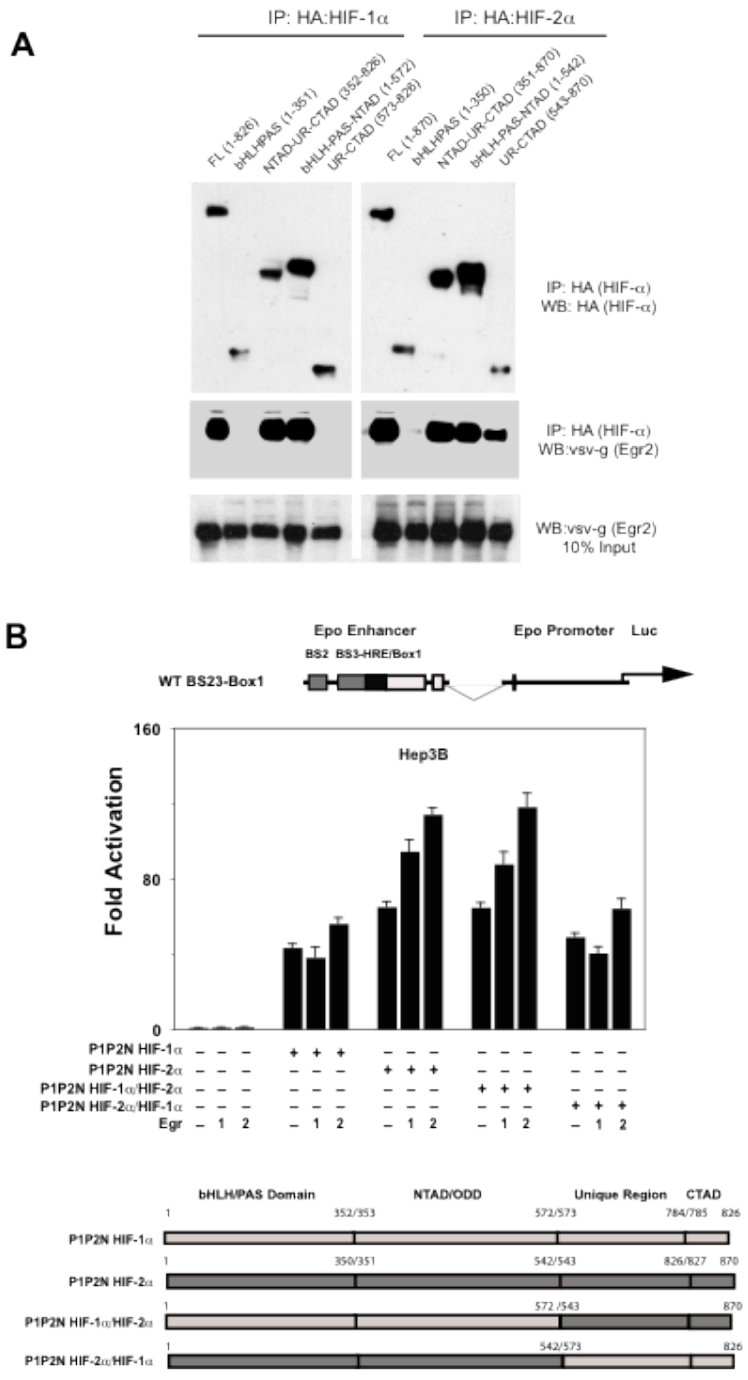


Figure 6. HIF-2α CTAD mediates interaction with EGR2. (**A**) CoIP of HA-tagged, exogenous HIF-1α or HIF-2α deletion constructs and vsv-g-tagged EGR2. (**B**) Contribution of HIF-α CTADs to EGR-augmented *Epo* expression. Hybrid HIF-α constructs coexpressed with EGR1 and EGR2, followed by real-time PCR analysis of *Epo* expression.

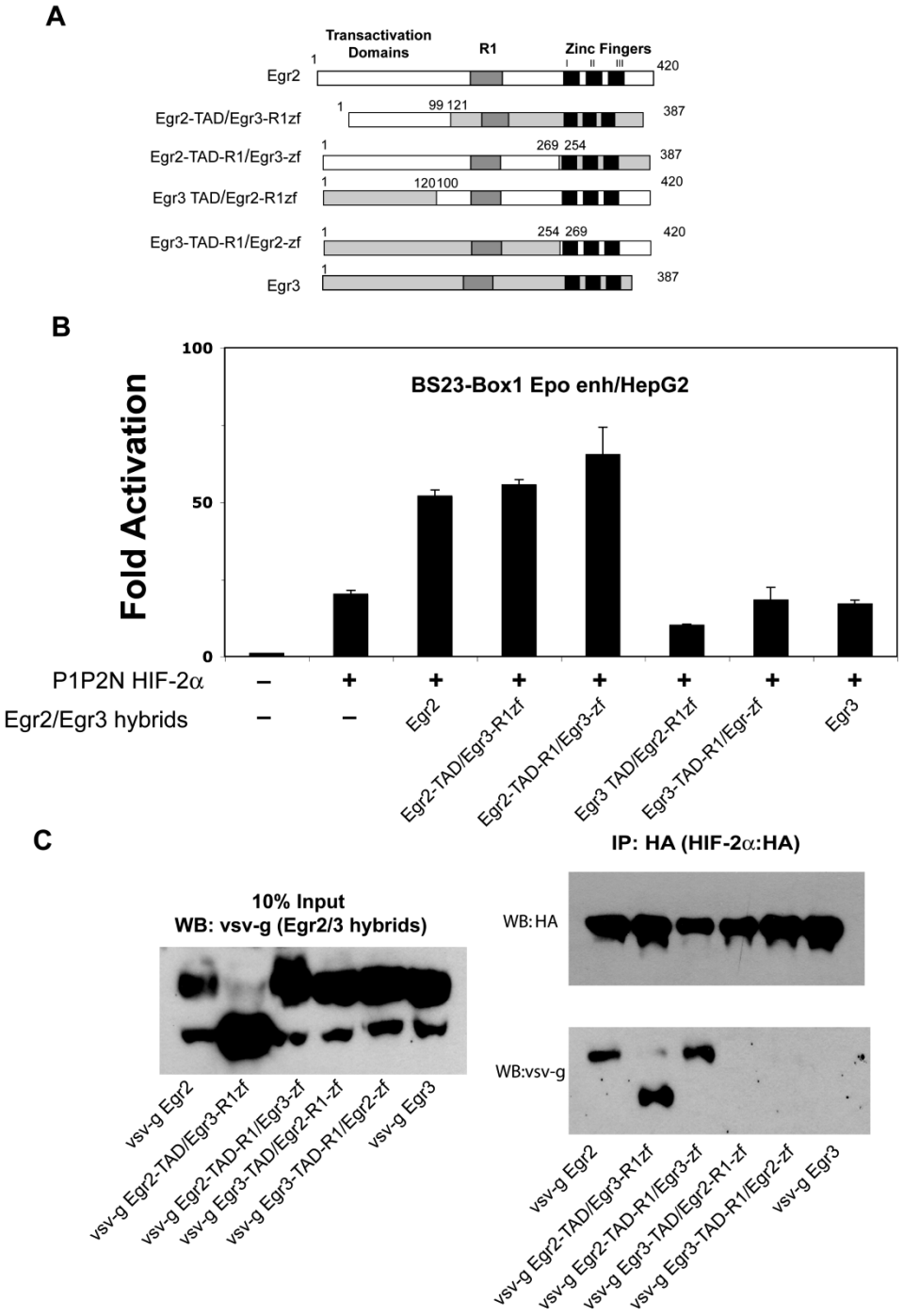


Figure 7. EGR2 TAD mediates interaction with HIF-2α. (**A**) Hybrid EGR2/EGR3 construct maps. TAD (transactivation domain), R1 NAB2 interaction domain, and zinc finger DNA binding domains analyzed through domain swapping. (**B**) Analysis of *Epo* expression after coexpression of P1P2N HIF-2α and EGR2/3 hybrids. (**C**) CoIP of exogenous, HA-tagged HIF-2α, followed by immunoblot for vsv-g-tagged EGR2/3 hybrids.

Discussion

EPO activation shows a preference for HIF-2α over HIF-1α. Though HIF-1α levels increase dramatically during hypoxia, similar to the increase in *EPO* expression, HIF-2α is the main regulator despite the minimal increase in protein levels, indicating unknown mechanisms of activation, such as the interaction of a coactivator. I have identified EGR1 and EGR2 as hypoxia-induced coactivators that confer HIF-2α-selective activity to the *EPO* enhancer. We have shown that EGR1 and EGR2 coactivate *EPO* expression during hypoxia and that this activity is dependent on HIF-2α activation, but EGR2 has a stronger effect. That neither EGR can activate transcription alone indicates a synergistic partnership with HIF-2α. We have identified an element within the *Epo* 3' enhancer required for EGR activity, labeled BS2. Interestingly, BS2 corresponds to a region identified previously (Semenza, Nejfelt et al. 1991), named OL-1 in that study, which bound strongly to an unknown factor in the nuclear extracts of anemic kidney and liver. Endogenous *EPO* expression during hypoxia is reduced after knockdown of EGR1 or EGR2 and increased after co-expression of HIF-2α and EGR2. Using endogenous CoIP, EGR2 was shown to physically interact with HIF-2α. This interaction is mediated through the HIF-2α CTAD, and replacing it with the HIF-1α CTAD results in loss of synergistic activation. The EGR2 transactivation domain is also necessary for interaction with HIF-2α and synergistic activation.

The data allude to a repressor element in Box 3 (Fig. 1B). This region has been analyzed previously, though no repressive function was detected(Fig. 1B, BK vs. BJ from (Semenza and Wang 1992)). The reporter used in that study contained an SV40 promoter from the pSVcat reporter vector, while our construct utilizes the endogenous *Epo* promoter.

The endogenous promoter has been shown to be required for full hypoxic induction of *Epo* (Blanchard, Acquaviva et al. 1992, Galson, Tsuchiya et al. 1995). The use of an exogenous promoter may have masked the contribution of the Box 3 region in that study.

The role of EGR1 in *EPO* induction has been previously investigated (Gess, Wolf et al. 1997). Though that study did not find a link between EGR1 and *Epo* activation, their data does not contradict ours. They determined that an up-regulation in *EGR1* expression, likely due to the stress involved in establishing a primary hepatocyte culture from rat livers, did not lead to an increase in *Epo* transcripts. This is in agreement with our finding that EGR1 and EGR2 are unable to activate *Epo* alone, as well as the data showing endogenous EGR2 has a more pronounced effect.

Ohigashi et al postulated the involvement of an autocrine mechanism in the hypoxia-induced, exponential increase in *EPO* production (Ohigashi, Yoshioka et al. 1996). In that study, they noted that endogenous *EPO* expression in Hep3B cells was increased after administration of recombinant human EPO in a dose-dependent manner. Although Epo receptor (EpoR) expression was unaffected, this upregulation was mediated through it. EGR2 has been shown to be positively regulated by EPO (Cervellini, Annenkov et al. 2013), and this effect occurs through the EpoR (Mengozzi, Cervellini et al. 2012). *EGR1* expression has also been shown to be activated by EPO through the EpoR (Schulze, Buchse et al. 2008, Inbar, Cohen-Armon et al. 2012). Taken together along with our data showing EGR-mediated upregulation of *EPO* expression, these studies suggest a positive feedback regulation mechanism of *EPO* through EGR proteins. EPO produced initially would act in an autocrine manner to stimulate *EGR1/EGR2* expression in EPO-producing cells. This in turn

would up-regulate *EPO* expression even further, contributing to the exponential increase in expression characteristic during hypoxia.

Normal *EPO* expression appears to be repressed *in vivo* during extended hypoxia (Chikuma, Masuda et al. 2000). The NAB2 data presented here may indicate a mechanism for this repression. NAB2 represses *EPO* expression when coexpressed with EGR1 or EGR2 in cell culture (Fig. 3A), though it is unknown if this happens *in vivo*. After an initial upregulation of *EPO* by HIF and EGR factors, NAB2 may be recruited to the locus by enhancer-bound EGR and effectively turn off expression. This mechanism would likely not be present in Hep3B cells as opposed to *in vivo*, as NAB2 protein levels diminish after early hypoxia, and *EPO* expression is not down-regulated (Fig. 5A). This putative mechanism may be a self-regulatory effect of EPO itself. As EPO has been shown to activate *EGR1* and *EGR2* expression, EGR1/2 in turn would activate NAB2, which would repress *EPO* through enhancer-bound EGR. This cascade would serve to first hyperactivate *EPO* expression and subsequently deactivate it.

While EGR factors significantly enhance HIF-mediated *EPO* transcription, and EGR1 and EGR2 are hypoxia-inducible, the precise mechanism for their involvement remains to be elucidated. NAB2 may first be recruited early during hypoxia and play a role in activation. NAB2 has been shown to act with EGR as a coactivator of luteinizing hormone (Sevetson, Svaren et al. 2000) and interleukin-2 (Collins, Wolfraim et al. 2006).

EGR binding may augment *EPO* induction by improving HIF-2α-specific DNA binding kinetics, thus conferring preference for HIF-2α over HIF-1α. Possibly, the complex formed by EGR2 and HIF-2α may bind to DNA more tenaciously than HIF-2α alone or HIF-

1α due to the additional DNA interaction point. Alternatively, EGR may alter DNA binding specificity. The binding site affinity of HOX proteins was shown to be affected by binding of cofactor EXD (Slattery, Riley et al. 2011). A similar mechanism may exist by which EGR2 binding with HIF-2α increases the affinity for the specific sequence of the *EPO* enhancer HRE.

We have shown that EGR1 and EGR2 can act synergistically with HIF-2α on *EPO* expression, but neither EGR can activate *EPO* alone. They may act to relax the chromatin environment, establishing euchromatin through remodeling complexes, but may not act to recruit the necessary factors to initiate transcription at that particular gene. Recruitment of chromatin remodeling factors by EGRs may be either direct or through an associated protein, such as NAB2. Local remodeling would enable HIF-2α and its cofactors easier access to the enhancer, increasing transcriptional activation, but would not be required for basal activation. Thus, HIF-2α would be the major transcription factor, able to activate transcription alone. But the establishment of euchromatin by EGR or EGR/NAB2 would increase its efficiency, allowing for the strong activation seen *in vivo*. Brahma and Brahma/SWI2-Related Gene 1 (BRM/BRG-1) are ATPase subunits of the SWI/SNF nucleosome remodeling complex and are possible candidates for EGR-mediated recruitment. Both have been shown to be recruited to the *EPO* enhancer and are required for full *EPO* induction (Wang, Zhang et al. 2004). And as with EGR, *EPO* expression is still activated in their absence. Though BRM has a compensatory effect during *BRG-1* knockdown, *BRM* knockdown causes a modest decrease in *EPO* levels, which is further reduced in a *BRM/BRG-1* double knockdown. In both cases, *EPO* expression is not lost completely. *BRM/BRG-1* knockdown results in a reduction in

RNA Polymerase II recruitment to the *EPO* promoter (Wang, Zhang et al. 2010). This supports their role of creating a more permissive chromatin environment. But this remodeling activity appears to be separate from the activity of acetyltransferases p300 and CBP, which are recruited through the HIF transactivation domains. Histone acetylation is unaffected by *BRM/BRG-1* knockdown, p300 knockdown has a minor effect on BRM/BRG-1 recruitment to the *EPO* enhancer, and knocking down both p300 and *BRM/BRG-1* reduces *EPO* induction further than either single knockdown. This apparent separation of activity indicates that each protein complex is recruited independently of the other. While HIF and HNF-4α recruit KAT cofactors, SWI/SNF recruitment may be mediated through EGR.

To test the hypothesis that EGR1 or EGR2 recruits the SWI/SNF chromatin remodeling complex to the *EPO* enhancer, co-immunoprecipitation would be used to detect endogenous interaction between the proteins under normoxic and hypoxic conditions. Under this hypothesis, an EGR1 or EGR2 would immunoprecipitate either BRG1 or BRM, but a hybrid containing the EGR3 TAD likely would not, as that construct is not synergistically active. ChIP experiments have shown that EGR1 and EGR2 (Fig. 3C), as well as BRG-1 and BRM (Wang, Zhang et al. 2004, Wang, Zhang et al. 2010), are recruited to the *EPO* enhancer during hypoxia. If EGR is required to mediate this recruitment, *EGR1* or *EGR2* knockdown using our stable cell lines should ablate BRM/BRG-1 recruitment. Additionally, I would expect the effect of knockdown or over-expression of SWI/SNF on *EPO* expression to be lost after *EGR1* or *EGR2* knockdown due to loss of recruitment.

CHAPTER FOUR
Results

HYPOXIA-INDUCED REGULATION OF HIF-2A ACETYLATION

An Acetate/ACSS2 switch signals acetylation-dependent HIF-2α activation

HIF-2α activity is dependent on a post-translational modification mechanism involving acetylation and deacetylation. During hypoxia, the lysine acetyltransferase CBP acetylates HIF-2α (Chen, Xu et al. 2012), which is subsequently deacetylated by the class III deacetylase SIRT1 (Dioum, Chen et al. 2009). Both activities are required for HIF-2α signaling. The activity of SIRT1, as an NAD+ dependent enzyme, depends on the redox state of the cell, which is affected by hypoxia and metabolic conditions. As HIF-2α is also responsive to these conditions, their effect on SIRT1 provides a mechanism that induces deacetylation-dependent modulation of HIF-2α activity. The mechanism that initiates CBP-catalyzed acetylation of HIF-2α is less clear. Asparaginyl hydroxylation in the CTAD of HIFs by FIH1, which blocks recruitment of KATs such as CBP, is inhibited by hypoxia (Lando, Peet et al. 2002, Lando, Peet et al. 2002) and high levels of ROS (Masson, Singleton et al. 2012). Yet, this mechanism is not significant in HIF-2α activity (Yan, Bartz et al. 2007), nor is the CTAD required for CBP recruitment during hypoxia (Chen, Xu et al. 2012).

