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The asparaginyl hydroxylase factor Inhibiting HIF-1alpha is an essential regulator of metabolism

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The Asparaginyl Hydroxylase Factor Inhibiting HIF-1alpha Is an Essential Regulator of Metabolism

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biology by Na Zhang

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The Dissertation of Na Zhang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

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ABSTRACT OF THE DISSERTATION

The Asparaginyl Hydroxylase Factor Inhibiting HIF-1alpha is an Essential Regulator of Metabolism

by

Na Zhang

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Professor Randall S. Johnson, Chair

Factor Inhibiting HIF-1α (FIH) is an asparaginyl hydroxylase. Hydroxylation of HIF-α proteins by FIH blocks association of HIFs with the transcriptional co-activators CBP/p300, thus inhibiting transcriptional activation. We have created mice with a null mutation in the FIH gene, and found that it has little or no discernable role in mice in altering classical aspects of HIF function, e.g., angiogenesis, erythropoiesis, or development. Rather, it is an essential regulator of metabolism: mice lacking FIH exhibit reduced body weight, elevated metabolic rate, hyperventilation, improved glucose and lipid homeostasis, and
are resistant to high fat diet-induced weight gain and hepatic steatosis. Neuron-specific loss of FIH phenocopied some of the major metabolic phenotypes of the global null animals: those mice have reduced body weight, increased metabolic rate, enhanced insulin sensitivity, and are also protected against high fat diet-induced weight gain. These results demonstrate that FIH acts to a significant degree through the nervous system to regulate metabolism.
Chapter 1

General Introduction
Oxygen-dependent regulation of HIF-1α

Hypoxia is a physiological condition where available oxygen levels are below 5%, triggering complicated cellular responses for adaptation such as angiogenesis, iron metabolism, glucose metabolism and cell proliferation/survival, etc. It is a common physiological phenomenon and also a key component of pathology such as heart attack, stroke and cancer [1]. In metazoan organisms, hypoxia inducible factor 1 (HIF-1) is the key regulator responding to changes in tissue oxygenation [2-4]. When the oxygen level in tissue is low, HIF-1 functions to maintain development, physiology, and disease pathogenesis [5]. HIF-1 is a heterodimeric transcription factor consisting of the basic helix-loop-helix PAS (Per/Arnt/Sim) domain proteins HIF-1α and HIF-1β (also called aryl hydrocarbon receptor nuclear translocator, ARNT). HIF-1α is regulated in an oxygen dependent manner, whereas HIF-1β is constitutively expressed. Although both HIF-1α and HIF-1β subunits are indispensable for HIF activity, HIF-1 functions are primarily regulated by the posttranslational modification of HIF-1α [2, 6, 7].

Two known different types of hydroxylases regulate HIF-1α protein stability and transcription activity respectively: prolyl hydroxylases (PHDs) and asparaginyl hydroxylase (FIH). They are dioxygenases that require oxygen, Fe (II), and 2-oxoglutarate as cosubstrates [8, 9]. Under normal oxygen conditions (normoxia), two conserved proline residues in a region referred to as the oxygen-dependent degradation domain (ODD) in HIF-1α protein are hydroxylated by
PHDs. The ODD with two hydroxylated proline residues are recognized by the product of the von Hippel-Lindau (pVHL) tumor suppressor gene, one component of a ubiquitin ligase complex that tags HIF-1α with polyubiquitin to induce HIF-1α degradation by the proteasome [10] (Figure 1.1 a). In addition to modulation of protein stability, the transcription activity of HIF-1α is also inhibited under normoxia, which is mediated by FIH (factor inhibiting HIF-1α). FIH hydroxylates the asparagine residue in the C-TAD (C-terminal transactivation domain). This modification blocks the binding of the 300-kilodalton coactivator protein (p300) and CREB binding protein (CBP). Consequently, HIF-1α transcription activity is inhibited in normoxia [11]. Because oxygen is necessary for PHDs and FIH to catalyze the reactions, prolyl hydroxylation and asparaginyl hydroxylation are blocked under hypoxia (low oxygen availability) (Figure 1.1 b). With no VHL-mediated proteasomal degradation, HIF-1α rapidly accumulates, translocates to the nucleus where it dimerizes with HIF-1β and binds to the hypoxia response element with a DNA sequence 5’-RCGTG-3’ (R, purine (A or G)) in HIF target genes. P300/CBP coactivators are recruited, thereby promoting the transcription of HIF-1α target genes, which include vascular endothelial growth factor (Vegf), glucose transporter-1 (Glut-1) and phosphoglycerate kinase (Pgk), etc. All these target genes act to minimize the effects caused by low oxygen in tissue [2, 6, 9].