Because CBP requires acetyl CoA as a substrate, acetyl CoA availability may be a regulatory factor. Multiple enzymes produce acetyl CoA in the cell. Pyruvate dehydrogenase (PDH) converts pyruvate into acetyl CoA for entry into the TCA cycle. But during hypoxia, this cycle is shut down in favor of lactic acid fermentation. PDH, the rate limiting enzyme for

the TCA cycle, is inhibited by PDK1, a HIF-1α target gene, during hypoxia (Kim, Tchernyshyov et al. 2006, Papandreou, Cairns et al. 2006), and therefore unlikely to play a role in hypoxic regulation of HIF-2α. Acetate-dependent acetyl CoA Synthetase 1 (ACSS1) is localized mainly to the mitochondrial matrix (Fujino, Kondo et al. 2001). ATP-citrate lyase (ACLY) (Zaidi, Swinnen et al. 2012) and Acetyl CoA Synthetase 2 (ACSS2) (Loikkanen, Haghighi et al. 2002) are both found mainly in the cytoplasm, but they are detectable in the nucleus (Wellen, Hatzivassiliou et al. 2009), likely to provide acetyl CoA substrate for histone acetylation. We have discovered that ACSS2 is the acetyl CoA synthetase that regulates HIF-2α acetylation during hypoxia and glucose deprivation (Xu, Nagati et al. 2014). I investigated the mechanism which signals ACSS2 regulation of HIF-2α stress signaling in *EPO* regulation during anemia in part 1, and in regulating tumor cell properties in part 2.

Results, part 1

ACSS2 is required for regulation of HIF-2α acetylation-dependent activity

Because HIF-2α activity is regulated by acetylation by CBP, the acetylation kinetics were first defined. Hep3B cells were exposed to a hypoxia time course and assayed for HIF-2α acetylation or interaction with CBP and p300. HIF-2α acetylation peaks at 2-4 hours of hypoxia (Fig. 8A), tapering off by 8 hours. In coincident with this, CBP association with HIF-2α is detected at 2 hours hypoxia, but not at 8 hours (Fig. 8B). P300, on the other hand, is coimmunoprecipitated with HIF-2α at 8 hours hypoxia, but not 2 hours. As HIF-2α activity is restricted to the nucleus, nuclear localization of acetyl CoA synthetase enzymes

was determined after incubation in low oxygen. Subcellular fractionation was performed at 2 and 8 hours of hypoxia treatment (Fig. 8C). ACLY was only detected in the cytoplasm at these times. ACSS2, on the other hand, was detected in the nuclear fraction after 2 hours of hypoxia, concurrent with HIF-2α acetylation and CBP complex formation.

To determine if acetyl CoA synthetase activity is required for HIF-2α acetylation, *ACLY*, *ACSS2*, and *ACSS1* were knocked down by siRNA, and HIF-2α acetylation status determined (Fig. 8D). Only knockdown of *ACSS2* resulted in loss of HIF-2α acetylation. ACSS2, but not ACLY, is required for CBP complex formation, as knockdown of ACSS2 results in loss of CBP coimmunoprecipitation with HIF-2α (Fig. 8E). Interestingly, p300 is detected in complex with HIF-2α after 2 hours of hypoxia when *ACSS2* is knocked down, and therefore CBP interaction is lost. This may indicate that under normal activation, CBP association displaces or blocks p300 interaction with HIF-2α. Functionally, hypoxia-driven *EPO* expression is significantly reduced by loss of ACSS2, but not ACSS1 or ACLY (Fig. 8F). Expression of other genes activated in part (*VEGFA*, *SERPINE1*, and *SLC2A1*) or solely (*MMP9*) by HIF-2α in Hep3B cells (Fig. S2A, B, C (Xu, Nagati et al. 2014)) is reduced by *ACSS2* knockdown, but not *ACSS1* or *ACLY* knockdown (Fig. S2D (Xu, Nagati et al. 2014)). ACSS2 activity is HIF-2α-specific. While *ACSS2* knockdown abrogated HIF-2α recruitment to the *EPO* enhancer, it did not affect HIF-1α occupancy to the *EPO* enhancer or that of HIF-1α-specific target *PGK1* (Fig. S1 (Xu, Nagati et al. 2014)). Additionally, PGK1 activation by is unaffected by *ACSS2* knockdown (Fig. S2E (Xu, Nagati et al. 2014)). Taken together, these data indicate that ACSS2 is the source of acetyl CoA for HIF-2α acetylation-dependent activity.

ACSS2 is required for in vivo HIF-2α acetylation and target gene activation

HIF-2α is acetylated in WT mouse kidney under hypoxia, but not in *Acss2* KO mice (Fig. 9A). Under hypoxia, HIF-2α is recruited and CBP co-recruited to the Epo enhancer in the kidney as determined by ChIP assay (Fig. 9B). Yet, in *Acss2* KO mice, recruitment of both factors is lost. Consequently, *Epo* expression (Fig. 9C) and serum EPO levels (Fig. 9D), both of which increase dramatically during hypoxia in WT mice, are severely reduced in *Acss2* KO mice.

EPO is upregulated during anemia to replenish red blood cell mass. *Acss2* KO mice have a significantly lower basal hematocrit level (Fig. 10A). When acute anemia is induced in mice using phenylhydrazine (PHZ), *Acss2* KO mice took twice as long to recover, despite their lower basal levels. This indicates that the *Epo* response is impaired in anemia, as well as hypoxia. Indeed, after PHZ induced anemia, serum EPO levels are significantly lower in *Acss2* KO mice compared to WT mice (Fig. 10B). Additionally, *Epo* expression in the kidney (Fig. 10C) and liver (Fig. 10D) is impaired during anemia in KO mice. Expression of the HIF-1α target gene *Pgk1* is unaffected by *Acss2* knockout. As during hypoxia, HIF-2α is acetylated during acute anemia, and this is lost in *Acss2* KO mice kidney (Fig. 10E) and liver (Fig. 10F).

Acetate signals HIF-2α acetylation

Cellular acetate levels increase during hypoxia (Yoshii, Furukawa et al. 2009). As acetate is a substrate for ACSS2 production of acetyl CoA, this increase may serve as a

signal for HIF-2α acetylation and thus regulate hypoxia-driven activity. To verify an increase in acetate, levels were determined under hypoxia or anemia. In Hep3B cells, acetate levels increase during hypoxia, peaking at two hours and tapering off by 16 hours (Fig. 11A). These time points correspond to peaks in HIF-2α acetylation and CBP recruitment. In the mouse kidney, acetate levels increased within two hours of hypoxia and remained elevated after 16 hours of treatment (Fig. 11B). Additionally, acetate levels were unchanged between WT and *Acss2* KO mice. Finally, in the acute anemia model, acetate levels in mouse kidney (Fig. 11C) and liver (Fig. 11D) were elevated after four days of PHZ treatment. As under hypoxia, there was no difference in acetate levels between WT and *Acss2* KO mice.

To determine if an increase in acetate could, in fact, signal HIF-2α acetylation-dependent activity, exogenous acetate was added to cell culture media. First, the addition of acetate, but not other short chain fatty acids butyrate or propionate, induced acetylation of HIF-2α (Fig. 12A). Because hypoxia-induced HIF-2α acetylation requires CBP and ACSS2, acetate was introduced after siRNA knockdown of these factors. Knockdown of *ACSS2*, but not ACSS1 or *ACLY*, and *CBP*, but not *p300*, resulted in loss of acetate-induced HIF-2α acetylation (Fig. 12B). Acetate supplementation induced CBP-HIF-2α complex formation, while butyrate and propionate did not (Fig. 12C). This complex formation required ACSS2 (Fig. 12D) and SIRT1 (Fig. 12E). Finally, acetate supplementation resulted in nuclear translocation of ACSS2 (Fig. 12F). Taken together, these data support a conclusion that an increase in acetate, due to hypoxia or exogenously administered, acts as a signal through ACSS2 for HIF-2α acetylation and complex formation with CBP.

ACSS2/acetate regulation of HIF-2α requires intact acetylation

To further verify the role of HIF-2α acetylation, a HIF-2α mutant in which the three lysines (K3) determined to be acetylated during hypoxia and critical for CBP- and SIRT1-dependent coactivity (Dioum, Chen et al. 2009, Chen, Xu et al. 2012) were mutated to arginine (R3) was employed. Both hypoxia (Fig. 13A) and acetate supplementation (Fig. 13B) failed to induce acetylation of the HIF-2α R3 mutant. CBP did not form a complex with the R3 mutant after hypoxia (Fig. 13C) or acetate addition (Fig. 13D). Knockdown of *HIF-2α* results in loss of *EPO* activation during hypoxia. The R3 mutant failed to rescue this loss indicating the importance of these residues for activity (Fig. 13E). While knockdown of *ACSS2* impaired the ability of endogenous HIF-2α, or a WT (K3) rescue contruct, to activate *EPO* expression, *ACSS2* knockdown had no effect on *EPO* levels after rescue with the R3 mutant. Thus, the observed regulation of WT HIF-2α activity by ACSS2 requires the lysine residues determined to be acetylation sites.

Acetate administration augments in vivo hematopoiesis during anemia

Because of the observed effect of acetate supplementation on HIF-2α acetylation and CBP interaction, we hypothesized that acetate administration may increase HIF-2α activity in anemic mice through activation of *Epo* expression. Thus, mice treated with PHZ to induce acute anemia were given acetate orally as glyceryl triacetate (GTA) or intraperitoneally as a buffered sodium acetate solution (Fig. 14A, top and bottom, respectively), and hematocrits were determined for 16 days. Mice treated with acetate recovered more quickly than untreated mice, or those treated with vehicle, butyrate, or propionate. To determine the *in*

vivo requirement of ACSS2 for acetate-induced recovery, WT and *Acss2* KO mice were compared. After PHZ-induced anemia, WT mice recovered more quickly than *Acss2* null mice (Fig. 14B). And while acetate administration hastened recovery of WT mice over vehicle, acetate did not affect the recovery of *Acss2* KO mice. *Epo* mRNA levels in response to PHZ-induced anemia were elevated in the kidney and liver (Fig. S6A, B (Xu, Nagati et al. 2014)). Acetate administration resulted in both higher levels and prolonged duration of *Epo* activation in these organs. Thus, acetate improves hematopoietic recovery from induced anemia, and this activity requires ACSS2.

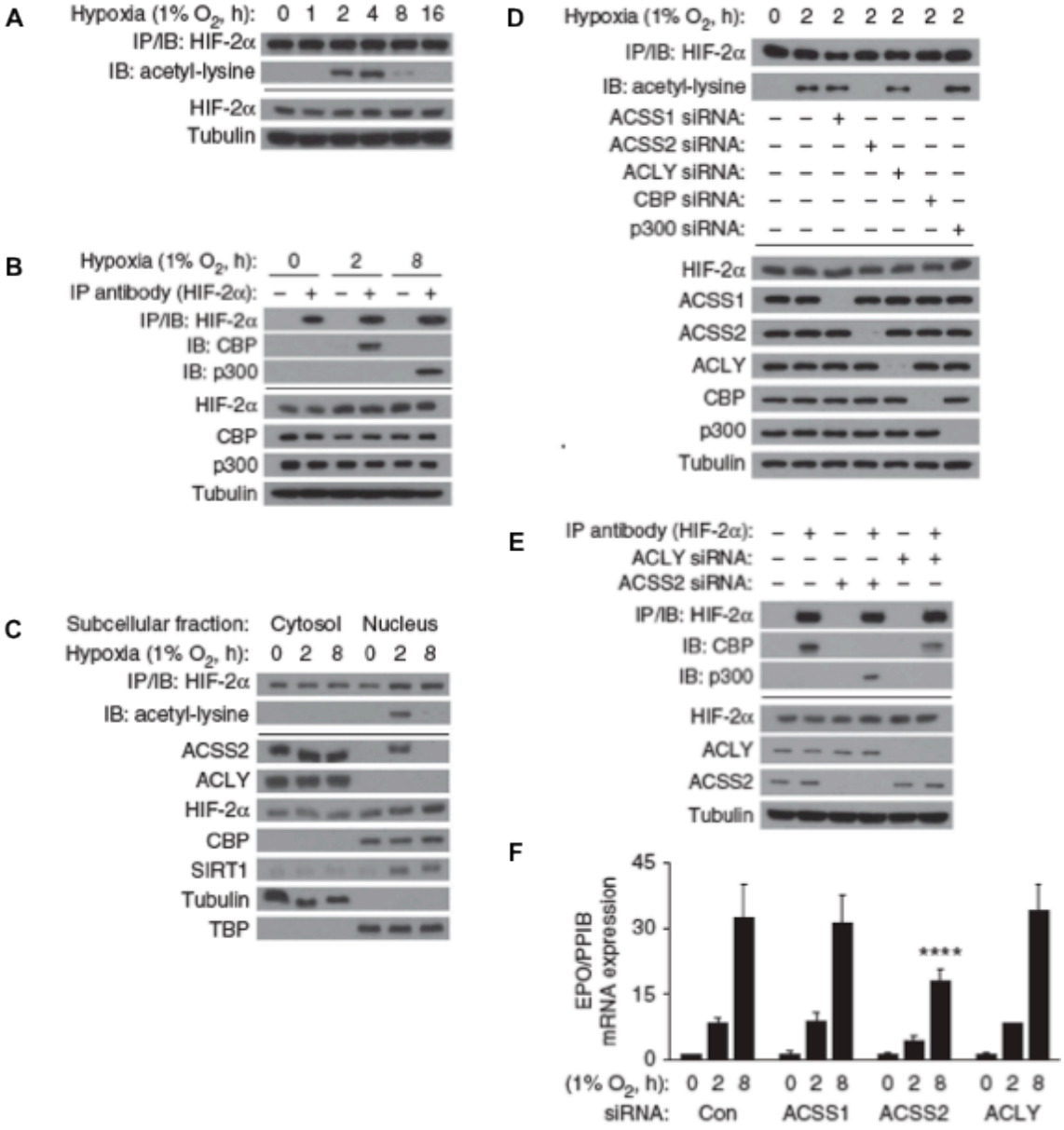


Figure 8. ACSS2 regulates hypoxa-induced acetylation response of HIF-2α. (**A**) Assay of acetylation status of endogenous HIF-2α in Hep3B cells during hypoxia time course, analyzed by IP of HIF-2α followed by immunoblot of acetyllysine. (**B**) CoIP detection of endogenous complex formation between HIF-2α and acetyltransferases CBP and p300. (**C**) Detection of nuclear localization of ACSS2 and ACLY during a hypoxia time course. (**D**) Assay of acetylation status of HIF-2α during hypoxia following siRNA-mediated knockdown of *ACSS1*, *ACSS2*, and *ACLY*. (**E**) Contribution of ACLY and ACSS2 to HIF-2α complex formation with CBP and p300 during hypoxia. (**F**) Real-time PCR analysis of hypoxia-induced *EPO* expression following siRNA-mediated knockdown of *ACSS1*, *ACSS2*, and *ACLY*.

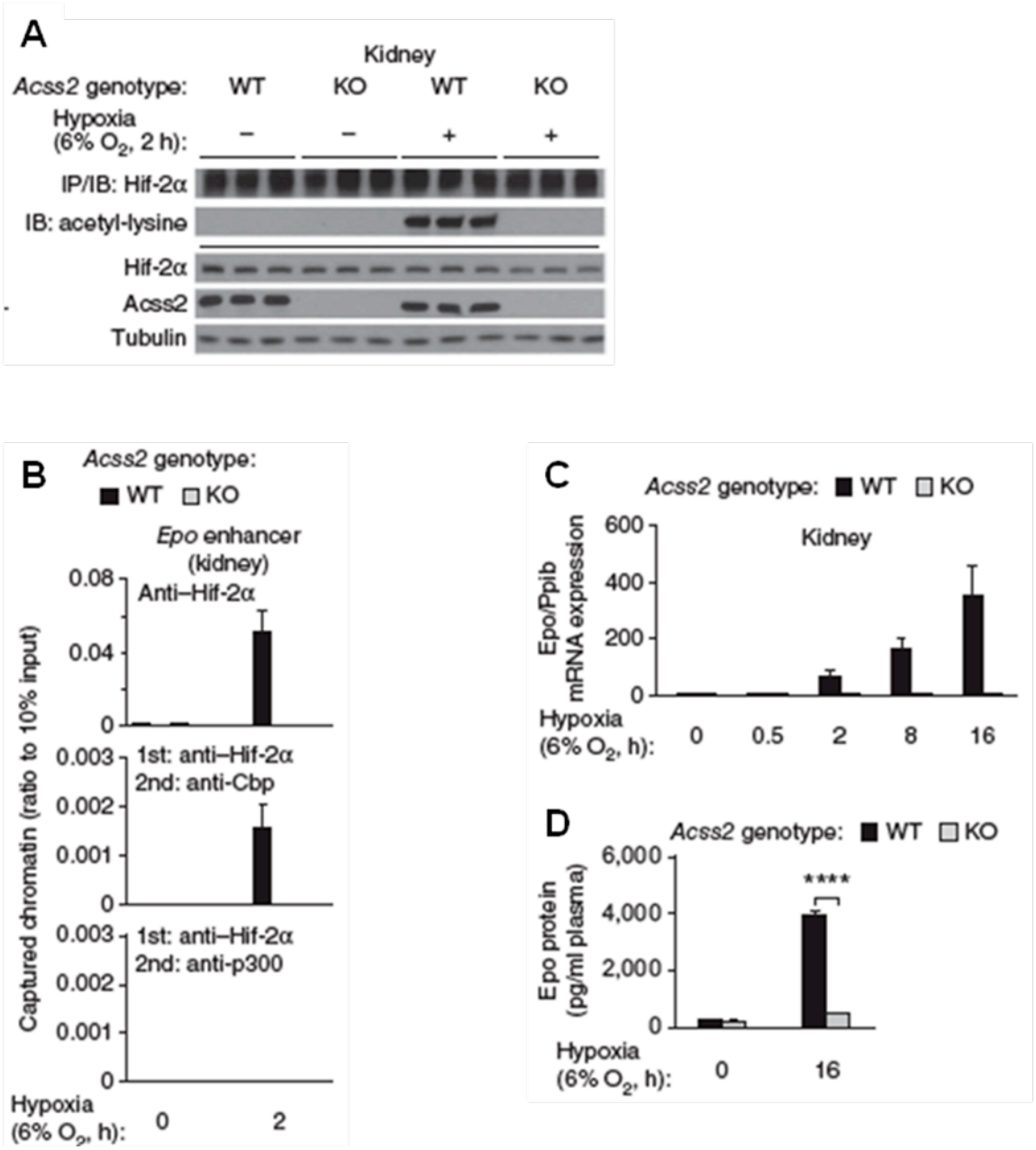


Figure 9. ACSS2 is required for hypoxia-induced, *in vivo* HIF-2α acetylation and *Epo* expression. (**A**) Assay of acetylation status of HIF-2α in WT and *Acss2* KO mouse kidney following hypoxia exposure. (**B**) Hypoxia-induced recruitment of HIF-2α and co-recruitment of CBP and p300 to the *Epo* enhancer, as determined by ChIP and sequential ChIP assays, in WT and *Acss2* KO mouse kidney. (**C**) Hypoxia-induced *Epo* expression in WT and *Acss2* KO mouse kidney. (**D**) Plasma EPO levels detected in WT and *Acss2* KO mice during normoxia and 16 hrs hypoxia.