HIF-1α has two paralogs: HIF-2α and HIF-3α. HIF-2α is a protein highly homologous to HIF-1α. It is also regulated by posttranslational proline and asparagine hydroxylation, dimerizes with HIF-1β and induces expression of a
group of target genes. They overlap with, but are distinct from those genes regulated by HIF-1α [12]. HIF-3α, also named as the inhibitory PAS domain protein (IPAS), is a dominant negative regulator of HIF-1. Transcriptional regulation of its expression by HIF-1 indicates that HIF-3α may be also involved in a negative feedback gene regulation in adaptive responses to hypoxia [13].

HIF-1α in cancer

A number of research using immunohistochemical analysis of human cancer biopsies revealed increased levels of HIF-1α protein in most of primary cancers and their distant metastases [14-17]. In some human cancers of the brain (oligodendroglioma), breast, cervis and oropharynx, there are significant correlations between HIF-1α overexpression and patient mortality [14, 18-21].

Genetic alterations and intratumoral hypoxia are two main causes for increased levels of HIF-1α protein in solid tumors, relative to the surrounding normal tissues (Figure 1.2) [6]. In the cases of genetic alterations, various mutations in cancer activate HIF-1α pathway in different mechanisms. Gain of function of oncogenes or loss of function of tumor suppressor genes leads to either decreased HIF-1α degradation or increased HIF-1α expression [6, 22, 23]. One of the most marked examples is observed in individuals with von Hippel-Lindau syndrome. These people are heterozygous for a germline loss-of-function mutation in the VHL tumor suppressor gene on the short arm of chromosome 3 (3p25-26), and are at high risks of developing renal carcinomas (RCCs) with a somatic inactivation of the remaining wild-type VHL gene. Due to impaired VHL-
mediated ubiquitylation and subsequent proteasomal degradation, HIF-1α protein is accumulated in a great amount, resulting in drastically increased HIF-1α activity even in non-hypoxic conditions [24, 25].

Although genetic alterations induce HIF-1α activation in some types of human cancer, intratumoral hypoxia turns out to be the major mechanism underlying the increased levels of HIF-1α in cancer. Intratumoral hypoxia is a critical hallmark of solid tumors and caused by imbalance between the poor supply of oxygen by tumor vasculature and the rapid consumption of oxygen by advanced tumor cells [26]. In normal breast, the median partial oxygen pressure (pO2) is 65mm Hg, whereas it is only 10mm Hg in locally advanced breast cancers [27]. Increased levels of HIF-1α protein, whose stabilization and activity are regulated in an oxygen-dependent manner, are obvious in hypoxic tumor regions. Immunohistochemistry for HIF-1α protein on human cancer biopsies revealed strong stainings in areas surrounding necrotic regions or those far from blood vessels [6, 14].

The direct consequence of accumulated and activated HIF-1α is induction of its target genes, which promote tumor progression in many critical aspects including angiogenesis, metabolic adaptation, apoptosis resistance, genetic instability, pH regulation, invasion and metastasis, etc [1, 26, 28-32].

One of the most important HIF-1α targets, VEGF induces tumor angiogenesis, which increases oxygen delivery to solid tumors and promotes tumor growth. Other critical targets of HIF-1α, such as Glut-1 and LDH (lactate
dehydrogenase), play critical roles in the process of metabolic adaptation. In hypoxic conditions, increased expression of glycolytic genes leads to higher glycolytic rate in tumor cells for energy production (the Warburg effect). Oxygen consumption is reduced by facilitation of glycolysis and inhibition of aerobic oxidation in hypoxic tumor cells.