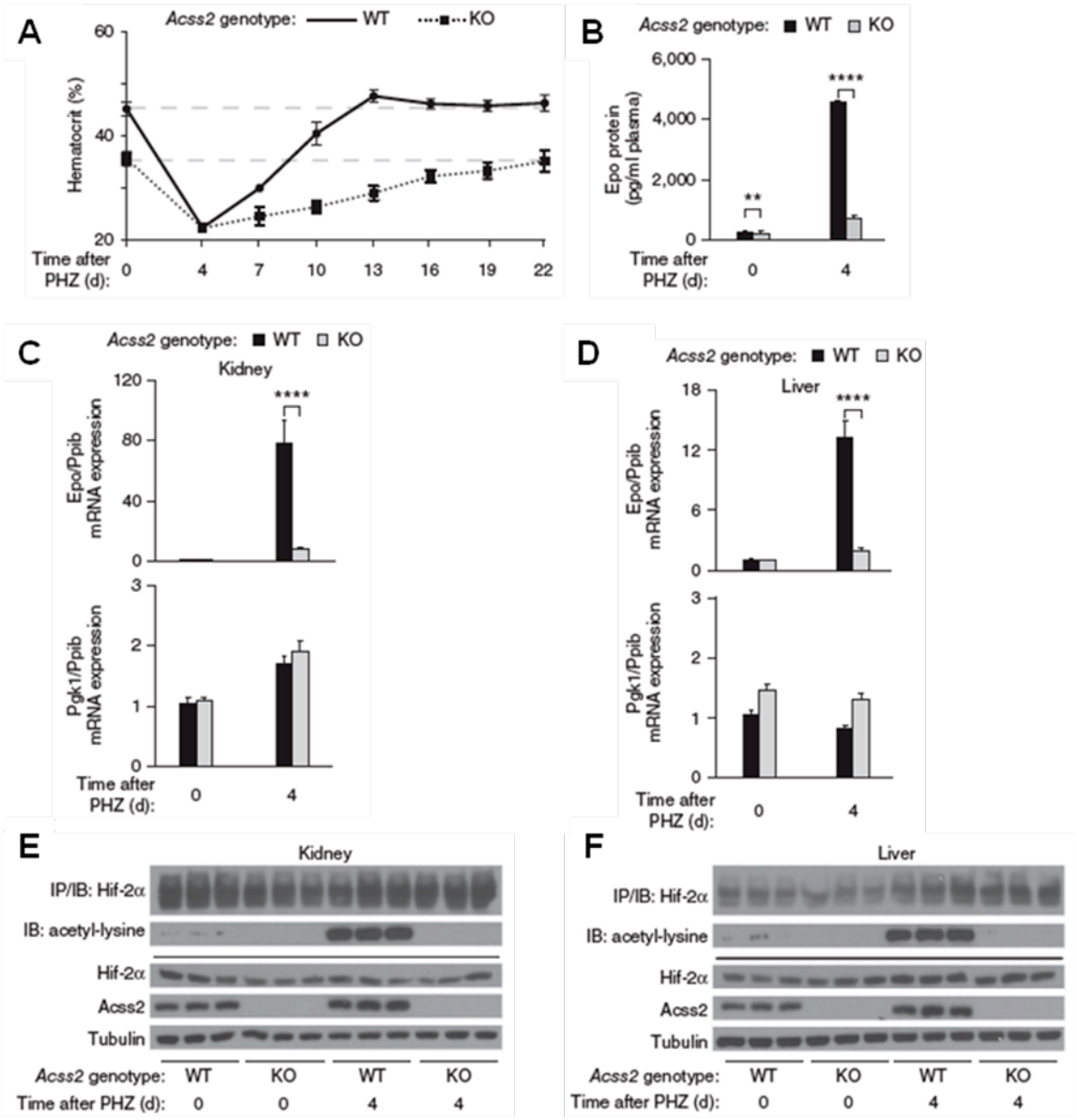


Figure 10. ACSS2 regulates *in vivo* response to acute anemia. (**A**) Hematocrit recovery analysis after phenylhydrazine (PHZ)-induced anemia in *Acss2* WT and KO mice. (**B**) Plasma EPO levels in *Acss2* WT or KO mice four days after PHZ treatment. (**C, D**) Real-time PCR analysis of *Epo* expression in kidney (**C**) or liver (**D**) of ACSS2 WT or KO mice after PHZ treatment. Expression of HIF-1α target gene *Pgk1* is unaffected by *Acss2* genotype. (**E, F**) Assay of acetylation status of HIF-2α in kidney (**E**) or liver (**F**) of *Acss2* WT or KO mice after PHZ treatment.

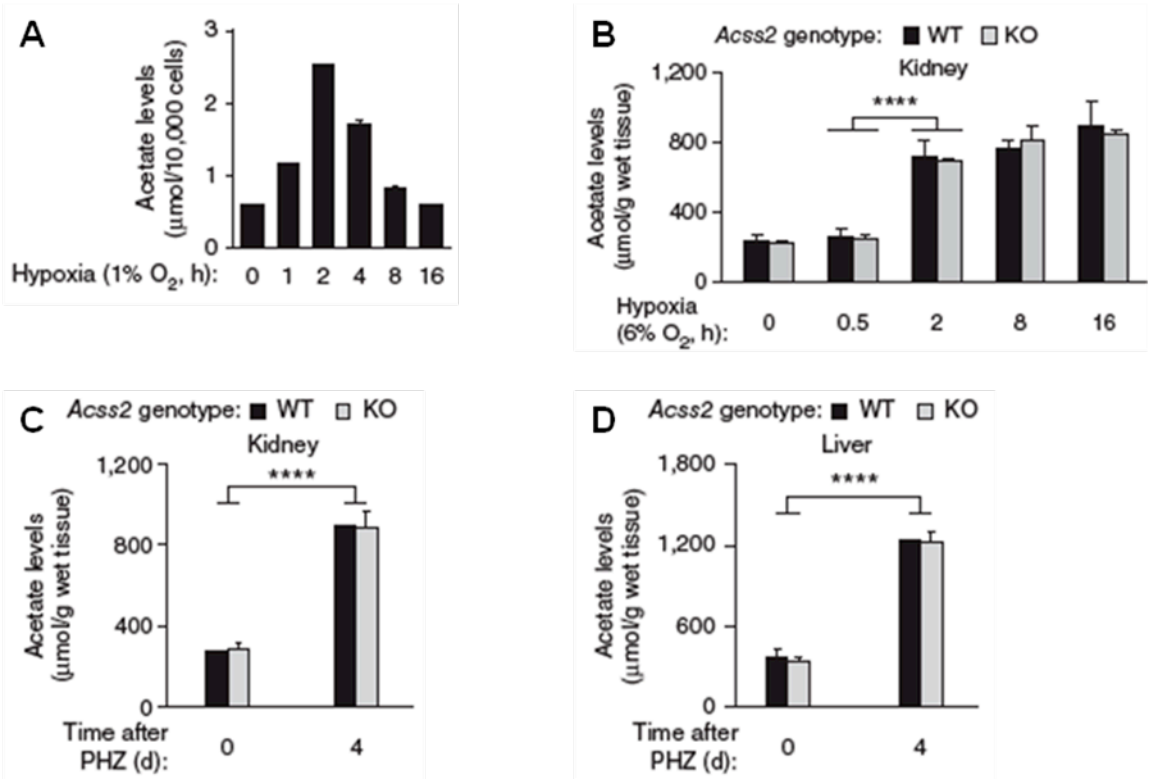


Figure 11. Cellular and *in vivo* levels of ACSS2 substrate acetate increase during hypoxia and anemia . (**A**) Acetate measurements in Hep3B cells during a hypoxia time course. (**B**) Acetate measurements in *Acss2* WT and KO mouse kidney during a hypoxia time course. (**C**, **D**) Acetate measurements in *Acss2* WT and KO mouse kidney (**C**) or liver (**D**) following PHZ-induced anemia.

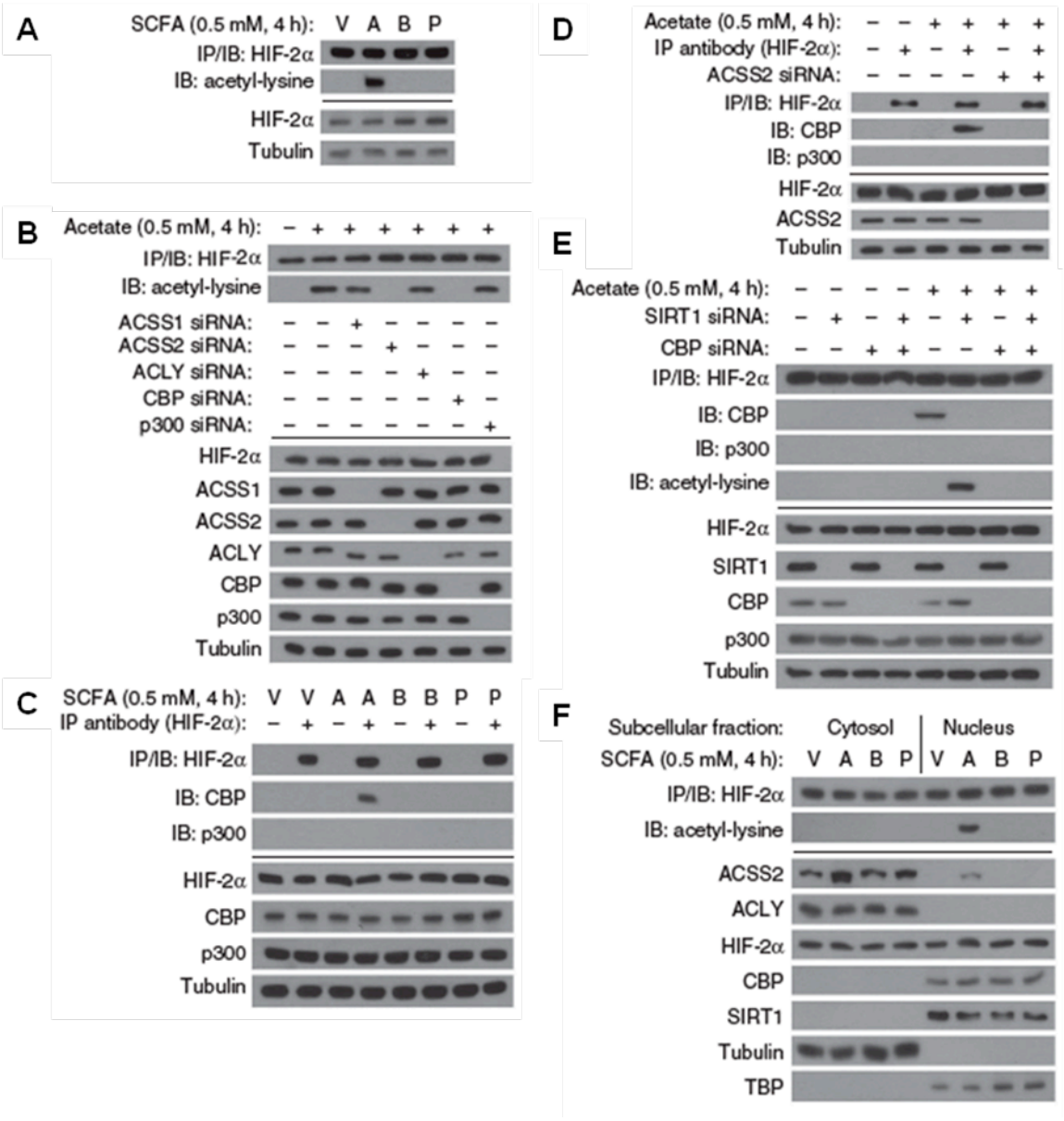


Figure 12. Acetate supplementation induces HIF-2α acetylation. (**A**) Assay of acetylation status of HIF-2α in Hep3B cells four hrs following media supplementation with vehicle (V) or short-chain fatty acids (SCFA) acetate (A), butyrate (B), and propionate (P). (**B**) Contribution of ACSS1, ACSS2, and ACLY to acetate-dependent HIF-2α acetylation, as determine by siRNA-mediated knockdown. (**C**) CoIP detection of SCFA-induced HIF-2α interaction with CBP. (**D, E**) Contribution of ACSS2 (**D**) or SIRT1 (**E**) to acetate-induced CBP-HIF-2α complex formation, as determined by *ACSS2* knockdown. (**F**) Analysis of subcellular localization of ACSS2 following SCFA supplementation.

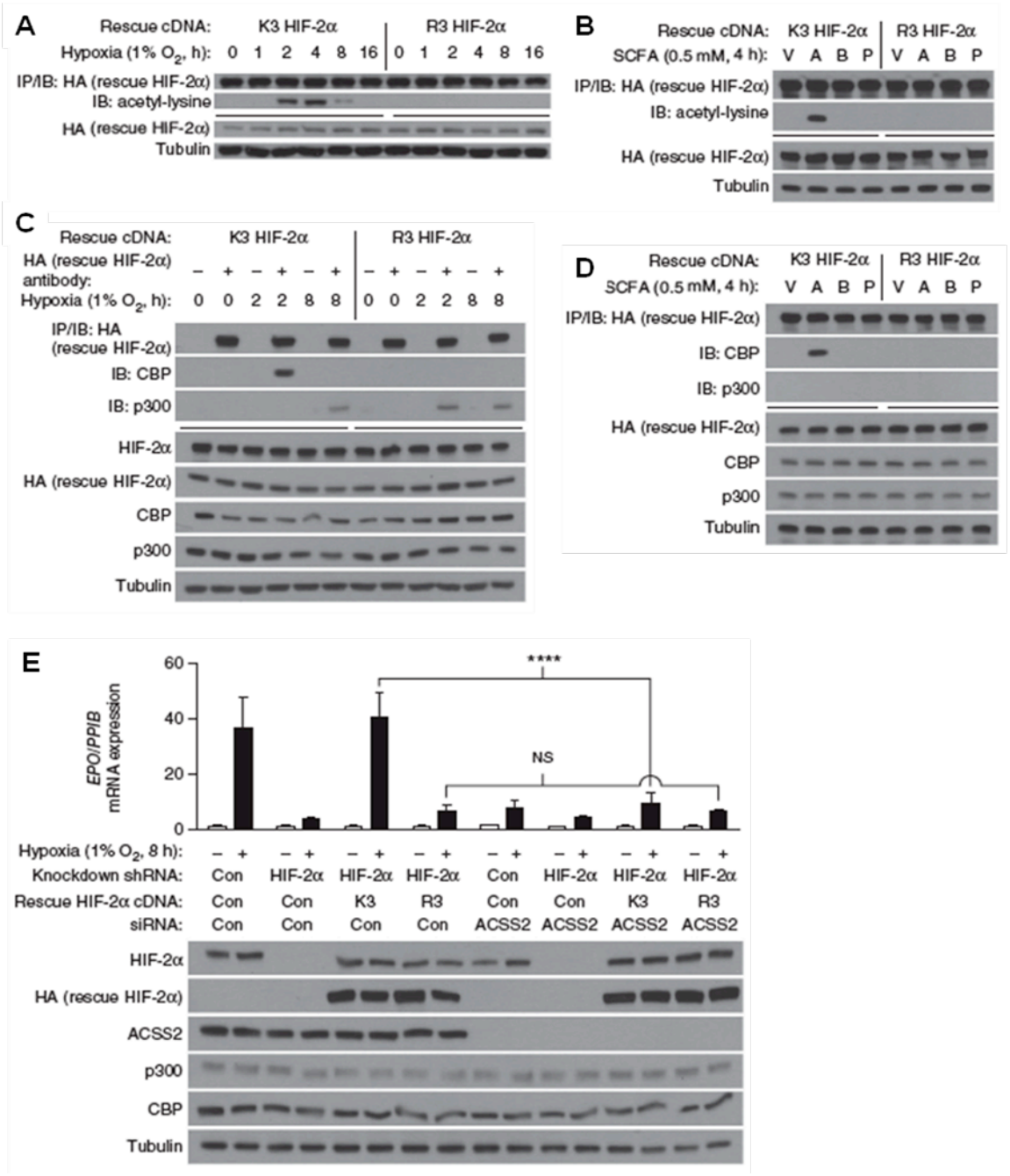
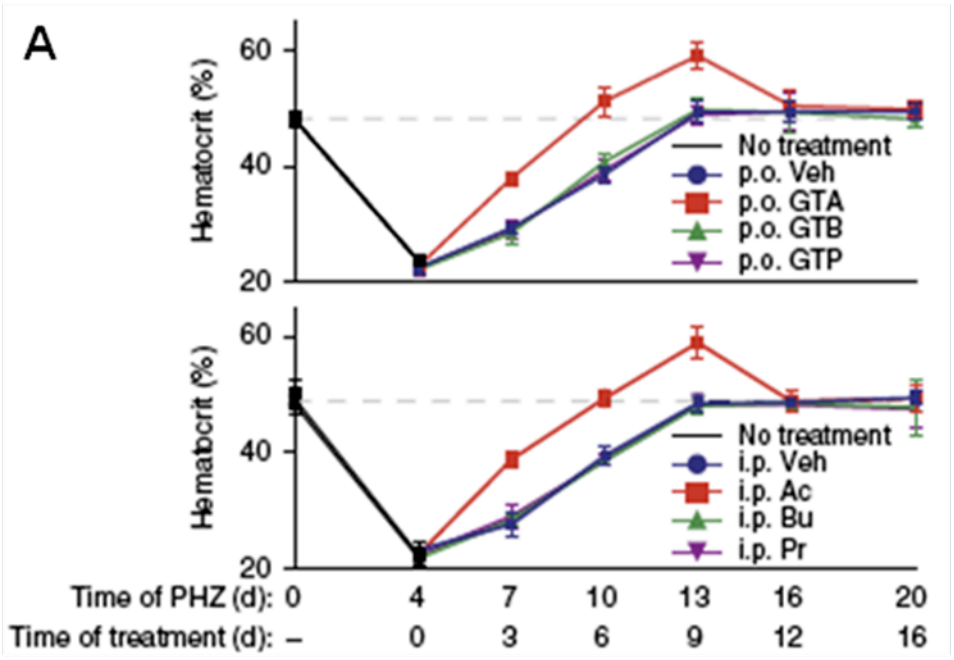