In addition, HIF-1α activation is also correlated with metastasis, one important aspect of cancer progression and the major cause of mortality in human cancer patients. Metastasis is a complicated multistep process and involves tumor cell invasion, intravasation, extravasation, colonization and proliferation. Activated HIF-1α protein promotes the formation of metastasis in distant organs via regulation of some key factors including lysyl oxidase (LOX) [33], E-Cadherin [34-36], C-X-C chemokine receptor 4 (CXCR4) and stromal-derived factor 1 (SDF-1/CXCR12) [37-41]. For example, chemokine receptor CXCR4 is highly expressed in metastatic cancer cells. Stromal cells at the metastatic sites such as lung, bone marrow and liver produce the cognate ligand SDF-1 for CXCR4. Both CXCR4 and SDF-1 have HIF-binding sites and their expression is induced by hypoxia and HIF. The paracrine loops between CXCR4-expressing cancer cells and SDF-1-expressing stromal cells play a critical role in directing migration of metastatic cancer cells.

**Polyoma Middle T (PyMT) breast cancer model**

Genetically manipulated mouse models are useful and powerful tools to study the underlying mechanism of human cancers including breast cancer. They
were developed by selective deletion of tumor suppressor genes, such as Trp53, Brca1 and Pten, or expression of oncogenes including Erbb2, Myc, Hras and PyMT in mammary epithelial cells. The mammary gland selectivity is accomplished using the mammary tumor virus long terminal repeat (MMTV-LTR) and whey acidic protein (Wap) promoters [42].

MMTV-PyMT model has been widely used for breast cancer research because it reflects the complexity of human breast cancer progression [43]. In this particular model, polyoma virus middle T antigen is driven by the MMTV-LTR and selectively expressed in mammary epithelium. PyMT is encoded by the small DNA mouse polyoma virus, which was discovered some 60 years ago and found to be induce formation of tumors when inoculated into young mice [44]. PyMT is a membrane protein, tethered to the cell membrane at its carboxyl-terminal end. It binds to several cellular proteins including protein phosphatase 2A (PP2A), c-SRC tyrosine kinase and phosphatidylinositol 3-kinase (PI3K) and stimulates several signal transduction pathways, such as the mitogen-activated protein kinase (MAPK) and PI3K cascades [43, 44]. Although PyMT is not expressed in human cancer cells, its interaction with these signaling pathways altered in human breast cancers makes it a potent oncogene. MMTV-PyMT mice develop mammary hyperplasia as early as 4-weeks of age [45, 46]. When mice were around 14 weeks old, the majority of these mice developed carcinoma and distant pulmonary metastases [45]. In this model, four distinct stages of tumor progression can be identified (Figure 1.3). They are hyperplasia, adenoma, early
carcinoma and late carcinoma, which are comparable to human breast cancer progression. Extensive histopathological analysis on PyMT mammary tumors revealed similar expression patterns of biomarkers associated with poor prognosis in human breast cancer patients. For example, overexpression of ErbB2/Neu observed in human breast cancer with poor prognosis is also found in PyMT mammary tumors [45]. Remarkably, there is a high frequency of pulmonary metastasis at the terminal stage, which does not occur in some of other transgenic mouse models. All these features demonstrate that PyMT model is a powerful and reliable tool for investigation of human breast cancer.

**Conditional deletion by Cre/loxP system**

The Cre/loxP recombinase system mediates site-specific DNA recombination and is being widely utilized to study gene function in vivo. It was first described in bacteriophage P1 [47]. LoxP (locus of X-over in P1) site is a 34-bp DNA sequence containing two 13-bp inverted repeats and an asymmetric 8-bp spacer region. Cre recombinase is a 343 amino acid protein. DNA sequence flanked by two loxP sites in same orientation will be excised by Cre recombinase. To generate tissue-specific conditional knockout mice, two lines of mice are needed. One line has a loxP-flanked gene of interest. The other mouse line contains Cre recombinase under the control of a tissue-specific promoter. These two mice are crossed to delete the gene of interest exclusively in the tissue with an active promoter driving Cre recombinase expression [48]. The Cre/loxP system is a great tool to target genes in a tissue/cell-specific manner. Moreover,
it can be combined with mouse models for human diseases. For example, to determine the roles of HIF-1α in endothelium during mammary tumor progression, HIF-1α\textsuperscript{df} female mice with exon 2 of HIF-1α gene flanked by two loxP sites were crossed with HIF-1α\textsuperscript{df}/Tie2-Cre/MMTV-PyMT male mice containing both MMTV-driven PyMT oncogene and Cre under the promoter of Tie2 [49, 50]. HIF-1α\textsuperscript{df}/Tie2-Cre/MMTV-PyMT and HIF-1α\textsuperscript{df}/MMTV-PyMT female mice were used for investigation (Figure 1.4).