Figure 13. ACSS2/acetate regulation of *EPO* is mediated through HIF-2α acetylation. (**A**) Assay of hypoxia-induced acetylation of HIF-2α in stably transduced, Hep3B cell lines. Endogenous HIF-2α is knocked down by shRNA and rescued by resistant WT (K3) or acetylation-deficient, lysine to arginine mutant (R3) HIF-2α. (**B**) Effect of SCFA supplementation on K3 or R3 HIF-2α acetylation. (**C, D**) CoIP detection of either K3 or R3 HIF-2α complex formation with CBP or p300 during a hypoxia time course (**C**) or SCFA supplementation (**D**). (**E**) Effect of *ACSS2* knockdown on hypoxia-induced *EPO* expression following HIF-2α knockdown/K3 or R3 rescue.



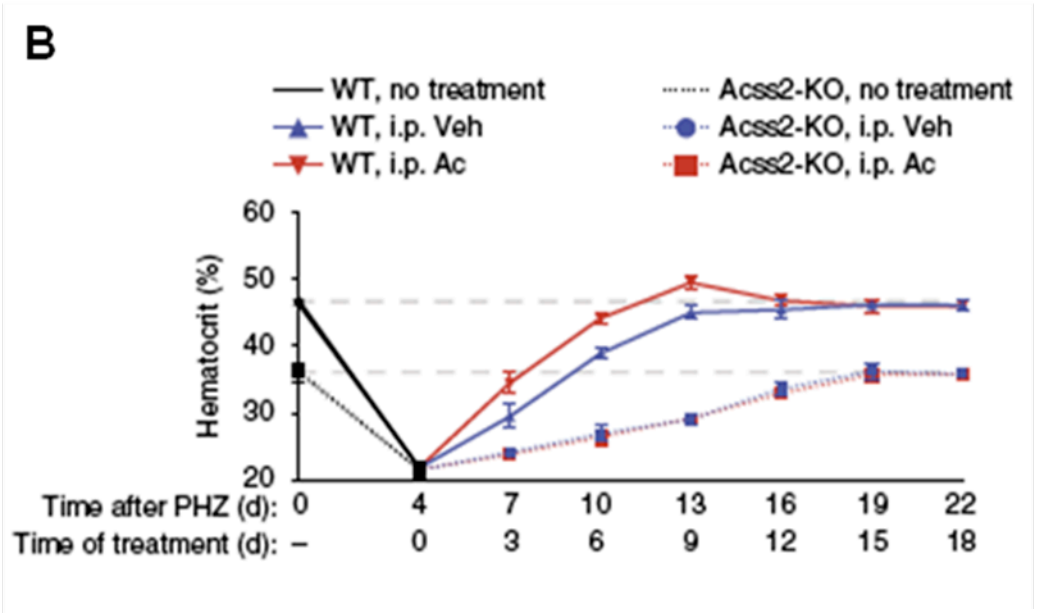


Figure 14. Acetate administration enhances ACSS2-dependent anemia recovery. (**A**) Hematocrit recovery analysis after phenylhydrazine (PHZ)-induced anemia in mice following administration of acetate, butyrate, and propionate either by oral gavage (top) as a glyceryl triester, or by intraperitoneal injection (bottom) in a buffered sodium salt solution. (**B**) Hematocrit recovery analysis in *Acss2* WT or KO mice after PHZ treatment with or without acetate administration.

Results, part 2

Glucose deprivation signals HIF-2α acetylation in a fibrosarcoma cell line

Because of the role HIFs play in tumor progression, regulation mechanisms of HIF-2α in cancer have high clinical significance. The tumor environment is characterized by stresses such as hypoxia and low glucose availability. In the human fibrosarcoma cell line HT1080, hypoxia results in increase levels of HIF-1α and HIF-2α (Fig. 15A). Similar to the pattern seen in Hep3B cells, HIF-1α levels increase dramatically under hypoxia from nearly undetectable at normoxia, while detectable HIF-2α protein increases more modestly. As regulators of glucose metabolism, HIF levels were also determined during glucose deprivation (Fig. 15B). HIF-1α remained undetectable during the entire 24 hour time course. HIF-2α levels, though detectable, also remained unchanged. But as seen with the augmentation of HIF-2α transcriptional activity in *EPO* expression during low oxygen events by acetylation (Xu, Nagati et al. 2014), HIF-2α activity may also be regulated during glucose starvation by acetylation. Therefore, this acetylation mechanism of HIF-2α was investigated in HT1080 cells. Hypoxia in Hep3B cells or mice led to an increase in acetate levels and a concurrent increase in HIF-2α acetylation and complex formation with CBP. Assay of acetate levels in HT1080 cells during hypoxia (Fig. 15C) and glucose deprivation (Fig. 15D) shows an increase in acetate concentration during these treatments, peaking at 4-8 hours or 24 hours respectively. HIF-2α was acetylated during these treatments, with peak acetylation occurring concurrently with peak acetate production (Figs. 15E and 15F). Additionally, as expected as the lysine acetyltransferase responsible for HIF-2α acetylation, CBP formed immunoprecipitates with HIF-2α in a similar temporal manner (Figs. 15G and 15H). Thus,

HIF-2α acetylation kinetics in HT1080 cells during hypoxia is similar to those seen in Hep3B cells (Xu, Nagati et al. 2014), while during glucose deprivation, however, acetylation occurs at later time points following the buildup of acetate.

ACSS2 regulates HIF-2α stress acetylation in HT1080 cells

ACSS2 generates acetyl CoA required for acetylation of HIF-2α in Hep3B cells (Xu, Nagati et al. 2014). To determine if it is also required in HT1080 cells during either hypoxia or low glucose conditions, the acetylation state of HIF-2α was determined during hypoxia (Fig. 16A) and glucose deprivation (Fig. 16B) after knockdown of Acss1, Acss2, or Acly with siRNA. Under both conditions, knockdown of Acss2, but not Acss1 or Acly, resulted in loss of HIF-2α acetylation. Additionally, knockdown of CBP, but not p300, abolished HIF-2α acetylation under hypoxia and glucose deprivation (Fig. 16A and 16B, respectively), indicating that CBP, not p300, acetylates HIF-2α in HT1080 cells. Knockdown of Acss2 followed by hypoxia (Fig. 16C) or glucose deprivation (Fig. 16D) also resulted in the loss of complex formation between HIF-2α and CBP. These data suggest that ACSS2-derived acetyl CoA is required for CBP-catalyzed acetylation under these conditions.

ACSS2 was determined to translocate to the nucleus of Hep3B cells during hypoxia concurrently with HIF-2α acetylation, apparently as a result of increased acetate levels (Xu, Nagati et al. 2014). To determine if this occurs in HT1080, cells were exposed to hypoxia (Fig. 16E) or low glucose (Fig. 16F), fractionated, and analyzed by Western blot. ACSS2 was detected in the nuclei of hypoxic cells after 4 hours and the nuclei of glucose-deprived cell after 24 hours. These time points indicate a shared mechanism of HIF-2α regulation in

hypoxic and glucose-deprived HT1080 cells, as well as hypoxic Hep3B cells, linking acetate concentration as a switch (Xu, Nagati et al. 2014) to inducing ACSS2 nuclear localization, CBP complex formation with HIF-2α, and the resulting acetylation of HIF-2α.

Acetate stimulates HIF-2α acetylation in HT1080 cells

To verify that acetate is indeed a switch regulating HIF-2α activity in HT1080, the cells were incubated in acetate-supplemented media. After a 4-hour treatment, acetate, but not other short chain fatty acids (SCFAs) propionate or butyrate, induced acetylation of HIF-2α (Fig. 17A). ACSS2 and CBP are required for acetate-induced acetylation, as knockdown of either, but not *ACSS1*, *ACLY*, or *p300*, abolished HIF-2α acetylation (Fig. 17B). CBP complex formation with HIF-2α was induced by supplementation with acetate, but not propionate or butyrate (Fig. 17C), and knockdown of Acss2 inhibited this interaction (Fig. 17D). Additionally, acetate treatment resulted in nuclear translocation of ACSS2 (Fig. 17E). SIRT1 is required for hypoxia- and acetate-mediated formation of the CBP-HIF-2α complex in Hep3B cells (Chen, Xu et al. 2012, Xu, Nagati et al. 2014). This requirement was confirmed for HT1080 cells by *SIRT1* knockdown analysis during hypoxia, low glucose, or acetate treatment (Figs. S2A, B, and C, respectively, (Chen, Xu et al. 2015)). Thus, high acetate concentrations do indeed result in HIF-2α acetylation regulation in HT1080 cells.

ACSS2, CBP and SIRT1 are required for stress-induced HIF-2α target gene activation

To determine if this acetylation mechanism is required for normal HIF-2α transcriptional activity in HT1080 cells, siRNA was used to knockdown *ACSS2*, *CBP*, and

SIRT1 during either hypoxia (Fig. 18A) or glucose deprivation (Fig. 18B), and the effect on expression of HIF-2α or HIF-1α target genes was determined. During hypoxia, expression of genes regulated by both HIF-1α and HIF-2α, *VEGFa* and *PAI1*, was partially lost during knockdown of either HIF. During glucose deprivation, on the other hand, only knockdown of HIF-2α affected expression of target genes. *PGK1*, which is solely regulated by HIF-1α, was activated during hypoxia, but not low glucose. This indicates that the HIF-2α pathway is activated during both hypoxia and low glucose, but HIF-1α is only active during hypoxia, as would be indicated by lack of detectable HIF-1α during glucose deprivation (Fig. 14B). Expression of genes regulated in part, or those regulated predominantly by HIF-2α, *MMP9* and *GLUT1*, in HT1080 cells is reduced after knockdown of *ACSS2*, *CBP*, or *SIRT1* during treatment with either hypoxia or low glucose. PGK1 is unaffected by knockdown of those proteins. Taken together, these results indicate that full HIF-2α signaling for several genes requires all components involved in the HIF-2α acetylation mechanism during both hypoxia and glucose deprivation.

Acetate regulation of HIF-2α transactivation is dependent on ACSS2 and CBP

To further investigate acetate-dependent regulation of HIF-2α activity, oxygen-stable HIFs were overexpressed in HT1080 cells, followed by knockdown of *ACSS2* and acetate supplementation (Fig. 19A). Expression of HIF target genes was then assessed. Both HIF-1α and HIF-2α activated expression of all genes, except *PGK1*, which is activated by HIF-1α. *ACSS2* knockdown resulted in reduced expression of all genes regulated by HIF-2α, while the addition of acetate resulted in higher expression of those genes. Acetate administration

did not enhance HIF-2α-dependent activation when ACSS2 was knocked down. This indicates that acetate does augment HIF-2α activity, and this requires the acetyl CoA generator ACSS2. Neither acetate supplementation nor *ACSS2* knockdown affected basal gene expression or HIF-1α-dependent activation, indicating that the acetate-ACSS2 signal is HIF-2α-specific. Gene expression induced by overexpression of oxygen stable HIF-2α followed by acetate supplementation was then assayed after knockdown of *p300*, *CBP*, and *ACSS2* (Fig. 19B). Knockdown of *CBP* and *ACSS2* resulted in a reduction in HIF-2α activation of gene expression. Acetate-mediated increase in expression of HIF-2α target genes was lost in *CBP* and *ACSS2* knockdown cells. Thus, both ACSS2 and CBP are required for acetate-dependent regulation of HIF-2α activity, and this mechanism applies to multiple HIF-2α target genes in HT1080 cells.

Tumor cell properties are regulated by ACSS2 and HIF-2α

These HIF-2α target genes frequently play a role in tumor progression. VEGFa enhances angiogenesis, while GLUT1 is a glucose transporter, helping to meet the increased metabolic demand of rapidly growing tumors. PAI1 and MMP9 play a role in cell invasion and migration during tumor metastasis. Due to its ability to augment HIF-2α signaling, ACSS2 may also contribute to cancer development. Thus, the tumor cell hallmarks of HT1080 cells were assayed after *ACSS2* knockdown by a stably integrated Lentiviral vector. As tumors are characterized by uncontrolled cell division, proliferation assays were performed. Under normal, unstressed growth conditions, knockdown of *ACSS2*, *HIF-1α*, or *HIF-2α* did not result in a change in cell proliferation from control cells (Fig. 20A). After

subjecting cells to the stresses typically seen in tumors, hypoxia (Fig. 20B) or low glucose (Fig. 20C), cell proliferation was reduced significantly in both *ACSS2* and *HIF-2α* knockdown cell lines, while *HIF-1α* knockdown cells did not differ from control cells. During metastasis, tumor cells migrate to different regions of the body and invade other tissues, forming new tumors. Cell migration assays show the ability of HT1080 cells to migrate increases during both hypoxia (Fig. 20D) and glucose deprivation (Fig. 20E). Increased migration capacity is lost during hypoxia after knockdown of either *HIF-1α* or *HIF-2α*, as well as *ACSS2* (Fig. 20D). ACSS2 and HIF-2α are also required for the low glucose-dependent increased cell migration activity, while HIF-1α, which is inactive under glucose deprivation, has no effect (Fig. 20E). Knockdown of *HIF-1α, HIF-2α*, and *ACSS2* reduces hypoxia-dependent augmentation of cell invasion, while only HIF-2α and ACSS2 are required for low glucose-enhanced invasion (Fig. 20F). Lastly, hypoxia- and low glucose-dependent colony formation activity is reduced in *HIF-2α* and *ACSS2* knockdown cell lines (Fig. 20G). Thus, ACSS2 mirrors HIF-2α requirement in each of these assays of tumor cell properties under both hypoxia and low glucose.

Acetate enhances ACSS2/HIF-2α-dependent tumor burden and metastasis

ACSS2 may play a role in HIF-2α-mediated tumor development *in vivo*. ACSS2 is required for full HIF-2α activation of *EPO* expression *in vivo*, and this is further augmented by acetate administration (Xu, Nagati et al. 2014). To determine if this mechanism regulates tumor growth, mice were given flank tumors by injection of HT1080 cells expressing luciferase and shRNA constructs targeting *ACSS2*, *HIF-1α*, or *HIF-2α*. This was followed by

oral administration of acetate in the form of triacetin (glyceryl triacetate or GTA). Tumors were analyzed by weight (Fig. 21A) or luciferase activity (Fig. 21B), as well as metastasis measured as luciferase activity in lung (Fig. 21C). Untreated HIF-1α knockdown tumors were larger than control tumors, in agreement with observations that HIF-1α has tumor suppressor activity. Conversely, *ACSS2* or *HIF-2α* knockdown resulted in smaller tumors overall, confirming their role in promoting tumor growth. Triacetin administration resulted in significantly increased primary and metastatic tumor burden in control or HIF-1α knockdown cells, but not in *ACSS2* or *HIF-2α* knockdown cells. Thus, acetate appears to increase tumor growth by a mechanism that requires HIF-2α and ACSS2.

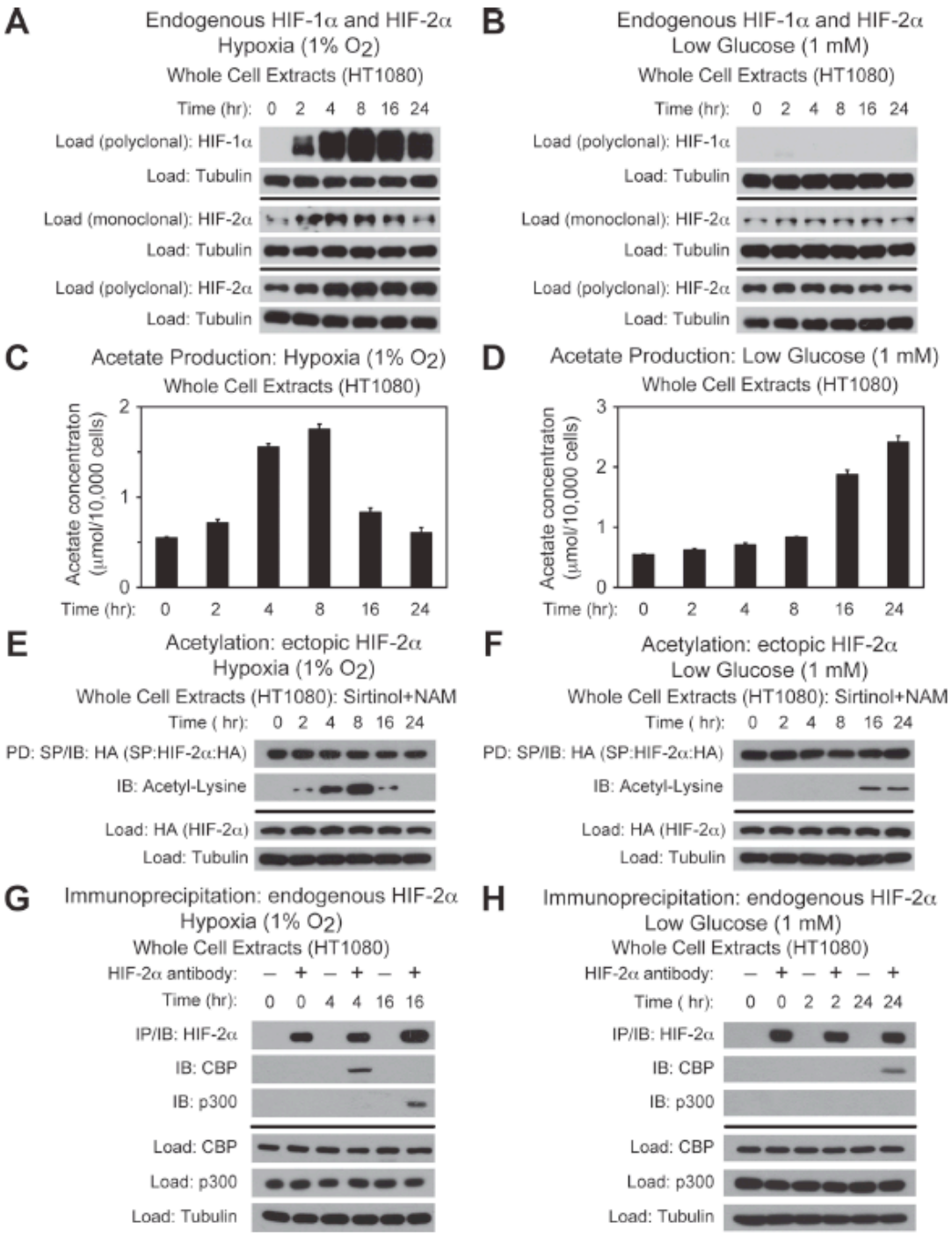


Figure 15. Glucose deprivation activates the HIF-2α acetylation response. (**A, B**) Western blots of HIF-1α and HIF-2α protein levels in HT1080 cells during hypoxia (**A**) or glucose deprivation (**B**) time courses. (**C**, **D**) Analysis of acetate concentration during hypoxia (**C**) and glucose deprivation time courses (**D**). (**E, F**) Assay of HIF-2α acetylation during hypoxia (**E**) or glucose deprivation (**F**). (**G, H**) CoIP detection of HIF-2α complex formation with CBP or p300 after hypoxia (**G**) or glucose deprivation (**H**).

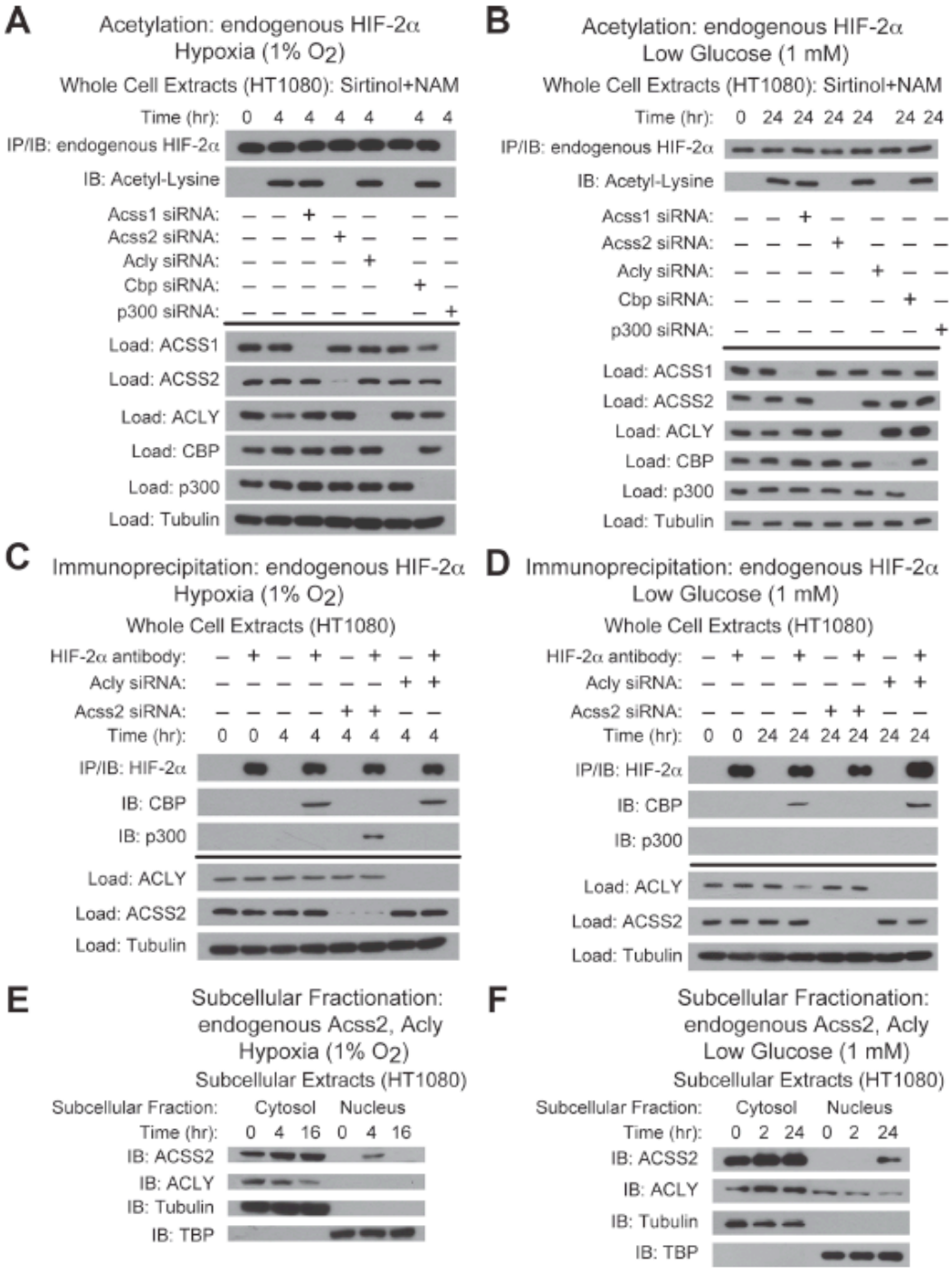


Figure 16. ACSS2 regulates the HIF-2α acetylation response to low glucose. (**A, B**) Contribution of ACSS1, ACSS2, and ACLY to HIF-2α acetylation during hypoxia (**A**) or glucose deprivation (**B**), as determined by siRNA-mediated knockdown. (**C**, **D**) CoIP detection of HIF-2α complex formation with CBP and p300 after ACSS2 knockdown, following 4 hours hypoxia treatment (**C**) or 24 hours glucose deprivation (**D**). (**E, F**) Subcellular localization of ACSS2 during hypoxia (**E**) or glucose deprivation (**F**).

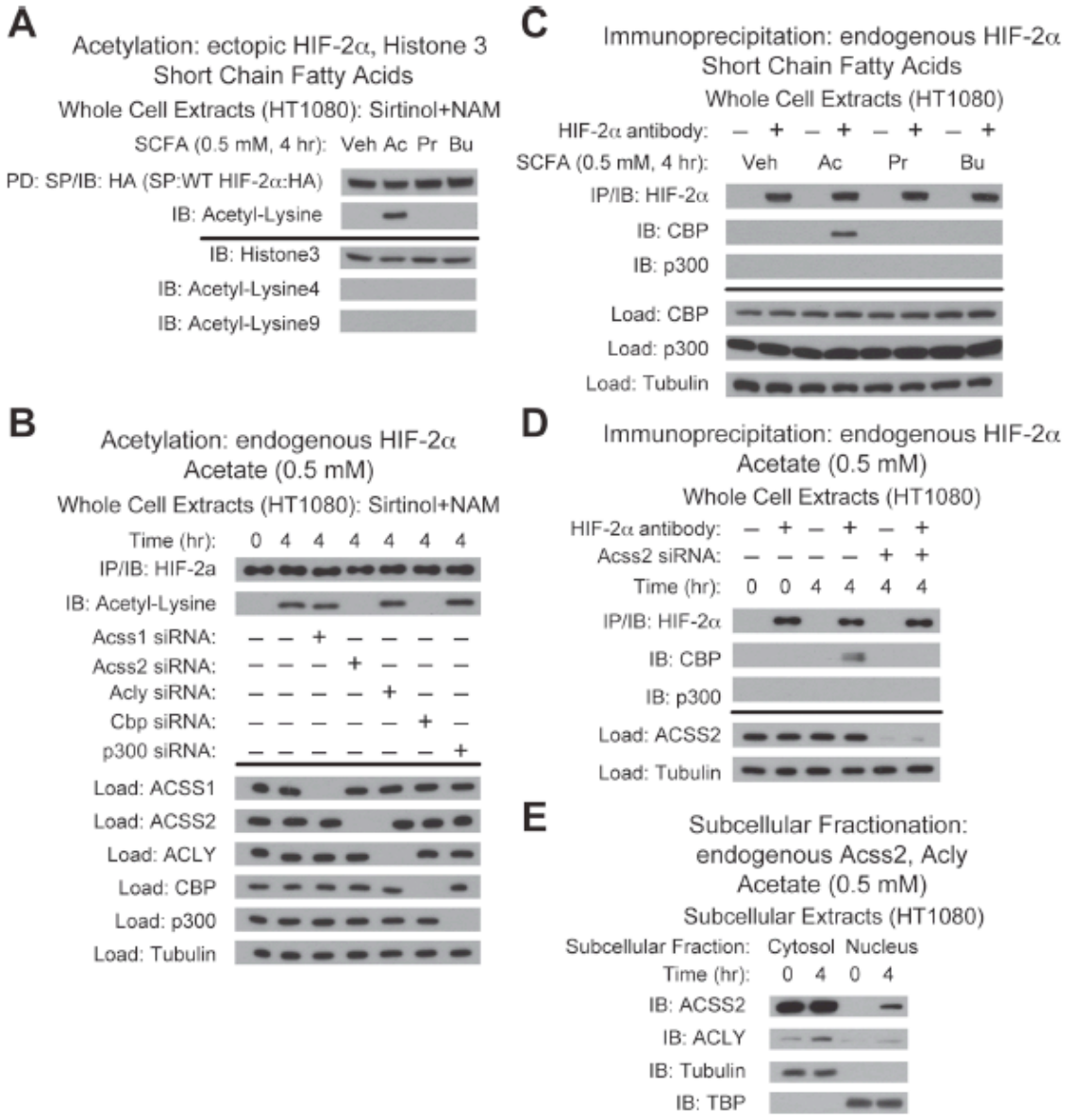


Figure 17. Acetate induces HIF-2α acetylation in HT1080 cells. (**A**) Assay of HIF-2α acetylation status in HT1080 cells after four hours SCFA supplementation. (**B**) Contribution of ACSS1, ACSS2, ACLY, CBP, and p300 to acetate-induced HIF-2α acetylation. (**C**) Detection of HIF-2α complex formation with CBP and p300 by CoIP after SCFA supplementation. (**D**) Requirement for ACSS2 in acetate-induced CBP-HIF-2α complex formation. (**E**) Subcellular localization of ACSS2 and ACLY after acetate supplementation.

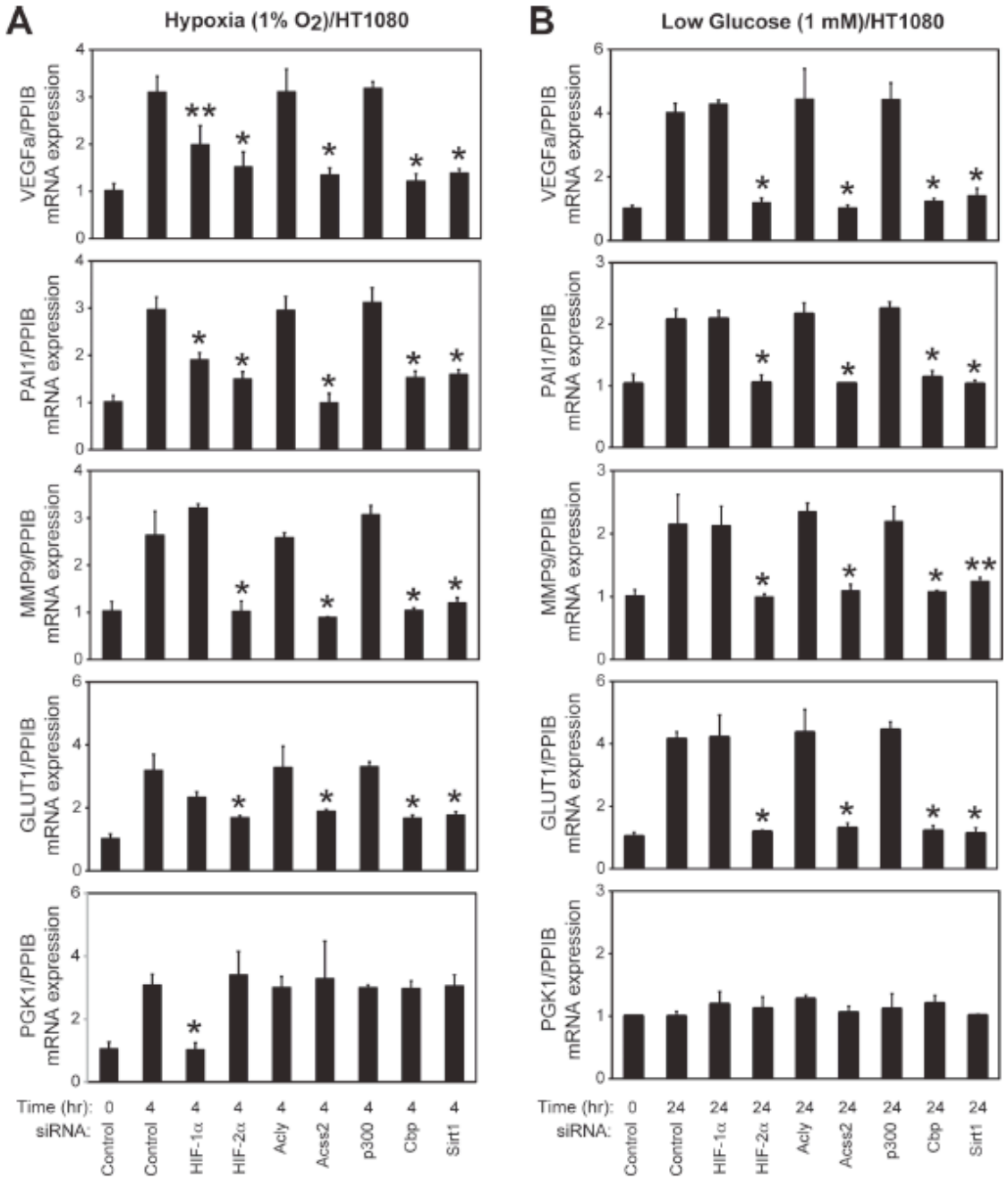


Figure 18. Glucose deprivation activates HIF-2α signaling and is dependent on ACSS2, CBP, and SIRT1. (**A, B**) Real-time PCR analysis of HIF-1α and HIF-2α target genes, *VEGFa*, *PAI1*, *MMP9*, *GLUT1*, and *PGK1*, after four hours hypoxia treatment (**A**) or 24 hours glucose deprivation (**B**). The contributions of HIF-1α, HIF-2α, ACLY, ACSS2, p300, CBP, and SIRT1 were determined through siRNA knockdown of these factors.