**Factor inhibiting HIF-1α (FIH)**

FIH is a 2-oxoglutarate dependent iron (II)-dioxygenase, which is encoded by the \textit{HIF1an} (hypoxia inducible factor 1 alpha subunit inhibitor) gene. It is a key component of the oxygen sensing system that regulates the stabilization and activity of HIF [10, 51].

FIH was first identified as a HIF-interacting protein and represses the transcriptional activity of HIF [52], and was later found to be an asparaginyl hydroxylase for HIF and its activity is dependent on the oxygen level [11, 53]. In the presence of sufficient oxygen, FIH hydroxylates the asparagine residue in the C-terminal transactivation domain of HIF-1α, which impairs the recruitment of the coactivators p300/CBP, leading to inhibition of HIF-1α transcriptional activity. As the oxygen level drops, FIH becomes progressively inactive, resulting in the activation of stabilized HIF-1α [10, 51]. Biochemical analysis on tissue culture cell lines revealed that FIH protein is widely expressed [54, 55]. The localization of
FIH is predominantly in the cytoplasm and not altered by hypoxia or hypoxia mimetics [54, 56-58].

PHDs and FIH serve as the critical oxygen sensors that control the activity of HIF-1α, the master transcriptional factor in responses to hypoxia. They negatively regulate HIF-1α in different pathways. They are also different in affinity for oxygen. Compared to PHDs, FIH has a higher affinity for oxygen. The estimated K_M values of FIH and PHDs are 90 µM and 100-250 µM, respectively [51, 59, 60]. FIH may still maintain its enzymatic activity at low oxygen levels, while PHDs are already inactive. FIH could thus serve to fine tune the HIF-1α response, however, there is no strong evidence yet to support this postulation.

Recent studies have identified a wide range of other substrates for FIH hydroxylation besides HIF-1α in vitro. They include proteins containing ankyrin repeat domains (ARD), such as the intracellular domain of Notch receptors [61, 62], p105, IkBα [63], SOCS (suppression of cytokine signaling) box protein 4 (ASB4) [64], MYPT1 [65], Tankyrase-2, Rabankyrin-5 and RNase L [66]. The identification of the widespread hydroxylation of ARD proteins by FIH revealed that the asparaginyl hydroxylation is a common posttranslational modification. To date, the functional outcome of FIH-mediated hydroxylation has not been well characterized. Some studies proposed that these ARD-containing substrates compete with HIF-1α for hydroxylation and thus contribute to a fine regulation on HIF-1α activation [67-69].
Previous studies described above implicate FIH is an inhibitor of HIF and mediates a wide range of posttranslational hydroxylation events. However, it still remains unclear about the physiological roles of FIH. Thus, in my research project, I used Cre/loxP system to generate the global and conditional knockout mice for FIH and performed a genetic examination of FIH function, which is essential to begin a determination of its actual role in vivo.
Figure 1.1. Oxygen-dependent regulation of hypoxia inducible factor-1α (HIF-1α) stabilization and activity

Modified from Schofield CJ and Ratcliffe PJ. *Nature Reviews Molecular Cell Biology*, 2004
Figure 1.2. Mechanisms and consequences of HIF-1 activity in cancer cells

Figure 1.3. Distinct stages of mammary tumor progression in MMTV-PyMT mice
Figure 1.4. Schematic of Tie2-Cre mediated deletion of HIF-1α in MMTV-PyMT breast cancer model
REFERENCES


Chapter 2

The asparaginyl hydroxylase factor inhibiting HIF-1α is an essential regulator of metabolism
INTRODUCTION

Metabolic response in animals is essentially the act of balancing demand against substrate availability. A key element of this balance for many animals and their constituent tissues is the variable of oxygen availability, which influences both rate and mechanism of substrate utilization for energy production [1]. The hypoxia inducible factors act as enhancers and regulators of transcriptional response to low oxygen. They are able to respond to a wide range of environmental oxygen concentrations, and coordinate responses at the cellular to organismal levels via alterations in at least 300 oxygen-responsive genes [2].

The HIF response has a series of complex controls. A key mechanism of regulation, however, is through enzymatic, oxygen-dependent modification of the HIF-1α and HIF-2α proteins [3]. The post-translational events involved in this depend on the availability of oxygen for the catalysis of two different modifications of HIF-α proteins. The first category of oxygen-dependent covalent changes involves the prolyl hydroxylases, or PHDs. These are required for hydroxylation of highly conserved prolines in the HIF-α transcription factors; these in turn ultimately regulate ubiquitination and turnover of the HIF-α proteins [1]. The second category requires a factor termed FIH (encoded by the Hif1an gene), and it catalyzes an asparagine hydroxylation step that controls the association of HIF-α transcription factors with CBP/p300 transcriptional co-activators [4, 5]. Genetic mutations in the PHDs and in the von Hippel-Lindau tumor suppressor gene (VHL) that mediate HIF-α ubiquitination have been
described: null mutations in these genes in mice cause mid-gestation lethality when PHD2 and VHL are deleted [6, 7]; mutations in the PHD1 and PHD3 genes have more subtle phenotypes [8], but all generally exhibit some of the archetypal features of increased HIF activation, e.g., increased vascularization and high levels of erythropoiesis [9].

The FIH gene has been associated with hydroxylation events in a range of other substrates as well, including those in the Notch pathway [10, 11] and the tankyrase protein [12, 13]; given this, a genetic examination of the role of the FIH gene is essential to begin a determination of its actual role in animal physiology. We have found that loss of FIH results in a wide-ranging derangement of physiological response, causes a hypermetabolic phenotype, but results in few of the classical effects of HIF activation in vivo. These data also indicate that targeting of this pathway could provide a unique mechanism for manipulation of metabolism.

RESULTS

Targeting of the FIH gene creates a null allele

As can be seen in Figure 2.1A, the FIH gene product is expressed in a wide range of tissues. Therefore, to better understand the role of the FIH factor in the overall regulation of oxygen homeostasis, we created a conditional null mutation of the gene, as shown in Figure 2.1B. This deletion was engineered in embryonic stem cells, with a targeting efficiency of 2 clones generated with the
designed event for approximately 1,800 ES cell clones screened (Figure S2.1A). Both isolated clones were able to produce germ line transmission of the altered allele. Following germline transmission of the conditional allele, animals were crossed with the EIIa-cre transgenic line in order to generate both complete nullizygous alleles (FIH\[^{A2}\]) as well as to generate conditional alleles (FIH\[^{flox}\]) as shown in (IV) and (V) of Figure 1B [14].

Crosses of the resulting FIH\[^{A2}\] heterozygous animals resulted in production of animals homozygous for the FIH null allele, which were verified for loss of protein production from the deletion by immunoblotting of whole embryo lysates derived from heterozygous crosses, as shown in Figure 2.1C. As can be seen, embryos genotyped as heterozygous have clearly reduced levels of FIH, and the embryo genotyped as nullizygous for the gene lacks any detectable FIH protein (Figure 2.1C). Genotypes were also confirmed by PCR analysis (Figure S2.1B).

The FIH gene encodes an asparaginyl hydroxylase that acts on the C-terminal activation domain (C-TAD) of the HIF-\(\alpha\) proteins [4]. To determine whether deletion of the FIH gene results in elimination of hydroxylation of the C-TAD of the HIF-\(\alpha\) proteins, a cloned and tagged C-TAD was stably transfected into MEFs generated from FIH\[^{df}\] embryos (exon2 of the FIH gene is flanked by two loxP sites). These cells were then treated with a Cre recombinase expression construct-containing adenovirus, and control and FIH null cells were created from untreated and treated cells, respectively. Mass spectrometric analysis of the C-TAD region was then carried out, and as seen in Figure 2.1D,
loss of FIH completely eliminates detectable hydroxylation of the asparagine residue within the C-TAD. This indicates that no redundancy exists in the pathway regulating this hydroxylation step, and that FIH is essential for this post-translational modification of the HIF-1α C-TAD.