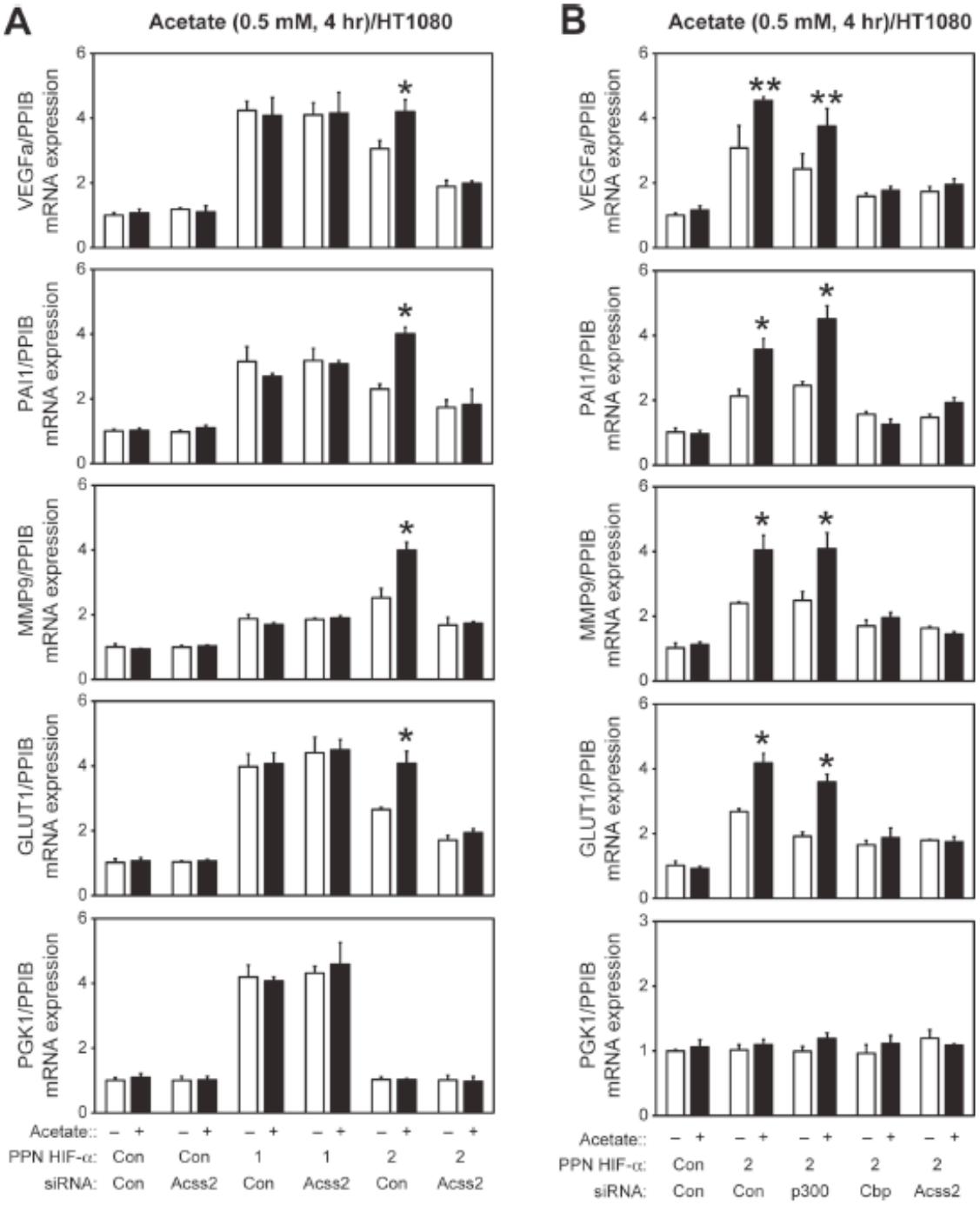


Figure 19. Acetate modulation of HIF-2α signaling requires CBP and ACSS2. (**A**) The effects of *ACSS2* knockdown and acetate supplementation on HIF target gene activation induced by expression of P1P2N HIF-1α and HIF-2α. Expression of *VEGFa*, *PAI1*, *MMP9*, *GLUT1*, and *PGK1* was determined by real-time PCR analysis. (**B**) Contribution of p300, CBP, and ACSS2 to HIF-2α-dependent, acetate-induced gene expression.

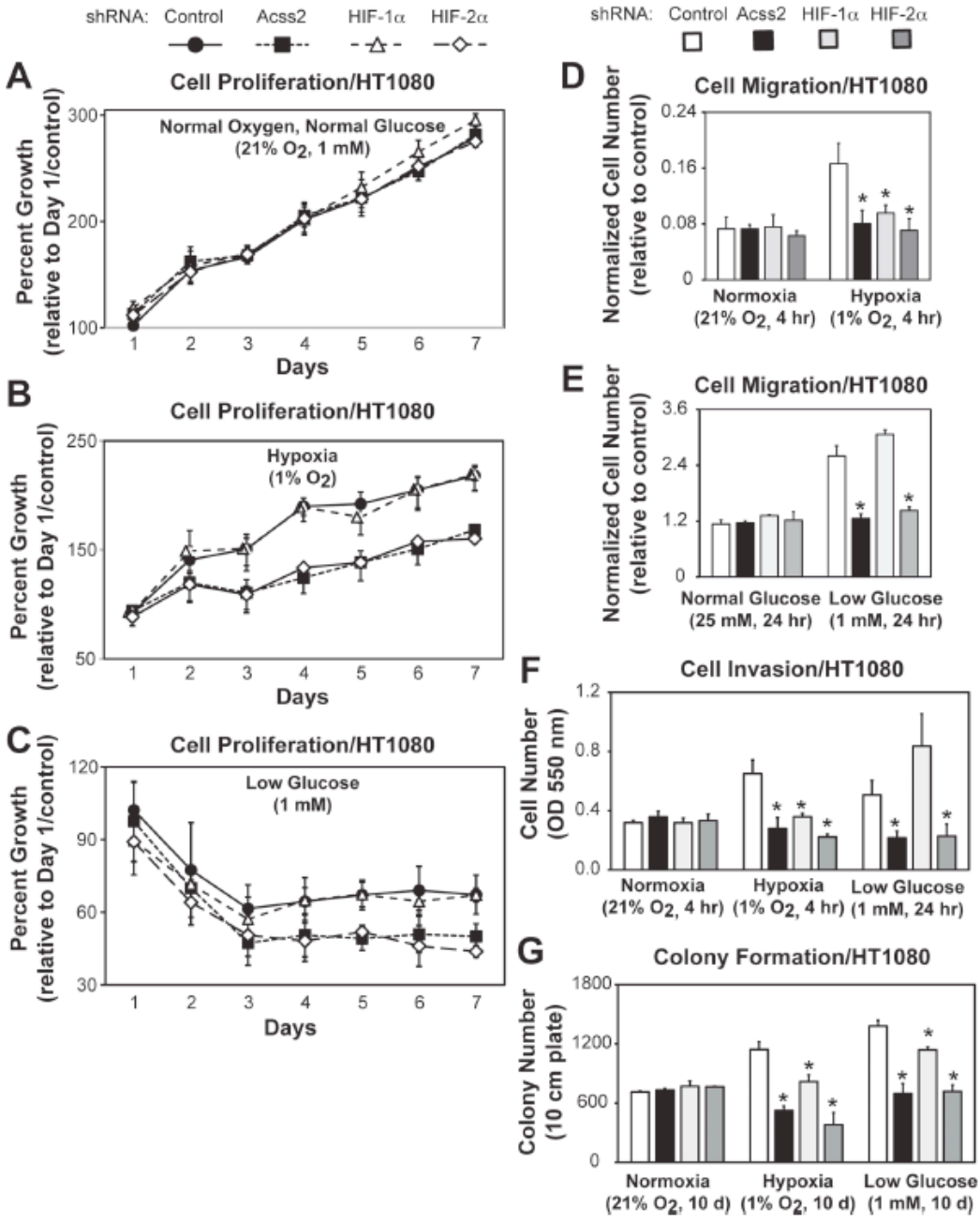


Figure 20. ACSS2 and HIF-2*α* regulate tumor cell properties of HT1080 cells. (**A, B, C**) Contribution of HIF-1α, HIF-2α, and ACSS2 to HT1080 cell proliferation under standard culture conditions (**A**), hypoxia (**B**), or glucose deprivation (**C**), as determined by stably transduced, knockdown cell lines. (**D, E**) Assay of cell migration after four hours of hypoxia (**D**) or 24 hours of glucose deprivation (**E**) in *ACSS2*, *HIF-1α*, and *HIF-2α* knockdown cell lines. (**F**) Cell invasion assayed after a four hour normoxia, hypoxia, or low glucose treatment in knockdown cell lines. (**G**) Colony formation determined after a 10 day normoxia, hypoxia, or low glucose treatment in knockdown cell lines.

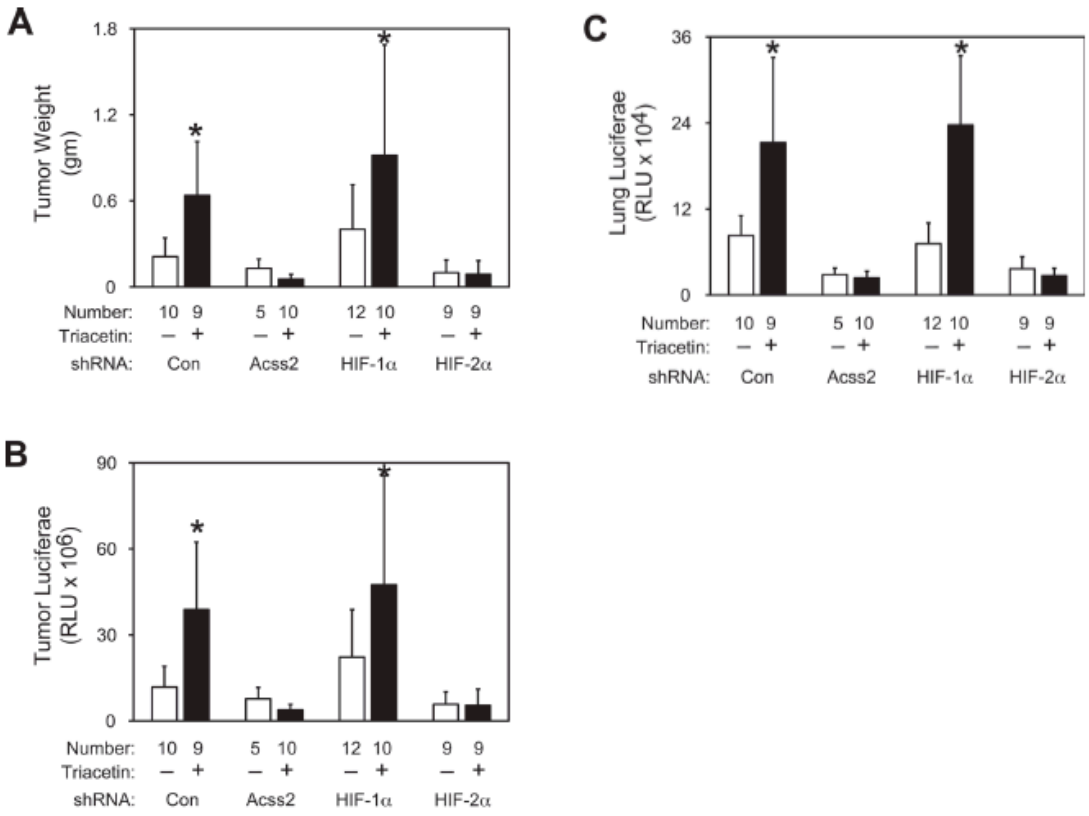


Figure 21. Acetate administration increases flank tumor burden and metastasis in a HIF-2α and ACSS2-dependent manner. (**A**) Weight of mouse flank tumors at experiment endpoint from mice receiving vehicle or acetate (as GTA). Tumors are injected, HT1080 cell lines with stably integrated shRNA constructs, either a non-targeting control, or targeting *ACSS2*, *HIF-1α*, or *HIF-2α*. (**B**) Tumor burden determined by luciferase activity. (**C**) Metastatic activity as measured by luciferase assay of lung tissue.

Discussion

HIF-2α activity is regulated by acetylation by CBP. During hypoxia, acetylation and deacetylation of HIF-2α results in increased activity (Dioum, Chen et al. 2009, Chen, Xu et al. 2012). Investigation of this mechanism revealed that ACSS2, which generates the CBP substrate acetyl CoA and is highly expressed in the EPO production sites the liver and kidney (Ellis, Bowman et al. 2015), is required for CBP and SIRT1 augmentation of HIF-2α activity during both hypoxia and glucose deprivation. Knockdown in Hep3B and HT1080 cell lines of *ACSS2* resulted in the loss of HIF-2α acetylation, loss of HIF-2α complex formation with CBP or SIRT1, and diminished activation of HIF-2α target genes during hypoxia and glucose deprivation. Knockout of *Acss2* in the mouse resulted in loss of HIF-2α acetylation, reduced *Epo* expression, lower basal hematocrit, and longer recovery from induced anemia. The ACSS2 substrate acetate, which is generated during hypoxia and glucose deprivation, serves as the switch to control ACSS2-driven HIF-2α acetylation. Supplementation of cell media with acetate promoted HIF-2α acetylation, increased complex formation with CBP, and enhanced expression of HIF-2α activated genes. This activity requires ACSS2, CBP, and SIRT1. Mice with PHZ-induced anemia recovered more quickly with acetate administration than those which received vehicle. This improved recovery was lost in mice lacking functional ACSS2. Additionally, acetate supplementation enhances tumor cell characteristics and increases tumor burden and metastasis in mouse xenografts, likely through upregulation of HIF-2α target genes involved in tumorigenesis. As with anemia recovery, acetate administration did not affect tumor characteristics in cell culture or tumor growth in flank tumors after depletion of ACSS2 with RNAi. These data elucidate a mechanism for

hypoxia/stress-induced HIF-2α-selective activation by acetylation. Acetate is generated under the stress conditions hypoxia and glucose deprivation. This drives ACSS2 to produce acetyl CoA, which is used in the CBP/SIRT1-dependent acetylation activation of HIF-2α.

Although ACSS2 produces acetyl CoA under normal conditions in the cytosol, HIF-2α signaling is not activated unless the cell is stressed. Hypoxia and acetate supplementation were shown to induce nuclear localization of ACSS2 concurrently with HIF-2α acetylation and CBP complex formation. As HIF-2α and CBP are mainly found in and function in the nucleus, nuclear-localized ACSS2 may be necessary to provide a local pool of acetyl CoA available for HIF-2α acetylation. This could be investigated with a mutant form of ACSS2 that lacks nuclear localization ability but retains enzymatic activity. If acetyl CoA produced by ACSS2 in the nucleus is required, the cytoplasm-restricted mutant would not be able to induce HIF-2α acetylation or CBP complex formation under hypoxia or acetate supplementation. Conversely, forced nuclear localization through an exogenous signal tag would enhance HIF-2α acetylation, complex formation with CBP and SIRT1, and target gene activation, likely even under normoxic conditions.

The observation that acetate administration to anemic mice improves recovery of hematocrit levels has therapeutic implications. If this pathway exists in humans, acetate, which is the acid present in culinary vinegar, may represent a cost effective way to improve recovery from anemia. Patients who refuse blood transfusions may benefit after blood loss from surgery or injury. Acetate may also accelerate adaptation to the low partial pressure of oxygen at high altitudes. Furthermore, EPO is known to have a neuroprotective effect (Sakanaka, Wen et al. 1998, Merelli, Czornyj et al. 2013), and its use may serve to improve

outcome after ischemic insult or trauma to the brain. Several studies have explored the efficacy of EPO administration on stroke and traumatic brain injury (TBI). Though results are mixed and may arguably suffer from bias and poor methodology, there is evidence that patients may benefit from the effect of EPO or EPO analogues (Minnerup, Heidrich et al. 2009, Jerndal, Forsberg et al. 2010, Peng, Xing et al. 2014). Because EPO is a peptide, it does not readily cross the blood brain barrier, and is only detected in the brain after administration of high doses, possibly due to breakdown of the barrier after injury (Lieutaud, Andrews et al. 2008). Acetate, on the other hand, crosses the blood brain barrier readily (Deelchand, Shestov et al. 2009). As EPO is produced during hypoxia in the brain (Marti, Gassmann et al. 1997, Bernaudin, Bellail et al. 2000) where it is regulated by HIF-2α (Chavez, Baranova et al. 2006), acetate administration may enhance *EPO* expression during brain injury and stroke through augmentation of HIF-2α acetylation, thus reducing cell death through EPO's neuroprotective properties. In fact, acetate administered as GTA has been shown to improve post-TBI performance on a Rotarod balance test (Arun, Ariyannur et al. 2010). Though attributed to amelioration of the loss of brain metabolite and acetate source N-acetylaspartate during TBI, it remains to be seen if acetate-influenced HIF-2α activity plays a role.