**Transcriptional activation of HIF target genes via C-TAD is controlled by FIH**

Assays of gene expression from a hypoxia response element (HRE)-driven luciferase construct indicated that loss of FIH in MEFs caused modest increases in transcription from an HRE reporter in both normoxic and hypoxic conditions (Figure 2.1E). Luciferase expression driven by a Gal-HIF1α-CTAD construct, where expression is dependent on C-TAD activity, shows that loss of FIH eliminates differences in hypoxic versus normoxic expression (Figure 2.1F). This clearly occurs through a mechanism dependent on an intact asparagine at residue 803 in the C-TAD, as mutation of this residue makes the construct insensitive to the absence of FIH (Figure 2.1F). These data demonstrate that the FIH mutation described here eliminates negative regulation of HIF C-TAD driven gene expression.

**Gene expression analysis demonstrates a selective clamp function for FIH**

Gene expression levels for a large number of genes were surveyed, but generally, effects on expression levels of HIF target genes were modest in cells nullizygous for FIH (Figure 2.2A). As shown, the HIF target genes like *vascular...*
endothelial growth factor (Vegf), phosphoglycerate kinase (Pgk) and glucose transporter-1 (Glut-1) were all minimally increased at normoxia, and their increased expression at levels of 1% atmospheric oxygen were increased at roughly similar levels (Figure 2.2A). These data indicate that the FIH gene likely acts as a clamp on gene expression levels, which are additionally controlled by other factors, including the VHL-mediated turnover of gene expression acting via prolyl hydroxylases.

As can be seen in Figure 2.2B, loss of the gene results in a small but consistent decrease in cell growth, evident in growth curves at 72 hours post-seeding. Although this is seen under both normoxia and hypoxia, the difference between the wild type and mutant cells is similar under both conditions.

To determine the effect of the two factors together on this process, we crossed animals carrying the conditional allele of the VHL gene (VHL$^{df}$) [15], and animals carrying the conditional allele of the FIH gene (FIH$^{df}$), and then derived MEFs (as described above) from the resultant embryos with conditional alleles (VHL$^{df}$/FIH$^{df}$) of both the VHL and FIH genes. Figure 2.2C documents that overall effects on HIF-1α levels are as expected, with an increase in HIF-1α levels primarily determined by VHL; little effect on HIF-1α protein levels is seen in VHL/FIH double null cells.

Figures 2.2D and 2.2E show the effects on gene expression in normoxia of loss of the two factors, VHL and FIH, concurrently; and demonstrates that their deletion has at least two classes of outcome. The first, seen in the HIF target
gene *Vegf*, is an approximately additive effect on gene expression of deletion of the two hydroxylase-mediated mechanisms of controlling gene expression (Figure 2.2D). The second and more dramatic difference is seen in a group of genes (Table S2.1) that includes the *carbonic anhydrase 9* gene (*Car9*); here, loss of either VHL or FIH alone create a small increase in gene expression, but loss of the two in tandem causes an extraordinary alteration in gene control, with an approximate 200-fold increase in *Car9* expression in the double deletion cells (Figure 2.2E). Figures 2.2F and 2.2G demonstrate that in the case of both the *Vegf* and *Car9* genes, a triple deletion of VHL, FIH and HIF-1α restores to wild type levels the increased gene expression caused by loss of VHL and FIH. It is interesting, given that HIF-2α is also regulated by VHL and FIH, and known to effect HIF-mediated gene expression, that loss of HIF-1α in MEFs alone represses the exaggerated gene expression caused by loss of VHL and FIH in tandem.

A gene expression matrix analysis was carried out, and the effects are shown graphically in Figure 2.2H. Here it is evident that loss of both factors does have an additive effect, and that there are both synergies and differential selectivity in the function of the two hydroxylase-dependent pathways of gene expression.

As shown in Figure 2.2I, loss of VHL has a deleterious effect on cell growth under both normoxia and hypoxia. As can be seen, loss of both factors has an additive negative effect on cell growth in normoxia and hypoxia. Here as
well, the loss of HIF-1α in cells that contain conditional alleles for VHL and FIH (VHL/FIH/HIF-1α triple null cells) restores plating efficiency to wild type control levels, and indicates that it is the over-activation of HIF-1α that is primarily deleterious for cell survival.