Though the hematopoietic effect of HIF-2α during anemia is crucial to recovery, HIF-2α exerts pro-tumorigenic influence. ACSS2 and HIF-2α regulate tumor cell properties, such as cell proliferation and migration. Acetate supplementation augments HIF-2α transactivation of genes that influence tumor growth and metastasis in cell culture and increases tumor burden and metastasis *in vivo*. ACSS2 has been noted by other groups for promoting tumor

growth. Yoshii et al showed ACSS2 enhances tumor growth and promotes cancer cell survival under extended hypoxia (Yoshii, Furukawa et al. 2009). They showed that acetate increases in tumor cells under hypoxia, though it was not linked to HIF-2α signaling at that time. Comerford et al also showed a reduction in tumor burden in ACSS2-null mice (Comerford, Huang et al. 2014). High ACSS2 expression was observed in breast, ovarian, and lung cancer tumor samples, while expression levels were negatively correlated with survival in a set of human breast cancers. Interestingly, ACSS2 was detected in the nucleus of some samples, suggesting that tumor conditions may induce nuclear localization, in parallel with our finding that hypoxia and low glucose induce nuclear localization. Acetate was determined to be a source of energy for tumor cells, converted to acetyl CoA by ACSS2 for entry into the TCA cycle (Comerford, Huang et al. 2014, Mashimo, Pichumani et al. 2014). ACSS2 may play a dual role in tumor growth, converting acetate into a metabolic substrate for energy production and activating tumorigenic gene expression through acetylation-mediated activation of HIF-2α. Thus, while acetate may prove to be clinically relevant in anemia recovery, it may be detrimental to outcome in cancer patients.

APPENDIX
Materials and Methods

(Adapted and modified from (Chen, Xu et al. 2015) and (Xu, Nagati et al. 2014))

Bioinformatics Analyses.

Erythropoietin enhancer sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) and UCSC (http://genome.ucsc.edu) genome browsers. The rVISTA algorithm program from the University of California in Santa Cruz (UCSC) was used to perform genome alignment analysis (Loots and Ovcharenko 2004). Elhadji Dioum visually analyzed the enhancer region using a ClustalW multiple sequence alignment program (http://www.ebi.ac.uk/cgi-bin/clustalw). The Tfsitescan/dynamic Plus server (Ghosh 2000) was used to determine putative binding sites in conserved regions.

Cell culture.

Hep3B cells (Cat. No. HB-8064, ATCC) and HT1080 cells (Cat. No. CCL-121) were maintained in complete DMEM medium (Cat. No. SH30022, HyClone), 10% FBS (Cat. No. S11150H, Atlanta Biologicals) with penicillin (100 U/mL)/streptomycin (100 μg/mL) (Cat. No. 15140-148, Gibco BRL) in a 5% CO_2, 95% air incubator. For passaging, cells were trypsinized with 1 ml 0.25% Trypsin/2.21 mM EDTA/HBSS (Cat. No. 25-052-CI, Corning) per freshly confluent 10-cm, and split according to usage. Cells used were at passage 12 to 25 in all experiments. For hypoxia treatments (1% O_2, 5% CO_2, 94% N_2), cells were incubate, manipulated, and harvested in a humidified environmental chamber (Coy Laboratory Products, Inc.). Cells were incubated with low glucose media (1 mM glucose) under normoxic conditions (21% oxygen, 5% carbon dioxide, 74% nitrogen) in a standard tissue

culture incubator. For short-chain fatty acid (SCFA) addition, we added the indicated sterile stocks of SCFAs (acetate, propionate, and butyrate) to complete medium (final concentration 0.5 mM) and harvested cells after the indicated time under normal oxygen conditions.

Reporter assays.

Reporter plasmids (30 ng per well), and expression plasmids (100 ng perwell) were used in transient transfection analyses. Hep3B cells were transfected at 50-60% confluency in 48-well plates using 30 ng/well reporter plasmid and 100 ng/well expression plasmids. Transfections were performed with 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, following additional hypoxia treatment, cells were harvested for luciferase assays. Media was aspirated from cells and replaced with lysis buffer. Aliquots of the lysis were transferred to a 96-well plate in triplicate, and a substrate buffer was applied. Luciferase activity was then measured on a luminometer.

Lentiviral generation.

The wild-type (WT) acetylase-sensitive (K3) and acetylase-insensitive (R3; K385R, K685R, K741R) human HIF-2α plasmids were previously described (Dioum, Chen et al. 2009, Chen, Xu et al. 2012). All human HIF-2α rescue cDNA constructs contained a carboxy-terminal hemagglutinin (HA) epitope tag. We generated wild-type (WT) and mutant (MUT) Acss2 from a mouse Acss2 cDNA. MUT Acss2 cDNA contains a deletion that removes coding exons 3 through 7 in the *Acss2* gene and the corresponding nucleotides in the *Acss2* mRNA (nt 439-898, RefSeq NM_019811.3). All Acss2 constructs contain an amino-terminal V5 epitope tag. The rescue human HIF-2α and mouse Acss2 cDNAs contain silent

mutations as detailed below that confer resistance to siRNA or shRNA directed against the endogenous human mRNA of interest.

The lentiviral knockdown constructs express multiple miR-30 shRNAs (Sun, Melegari et al. 2006) using Pol II promoters (Stegmeier, Hu et al. 2005) for efficient knockdown. For knockdown-only studies (control and Acss2), lentiviral (LTV) shuttle expression vectors (derivatives of pLenti6/V5-GW/lacZ, Invitrogen), blasticidin resistant for HT1080 cells and neomycin resistant for Hep3B cells, harbor a firefly luciferase (control and Acss2) or GFP cDNA (control, HIF-1α, HIF-2α, Egr1, Egr2,) followed by a concatamer of four different shRNAs directed against the gene of interest. For knockdown/rescue studies, LTV shuttle expression vectors encode an siRNA/shRNA-resistant mouse Acss2 or human HIF-2α cDNA encompassing the translated portion followed by an IRES:firefly luciferase:shRNA concatamer cassette (WT and MUT Acss2) or an IRES:DsRed:shRNA concatamer cassette (K3 and R3 HIF-2α).

The seed sequences for the four human Egr1 shRNAs are as follows: 5'-GATGAACGCAAGAGGCATA-3', 5'-CGACAGCAGTCCCATTTAC-3', 5'-GGACATGACAGTAACCTTT-3', 5'-GACCTGAAGGCCCTCAATA-3'. The seed sequences for the four human Egr2 shRNAs are as follows: 5'-gaaggcataatcaatattg-3', 5'-CTACTGTGGCCGAAAGTTT-3', 5'-GAAACCAGACCTTCACTTA-3', 5'-GAGAAGAGGTCGTTGGATC-3'. The seed sequences for the four human ACSS2 shRNAs are as follows: 5′-GTAATAGCCATCCTGGTCCCGC-3′, 5′-AACTTGGTCACCTTGTATTTGT-3′, 5′-TGGTATGTGATCTGAGTGGTCT-3′, 5′-GTATCCAGGAAACTTCTTAAAG-3′. The corresponding sequences for the

siRNA/shRNA-resistant mouse Acss2 cDNA with changes from the human ACSS2 mRNA indicated in lower case are as follows: 5′-aTAgTAtCCgTCtTGaTCtctt-3′, 5′-AAtTTtGTgACtTTaTAcTTaT-3′, 5′-ctaTAgGTaATtTtgGTtGTtT-3′, 5′-aTAgCCtGGgAAtTTtTTgAAa-3′. The seed sequences for the four human HIF-1α shRNAs are as follows: 5'-GAACAAATACATGGGATTA-3', 5'-AGAATGAAGTGTACCCTAA-3', 5'-GATGGAAGCACTAGACAAA-3', 5'-CAAGTAGCCTCTTTGACAA-3'. The seed sequences for the four human HIF-2α shRNAs are based on previously designed siRNAs (Sowter, Raval et al. 2003, Aprelikova, Chandramouli et al. 2004, Warnecke, Zaborowska et al. 2004, Carroll and Ashcroft 2006) and are as follows: 5′-ACTGCTATCAAAGATGCTGTTC-3′, 5′-TCTGTGTCCATGGCGAAGAGCT-3′, 5′-TTCATACTCCAGCTGTCGCTTC-3′, 5′-TAAGTCTATCCGGGCTTACTAA-3′ with this last shRNA targeting the 3′ untranslated region of the mRNA. The corresponding sequences for the siRNA/shRNA-resistant human HIF-2α cDNA with changes from the human HIF-2α mRNA indicated in lowercase are as follows: 5′-tgacgagTCgAAaATcgaaTTC-3′, 5′-TCaGTaTCCATcGCaAAcAatT-3′, 5′-cTCgTAtTCtAatTGcCttTTg-3′.

We used the LTV packaging plasmid psPAX2 (Addgene plasmid 12260) and the envelope plasmid pMD2.G (Addgene plasmid 12259) in conjunction with the shuttle expression plasmids to generate lentivirus. We generated concentrated lentiviral stocks from a 10-cm plate of HEK 293T cells, co-transfected with 3 μg LTV shuttle expression vector, 2.25 μg plasmid psPAX2 and 0.75 μg pMD2G using Lipofectamine 2000 (Cat. No. 11668019, Invitrogen), using Lenti-X Concentrator (Cat. No. 631232, Clontech). The virus was aliquoted immediately and stored it at −80 °C until use.

Stable cell line generation.

The day before transduction, 2×10^5 Hep3B or HT1080 cells were plated per well in 1 mL complete culture medium overnight in a 6-well plate. On the day of transduction, the medium was replaced with 1 mL of complete medium with 10 μg mL^{-1} polybrene (Cat. No. 107689, Sigma). The frozen lentiviral particles were thawed on ice, mixed gently and added to cells at an estimated MOI = 30. After 12 h, media was replaced with 2 mL of complete medium containing 400 μg/mL G418 (Cat. No. SV3006901, HyClone) for Hep3B lines, or 10 μg/ml blasticidin S (Cat. No. ant-bl, Invivogen) for HT1080 lines, and replaced every 2 days until 1 week after all control cells died. We maintained the initially positive-selected cells in 100 μg/mL G418 or 1 μg/ml blasticidin S for 2 weeks, harvested cells into a smaller dish until confluent, split cells at 1:3 ratio and grew them for at least ten passages under drug selection and then froze the cells down until use. For experiments, cells were thawed and maintained in 100 μg/mL G418 or 1 μg/ml blasticidin S for at least three passages before use. Antibiotic selection was removed after passaging for use in experiments.

siRNA/shRNA knockdown.

For transient siRNA knockdown experiments, the following siRNAs were used (Thermo Fisher Scientific, Lafayette, CO): nontargeting control (Cat. No. D-001810-10-20), ACSS1 (Cat. No. L-008549-01-0005), ACSS2 (Cat. No. L-010396-00-0005), ACLY (Cat. No. L-004915-00-0005), p300 (Cat. No. L-003486-00-0005), CBP (Cat. No. L-003477-00-0005), SIRT1 (Cat. No. L-003540-00-0005), HIF-1α (Cat. No. 004018-00-0005) and HIF-2α (Cat. No. L-004814-00-0005). The siRNA were transfected into Hep3B and HT1080 cells using DharmaFECT1 (Cat. No. T-2001-03, Thermo Fisher Scientific) as quadruplicate

biological replicates in a 12-well plate for mRNA analysis or a 6-cm plate for protein analysis.

For transient lentiviral transduction knockdown (control, ACCS2)/rescue (WT and MUT Acss2) experiments, we added 100 μL of concentrated lentiviral preparations to a 60-mm Hep3B plate, waited 6 h and then changed the medium to complete medium. After an additional 36 h, the indicated experiments were performed.

Immunoblotting.

Immunoblots were performed with 20 μg Hep3B whole-cell extracts, 10 μg HT1080 whole-cell extracsts , 20 μg nuclear or cytosolic extracts and 10 μg mouse kidney or liver extracts. Whole-cell extracts from cells were prepared with CytoBuster protein extraction reagent (Cat. No. 71009, Novagen) containing 1× protease inhibitor cocktail (Cat. No. P8340, Sigma) and 1 mM PMSF (phenylmethylsulfonyl fluoride) (Cat. No. P7626, Sigma), and stored at −80 °C until use. Nuclear extracts were prepared with the NE-PER nuclear and cytoplasmic extraction kit (Cat. No. 78833, Pierce) according to the manufacturer's protocol and stored at −80 °C until use. Extracts from liver or kidney were prepared with CytoBuster protein extraction reagent containing 1× protease inhibitor cocktail and 1 mM PMSF and stored at −80 °C until use.

The following antibodies were used: human Egr1 (Cat. No. sc-110, Santa Cruz); human Egr2 (Cat. No. PRB-236P, Covance); human p300 (1:500 dilution; Cat. No. sc-584, Santa Cruz Biotechnology), human CBP (1:500 dilution; Cat. No. 7389, Cell Signaling Technology), human SIRT1 (1:1,000 dilution; Cat. No. 07131, EMD Millipore), human ACLY (1:1,000 dilution; Cat. No. 3378, Cell Signaling Technology), human ACSS1

(1:1,000 dilution; Cat. No. SAB1400745, Sigma), human ACSS2 (1:500 dilution; Cat. No. ab66038, Abcam), human HIF-1α (1:1,000 dilution; Cat. No 610958, BD Biosciences), human HIF-2α (1:1,000 dilution; Cat. No. NB10-132, Novus Biologicals), α-tubulin (1:10,000 dilution; Cat. No. T9026, Sigma), TATA-binding protein (TBP) (1:1,000 dilution; Cat. No. sc-204, Santa Cruz Biotechnology), acetyl-lysine (1:1,000 dilution; Cat. No. 9814, Cell Signaling Technology), HA epitope (1:5,000 dilution; Cat. No. H9658, Sigma) and V5 epitope (1:10,000 dilution; Cat. No. R960-25, Invitrogen).

Acetate measurements.

For cellular acetate measurements, cell were grown at 80% confluency as triplicate biological replicates in 60-mm plates overnight in an incubator within the hypoxia workstation until the indicated time point. At the time of harvest, media was aspirated, and cells were scraped into 1 mL ice-cold 0.1 N HCl. The lysate was handled on ice and pelleted at 16,000xg, 4 °C, 10 min. The pH of 0.9 mL supernatant was adjusted to 6.5–7.0 with 5 N NaOH. Acetate concentrations of freshly prepared extracts were determined in duplicate using the Megazyme Acetic Acid Rapid Kit (Cat. No. K-ACETRM, Megazyme).

For hypoxic mouse kidneys, mice were exposed to room air (21% O_2) or hypoxia (6% O_2) for 2 h and then euthanized mice under normoxic or hypoxic air mixtures to harvest kidney samples. For anemic mouse kidneys and livers, we euthanized mice under normoxic air mixtures to harvest kidney samples. To measure tissue acetate levels, we placed (~0.1 g) weighed, freeze-clamped, powdered tissue into a cold microfuge tube on dry ice and slowly added 500 μL ice-cold 1 N perchloric acid. The samples were slowly thawed on ice, followed

by homogenization (PowerGen 700D, Fisher Scientific). Neutralization and acetate analysis were then performed as with cellular extracts.

Endogenous HIF-2α acetylation (cells).

Hep3B and HT1080 cells were cultured in a single 100-mm plate in complete medium supplemented with 5 μM sirtinol plus 10 mM NAM for 6 h under either normoxia, hypoxia, or low glucose. For acetylation assays using whole-cell extracts, cells were lysed with CytoBuster protein extraction reagent supplemented with 1× protease inhibitor cocktail, 1 mM PMSF, 10 mM NAM and 5 μM sirtinol. For acetylation assays with nuclear or cytosolic protein extracts, cells were fractionated cells and nuclear protein extraction performed using a kit (Cat. No. 40010, Active Motif) supplemented with 1 × protease inhibitor cocktail, 1 mM PMSF, 10 mM NAM and 5 μM sirtinol. To immunoprecipitate endogenous HIF-2α, extracts were incubated with a monoclonal human HIF-2α antibody (Cat. No. NB100-132, Novus Biologicals) for 1 h, and then immunoprecipitated using magnetic protein G beads (Cat. No. 54002, Active Motif). Immunoblot for endogenous HIF-2α or acetyl lysine was performed as described (Dioum, Chen et al. 2009, Chen, Xu et al. 2012).

For short-chain fatty acid (SCFA) experiments, cells were cultured for 2 h in complete medium with 5 μM sirtinol plus 10 mM NAM under normoxia and then incubated for 4 h with medium containing vehicle, sodium acetate, sodium proprionate or sodium butyrate with 5 μM sirtinol plus 10 mM NAM. For transient transfection siRNA knockdown experiments, cells were transfected with siRNA, for 42 h under normoxia with complete media before initiating treatment.

Ectopic HIF-2α acetylation (cells).

Cells of stably transformed cells expressing HIF-2α under normoxia for 6 h in complete medium containing 5 μM sirtinol plus 10 mM NAM and then treated cells with normoxia, hypoxia, or low glucose for the indicated period. At the end of the incubation period, cells were lysed with CytoBuster protein extraction reagent supplemented with 1× protease inhibitor cocktail, 1 mM PMSF, 10 mM NAM and 5 μM sirtinol. Ectopic HIF-2α was immunoprecipitated using HA-agarose pulldown (Hep3B) or SP-agarose pulldown (HT1080), then immunoblotted using antibody recognizing acetylated lysine, followed by immunoblotting using antibody recognizing HIF-2α or the HA epitope.

Endogenous HIF-2α acetylation (mice).

Mouse liver (~50 mg) or kidney samples (~100 mg) were lysed at the time of harvest in 0.5 mL CytoBuster protein extraction reagent supplemented with 1× protease inhibitor cocktail, 1 mM PMSF, 10 mM NAM and 5 μM sirtinol. Protein extracts (500 μg) were incubated with a monoclonal human HIF-2α antibody for 1 h to bind endogenous HIF-2α protein and immunoprecipitated the complex using magnetic protein G beads (Cat. No. 54002, Active Motif), then immunoblotted for endogenous HIF-2α or acetyl lysine.

Immunoprecipitation experiments

Endogenous HIF-2α, Egr1, or Egr2, or exogenous HIF-2α rescue proteins were immunoprecipitated from a single 100-mm plate using a Universal Magnetic Co-IP kit (Cat. No.54002, Active Motif) according to the manufacturer's protocol. Whole-cell extracts (500 μg) were pre-cleared with magnetic protein G beads, then incubated with antibodies to HIF-2α, Egr1, or Egr2 (endogenous) or HA (rescue HIF-2α protein), or with normal mouse IgG

(Cat. No. sc-2025, Santa Cruz Biotechnology) for 2 h before addition of magnetic protein G beads. The beads were washed and eluted according to kit instructions and immunoblotted with antibodies to human p300, human CBP, HIF-1α or HIF-2α. For HIF-EGR hybrid CoIP experiments, exogenous HIF-α was immunoprecipitated from transfected HEK293 cell extract using a μMACS HA Tag Protein Isolation Kit (Cat. No. 130-091-122, Miltenyi Biotec) according to kit instructions, followed by immunoblotting. HIF-α was detected with antibodies to HA. EGR was detected with an anti-vsv-g antibody (Cat. No. ab18612, Abcam).

Real-time PCR analyses.

Gene expression of endogenous *EPO*, *VEGFA*, *SERPINE1 (PAI1)*, *MMP9*, *SLC2A1 (GLUT1)*, *PGK1* and *PPIB* was determined from a single pooled sample made from three individually transfected wells (biological replicates) of cells in a 12-well plate for each condition or of endogenous *Epo* and *Ppib* from the indicated number of individual mouse organs (biological replicates). cDNA was generated by reverse transcription of total RNA followed by real-time PCR analysis (real-time RT-PCR) performed and measured in triplicate (three technical replicates) using human or mouse specific primer pairs as previously described. The following human real-time RT-PCR primer pairs were used: *EPO* (forward) 5′-GAGGCCGAGAATATCACGACGGG-3′, *EPO* (reverse) 5′-TGCCCGACCTCCATCCTCTTCCAG-3′; *VEGFA* (forward) 5′-AGTAGCTGCGCTGATAGACATCCATGA-3′, *VEGFA* (reverse) 5′-CACCCATGGCAGAAGGAGGAGGGCAGAA-3′; *SERPINE1* (forward) 5′-ATTCAAGCAGCTATGGGATTCAA-3′, *SERPINE1* (reverse) 5′-

CTGGACGAAGATCGCGTCTG-3′ (PrimerBank ID 10835159a2); *MMP9* (forward) 5′-GGGACGCAGACATCGTCATC-3′, *MMP9* (reverse) 5′-TCGTCATCGTCGAAATGGGC-3′ (PrimerBank ID 4826836a2); *SLC2A1* (forward) 5′-CTTTTCTGTTGGGGGCATGAT-3′, *SLC2A1* (reverse) 5′-CCGCAGTACACACCGATGAT-3′ (PrimerBank ID 5730051a2); *PGK1* (forward) 5′-TTAAAGGGAAGCGGGTCGTTA-3′, *PGK1* (reverse) 5′-TCCATTGTCCAAGCAGAATTTGA-3′; *PPIB* (forward) 5′-ATGTGGTTTTCGGCAAAGTTCTA-3′, *PPIB* (reverse) 5′-GGCTTGTCCCGGCTGTCT-3′. We used the following mouse real-time RT-PCR primer pairs: *Epo* (forward) 5′-GAGGCAGAAAATGTCACGATG-3′, *Epo* (reverse) 5′-CTTCCACCTCCATTCTTTTCC-3′; *Pgk1* (forward) 5′-CTCCGCTTTCATGTAGAGGAAG-3′, *Pgk1* (forward) 5′-GACATCTCCTAGTTTGGACAGTG-3′; *Ppib* (forward) 5′-ATGTGGTTTTCGGCAAAGTTCTA-3′, *Ppib* (reverse) 5′-GGCTTGTCCCGGCTGTCT-3′.

Chromatin immunoprecipitation assays (cells).

For each Hep3B sample analyzed in sequential ChIP experiments, three individual 150-mm plates of Hep3B cells were exposed to normoxia or hypoxia and then pooled together for chromatin preparations. Sequential chromatin immunoprecipitation assays (Re-ChIP) were performed using the Re-ChIP-IT magnetic chromatin re-immunoprecipitation kit (Cat. No. 53016, Active Motif). We used the following antisera for the first chromatin immunoprecipitation reaction: normal mouse IgG (Cat. No. 2027, Santa Cruz Biotechnology, Inc.), normal rabbit IgG (Cat. No. NI01, EMD Chemicals, Inc.), anti-human HIF-1α, anti-human HIF-2α, anti-human p300, or anti-human CBP. We used the following antisera for the

second chromatin immunoprecipitation reaction: anti-human p300, anti-human CBP, anti-human HIF-1α, or anti-human HIF-2α.

The ChIP-IT Express Magnetic assay kit (Cat. No. 53009, Active Motif) was used for EGR ChIP experiments. The procedure was performed according to manufacturer's instructions using the following antibodies: anti-human HIF-2α, anti-human Egr1, human anti-Egr2, and normal mouse IgG.

After the ChIP or sequential ChIP, the precipitated genomic DNA was analyzed by quantitative PCR using an Applied Biosystems ABI Prism 7000 thermocycler (Applied Biosystems) and Power SYBR Green Master Mix (Cat. No. 4367659, Applied Biosystems) with the following human *EPO* enhancer primers: 5′-CTCTGTCCCACTCCTGGCAGCAGTG-3′ (forward) and 5′-CCTTGATGACAATCTCAGCGCACTG-3′ (reverse), or human *PGK1* promoter primers: 5′- GGATCTTCGCCGCTACCCTTGTG -3′ (forward) and 5′-CTATTGGCCACAGCCCATCGCGGTC -3′ (reverse), or human *RPL13A* promoter primers: 5′-GAGGCGAGGGTGATAGAG-3′ (forward) and 5′-ACACACAAGGGTCCAATTC-3′ (reverse). We normalized captured genomic DNA to input material and compared the normoxic and hypoxic samples.

Chromatin immunoprecipitation assays (mice).

For mouse ChIP experiments, we exposed 4 wild-type and 4 Acss2-knockout mice to room air (21% oxygen) or hypoxia (6% oxygen) for 2 h as we have done previously and euthanized the mice under normoxic or hypoxic air mixtures to harvest the kidney samples. We minced 30 mg of fresh tissue to 1–3 mm^3, transferred tissue into 10 mL of PBS plus 100

µl 1× protease inhibitor cocktail (Cat. No. P8340, Sigma), added formaldehyde (final concentration 1%) and rotated tubes at room temperature for 15 min. We stopped cross-linking with fresh glycine (final concentration 0.125 M). After 5 min at room temperature, we pelleted the samples in a centrifuge at 420*g* at 4 °C, washed once with cold PBS plus protease inhibitors and then repelleted. We resuspended the washed pellet in 1 mL of PBS on ice and ground the tissue using a micro-tissue grinder on ice. We pelleted cells again as above at 4 °C. We carried out ChIP using the ChIP-IT Express Magnetic assay kit (Cat. No. 53009, Active Motif). For sequential ChIP experiments, we prepared and analyzed wild-type and Acss2-knockout kidney samples using the reagents as described above. We analyzed precipitated genomic DNA by quantitative PCR in triplicate measurements for each sample using the following mouse *Epo* enhancer primers: 5′-CTGTACCTCACCCCATCTGGTC-3′ (forward) and 5′-CCCAGCTCACTCAGCACTTGTCC-3′ (reverse), or mouse *Pgk1* promoter primers: 5′-GGCATTCTGCACGCTTCAA-3′ (forward) and 5′-GAAGAGGAGAACAGCGCGG -3′ (reverse). We normalized captured genomic DNA to input material and compared the normoxic and hypoxic samples.

Generation of Acss2-knockout mice.

For Acss2-knockout mouse studies, we created Acss2-knockout mice using SM-1 mouse embryonic stem cells (129S6/SvEvTac) by disruption of the *Acss2* gene using a targeting construct containing ~5 kb of intronic DNA just upstream of exon 3 and ~1 kb of DNA just downstream of exon 16 separated by a neomycin resistance drug cassette, *Pol II-neo-Bovine pA* and two copies of *HSV-TK* outside the 3′ end of the targeting construct (Soriano, Montgomery et al. 1991). The techniques used for gene targeting and creation of

chimeric mice have been previously described (Shimano, Shimomura et al. 1997). We mated chimeric founder mice with C57BL/6J mice to generate mixed-strain heterozygous Acss2 progeny, which were maintained by random matings of heterozygous Acss2 mice for more than 10 generations. We then crossed the line with C57BL6J/129 F1 hybrid mice and maintained the knockout allele on this mixed background for more than 10 generations. We generated mixed-strain wild-type and homozygous Acss2-knockout mice from matings of heterozygous Acss2 mice, which were fertile and were subsequently used to generate progeny for study use. We maintained mice under standard conditions (7 a.m./7 p.m. light/dark cycle) in the University of Texas Southwestern Medical Center animal facilities and fed them *ad libitum* with standard chow. The University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee approved all experiments.

Mouse experiments: hypoxia exposure.

For hypoxia exposure experiments, we examined equal numbers of 10- to 11-week-old wild-type versus homozygous Acss2 knockout mice under normoxia (room air, 21% oxygen) or the indicated period of short-term hypoxia exposure (6% oxygen; 0.5, 2, 8, 16 h) using modified mouse cages. After treatment, we euthanized mice and harvested kidneys for further characterization. We derived baseline hematocrits from mice exposed to normoxia. The University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee approved all experiments.

Mouse experiments: phenylhydrazine-induced anemia.

For acute anemia experiments in mice, we used phenylhydrazine injections to induce a hemolytic anemia as previously described (Itano, Hirota et al. 1975). For anemia studies

using Acss2-knockout mice, after measuring baseline hematocrits via the tail vein, we injected 3- to 4-month-old wild-type or Acss2-knockout mice (6 mice per group) with 40 mg kg^{-1} of phenylhydrazine hydrochloride (PHZ; Cat. No. 114745-5G, Sigma) in normal saline or an equal volume of normal saline two times at 24 h intervals, with equal numbers of male (4) and female (2) mice in each group. We bled mice from tails to measure hematocrits 48 h (day 2) and 96 h (day 4; nadir) after the initial PHZ injection. For acetate supplementation, we administered 400 μL of a sterile 0.5 M stock solution of either PBS (vehicle) or sodium acetate/PBS (A, Ac, NaAc) as a single dose at approximately 9 a.m. on the day of treatment by i.p. injection to PHZ-treated wild-type or Acss2-knockout mice (three mice per group, all males) with hematocrits between 21% and 25%, starting on day 4 following the initial PHZ dose. We measured hematocrits every 3 d beginning on day 4 after the initial PHZ injection and on the final day of euthanasia when we euthanized mice for organ harvesting. The University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee approved all experiments.

For CD1 mice/acetate treatment acute anemia studies, we injected 7- to 8-week-old (20–24 g) CD1 female mice i.p. (Charles River Laboratories) with 60 mg kg^{-1} of PHZ in normal saline (PHZ) or normal saline (control) two times at 24 h intervals. We bled mice from the tails to measure hematocrits 96 h (day 4) after the initial injection. For the PHZ treatment group, we first selected mice according to an acceptable range of anemia (~21–24%). We then subdivided mice into groups of 5 mice (plus up to 3 extra mice to allow for losses or harvesting of mice during the protocol) for every collection day with similar starting mean hematocrit levels for each group. We gavaged anemic mice per os (p.o.) with vehicle

(water), glyceryl triacetate (GTA; 90 μL/25 g body weight; Cat. No. W200700, Sigma-Aldrich Chemicals) glyceryl tributyrate (GTB; 140 μL/25 g body weight; Cat. No. W222305, Sigma) or glyceryl tripropionate (GTP; 115 μL/25 g body weight; Cat. No. W328618, Sigma) using a 20-gauge stainless steel animal feeding tube (Cat. No. FTSS-20S-25, Instech Solomon). We performed gavage once a day in the morning from day 4 to day 20 until euthanasia. We euthanized mice and harvested organs for further characterization on days 4, 7, 10, 13, 16 and 20 after the initial PHZ injection, corresponding to days 0, 3, 6, 9, 12 and 16 of gavage. We gave a control treatment group (solvent injection), which had approximately equal baseline hematocrits (~49%), acetate (GTA) or vehicle (water) by oral gavage once a day starting on day 4 following solvent injection and we harvested the mice on days 4, 7, 10, 13, 16 and 20 after the initial solvent injection. We noted no differences in hematocrit levels in this control group (data not shown).

For intraperitoneal (i.p.) short-chain fatty acid (SCFA) treatments, we prepared a sterile 0.5 M stock solution of each SCFA using sterile PBS and filter-sterilized using a 0.2 μM filter (Cat. No. 190-2520, Thermo Scientific). We injected mice intraperitoneally with the indicated SCFA/PBS or vehicle (sterile PBS alone) solution. We did not blind investigators as to PHZ or gavage treatments because of institutional animal monitoring requirements, although we performed harvesting and blood draws of mice using only eartag identifiers. The University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee approved all experiments.

Plasma Epo protein and hematocrit measurements.

To harvest plasma, we mixed 200 μL eye blood with 7.8 μL heparin (Cat. No. NC9593879, Fisher Scientific), incubated 30 min on ice and spun down at 14,000*g* for 10 min. We then removed the supernatant and stored in −80 °C until analysis. For plasma Epo protein measurements, we assayed 25 μL of plasma in duplicates using a Mouse Erythropoietin Quantikine ELISA Kit (Cat. No. MEP00B, R&D Systems).
We obtained spun hematocrits from tail-vein or eye bleeds.

Colony Formation

Five hundred HT1080 cells were seeded in triplicate 10 cm plates and allowed to attach for 24 hr. After 24 hr, cells were treated with complete media at 1% oxygen or media containing 1 mM glucose or 5 mM acetate at 21% oxygen for 10 days. Media was not changed throughout the experiment. Colonies were then stained with 1% crystal violet dissolved in ethanol/PBS (15%/85%). Cells were imaged and colony number determined using ImageJ software.

Cell proliferation assays

1 x 10^3 HT1080 cells/well were seeded in a 96-well plate with each cell line in replicate sets of eight. After 24 h, cells were exposed for 1 week to 1% oxygen, or exposed to 21% oxygen with either standard glucose media (25 mM glucose), or low glucose media (1 mM glucose). Media was changed every 48 hr with comparable media. Cell proliferation was detected every day with the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Kit (Cat. No. G5421, Promega) according to the manufacturer's protocol. The absorbance was recorded at 490 nm with a microplate reader.

Cell migration and cell invasion assays

For cell migration assays, HT1080 cells were serum-starved in 0.5% FBS/DMEM media overnight. After 12 hr, 1.5 x 10^5 HT1080 cells in serum-free media were transferred into a transwell insert. For cells maintained under normal conditions, cells were incubated with complete media and exposed to 21% oxygen for 4 h. For cells maintained under hypoxic conditions, cells were incubated with complete media and exposed to 1% oxygen for 4 hr. For cells exposed to low glucose conditions, cell were incubated with low glucose (1 mM) media at 21% oxygen for 24 hr and compared to control cells maintained under standard glucose (25 mM) conditions for 24 hr. Cell migration was detected from triplicates for each treatment after crystal violet staining. The absorbance was recorded at 560 nm with a microplate reader.

For cell invasion assays, we used a commercially available kit containing wells pre-filled with Matrigel (CytoSelect 24-Well Cell Invasion Assay Kit; Cat. No. CBA-110, Cell Biolabs). HT1080 cells were serum-starved in 0.5% FBS/DMEM media overnight. After 12 hr, 1.5 x 10^5 HT1080 cells in serum-free media were transferred into the transwell insert as above after pre-incubating the transwell insert for 1 hr with serum-free media at room temperature. Cell migration was determined from triplicates for each treatment according to the manufacturer's protocol.

***In vivo* nude mice flank tumor experiments**

All animal experiments were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee. Female mice were used exclusively for HT1080 tumor cell implantation. For flank tumor studies, mice were injected subcutaneously on the left dorsal flanks with 5×10^6 luciferase-expressing stably transformed

HT1080 cells (control, ACSS2, HIF-1α, or HIF-2α knockdown) grown using 10% FBS were resuspended in 0.5 ml DMEM. Tumor sizes were measured using calipers every other day beginning on the fourth day after cell injections. Beginning six days after injection of cells, mice were given an acetate ester, triacetin (5.8 gm/kg body weight in PBS; Cat. No. W200700, Sigma-Aldrich Chemicals), or vehicle (PBS, 0.01 mL/g body weight) by oral gavage once per day. Mice were harvested when the mean tumor volume of any group approached 2 cm^3. All experiments were terminated at this time-point.

***Ex vivo* nude mice flank tumor experiments**

At the completion of the tumor study, mice were sacrificed and lung as well as tumors removed for biochemical luciferase activity determination as described (Promega). Individual tissues were weighed and homogenized using a PowerGen 700D homogenizer (ThermoFisher Scientific) in lysis reagent (25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2mM 1,2 diaminocyclohexane-N,N,N,N-tetra-acetic acid, 10% glycerol, 1% NP-40) containing soybean trypsin inhibitor (0.2 mg/ml) and bovine serum albumin (0.2 mg/ml). Samples (2 µl tumor or 20 µl lung lysates) were diluted in 100 µl lysis reagent containing 2.5 mM $MgCl_2$. Immediately prior to measurement, 50 µl luciferin reagent (20 mM tricine, 1 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33 mM DTT, 0.27 mM coenzyme Q, 0.47 mM luciferin, 0.53 mM ATP, pH 7.8) was added and measurement performed for 10 sec in a single-tube luminometer (Sirius, Berthold Detection Systems).

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