**Synergistic effects of VHL and FIH in vivo**

To study the synergistic effects of VHL and FIH in vivo, I generated two conditional knockout mouse lines: in liver (Albumin-Cre) and skin (K14ER-Cre). Mice homozygous for the VHL mutation (VHL^{df}/Alb-Cre) in the liver were small and died between 5~7 weeks of age with severe hepatomegaly [16]. They had high hematocrit, consistent with high plasma EPO level. Plasma bilirubin level was also elevated. When both VHL and FIH were deleted in the liver, mice were even smaller and sicker (Figures S2.8A and B). Double mutant mice (VHL^{df}/FIH^{df}/Alb-Cre) had similar hematocrit and plasma EPO level to the VHL single mutants (data not shown). But they exhibited significantly higher plasma bilirubin level (Figure S2.8C). It was consistent with the jaundice appearance of double mutant mice. In addition, qPCR analysis revealed that some HIF target genes like *Glut-1* were significantly further up-regulated in double mutant livers (Figure S2.8D).

In skin-specific knockout mice, one obvious phenotype of mice homozygous for the VHL single mutation (VHL^{df}/K14^{ER}-Cre) was the red skin, which was caused by dilation of blood vessels [17]. When both VHL and FIH were deleted in the skin (VHL^{df}/FIH^{df}/K14^{ER}-Cre), mice had a redder appearance.
Double mutant mice had redder paws (Figure S2.8E). The more severe dilation of blood vessels could be seen in the back skin (Figure S2.8E) and in the ear (data not shown). The results from two different conditional knockout mouse lines indicate that VHL and FIH have synergistic effects in vivo.

**Loss of FIH causes an increase in cellular ATP levels and suppresses AMPK activation**

To investigate possible effects on cellular metabolism, we carried out assays on intracellular ATP levels in wild type and mutant cells maintained at normoxia and normoglycemia. As can be seen in Figure 2.3A, loss of FIH causes an increase in cellular ATP levels. Deletion of both FIH and HIF-1α results in decreased ATP levels (Figure S2.2A). To investigate further effects on cellular metabolism, total levels of the AMPK protein, as well as levels of activated AMPK were determined in normal as well as hypoxic and hypoglycemic cultures (Figure 2.3B and Figure S2.2B). This showed that loss of FIH acted to suppress AMPK activation under hypoglycemic conditions, whereas the suppression was diminished when both FIH and HIF-1α were deleted. A further investigation of AMPK activation during development showed that individual lysates of mid-gestation FIH null embryos also had lower levels of AMPK activation (Figure 2.3C); this indicates that the altered metabolic state which gives rise to increased cellular ATP in culture arises in development.

**Loss of FIH causes a hypermetabolic state in vivo**
We next investigated metabolic rates in adult FIH mutant mice. It has been found that activation of HIF in animals can increase glycolysis, reduce oxygen consumption and elevate the respiratory exchange ratio (RER=\(\frac{VCO_2}{VO_2}\)). However, we found that FIH mutant mice had an elevated consumption of oxygen and increased evolution of carbon dioxide under normoxic conditions (Figures 2.3D and 2.3E). RER is not changed in these mice, and this demonstrates that glycolysis has not increased in FIH mutant mice (Figure 2.3F).

This observation is important, as it indicates that the HIF-mediated control of glycolytic rates is not a key factor in the increased level of respiration in mutants; ratios of glycolysis relative to other metabolic pathways are unchanged (as indicated by the unchanged RER) in spite of the significant increase in mutant metabolism. As expected from elevations in oxygen consumption, loss of FIH causes an approximate 20% increase in heat production as expressed in calories per hour per unit body mass (Figure 2.3G). This is accompanied by an increased mutant heart rate (Figure 2.3H).

Increased oxygen consumption could be caused by changes in uncoupled mitochondrial respiration. We examined the expression levels of mitochondrial uncoupling genes in brown adipose tissue (BAT), white adipose tissue (WAT) and skeletal muscle (SM) and found that the \textit{uncoupling protein 1 (UCP1)} mRNA level in BAT from FIH nullizygous mice was significantly increased (Figure 2.3I). Levels of peroxisome proliferator-activated receptor \(\gamma\) coactivator-1\(\alpha\) (PGC-1\(\alpha\)) mRNA were also increased in BAT (Figure 2.3J); PGC-1\(\alpha\) is an important
regulator of thermogenesis (Uldry et al., 2006). Expression levels of the *uncoupling protein 2* (*UCP2*) and *uncoupling protein 3* (*UCP3*) genes in BAT, WAT and SM and the *PGC-1α* gene in WAT were similar between genotypes (Figure 2.3I and Figures S2.3A, S2.3B and S2.3C).

**Mice lacking FIH are hyperventilatory and exhibit alkalosis**

Ventilation is strongly affected in hypoxia; both tidal volume and respiratory frequency increase when mammals are exposed to low oxygen [18], and this can have significant effects on blood oxygenation. To investigate a potential role for FIH in hypoxic ventilation, we first examined global respiratory function in FIH null animals. In this context, we undertook plethysmography of the mutants (Figures 2.4A, 2.4B and 2.4C). These analyses demonstrated, first, that the frequency of breathing at normoxia is not elevated significantly in FIH mutants (Figure 2.4A), but that the tidal volume is significantly higher, with a mean increase of close to 30% (Figure 2.4B).

Taken together, these data argue that the large increase in tidal volume (Figure 2.4B) seen in wild type mice under hypoxic conditions is already present at normoxia in FIH mutants. Hyperoxia suppresses mutant tidal volume, such that at 30% oxygen their tidal volumes are not significantly different from those found in wild type mice. This indicates that FIH regulates a set point for mediating oxygen-regulated changes in tidal volume; and that this set point has been shifted in mutants to a higher oxygen concentration.
The elevation in frequency of respiration relative to wild type animals is only significant during hypoxia (Figure 2.4A); this indicates that a second level of compensation for hypoxia is triggered in FIH mutant animals, who presumably have little capacity to increase tidal volume further, and thus respond to decreased oxygenation with an increased respiratory frequency. This increase in respiratory frequency, in the presence of an increased tidal volume, would generate increased levels of blood oxygenation relative to wild type animals. Evidence of hyperoxygenation at normoxia is seen in Table S2.2, where both $pO_2$ (Partial Pressure of Oxygen) and $SO_2$ (oxygen saturation) are elevated in arterial blood gas analysis of mutants.

Analysis of minute volume ($Vi$, in Figure 2.4C), which is a measurement of total inspired air over time, and thus reflects both tidal volume and frequency of respiration, shows that the greatest discrepancy between wild types and mutants is at normoxia (Figure 2.4C). At hyperoxia, the genotypic differential in $Vi$ is no longer statistically significant.

Increased ventilation can be due to hyperpnoea, or respiratory adaptation to an increased metabolic rate; this would be accompanied by a plasma acidosis or, if compensated, a normal blood pH. If hyperventilation is occurring, however, the increased exhalation of carbon dioxide in the absence of a metabolically driven production of $CO_2$ causes a drop in $pCO_2$ (Partial Pressure of Carbon Dioxide) and an accompanying alkalosis. This is seen in FIH null mutants, which
have a clear hypocapnia (decreased pCO$_2$) and respiratory alkalosis (increased blood pH) characteristic of hyperventilation (Table S2.2).

**Mice lacking FIH have decreased EPO levels in response to hypoxia**

To further determine how hypoxic physiology is regulated by the FIH enzyme, we undertook examination of a basal response to hypoxia, the increased expression of the erythropoietic hormone erythropoietin (EPO) [1]. Consistent with a lack of HIF-related changes in vascularization in the FIH null mutants (Figure S2.4C), basal levels of EPO, hematocrits, and reticulocyte counts are not different from those of wild type animals (data not shown). This also differs from a wide range of tissue-specific deletions of both the PHDs and VHL, which exhibit increased EPO expression and varying degrees of polycythemia [9, 15, 17, 19, 20].

We found that global loss of FIH does alter EPO expression after 14 hours of exposure to hypoxia (9% O$_2$); levels of plasma EPO in FIH null animals are reduced by approximately 40% (Figure 2.4D). Renal expression of EPO mRNA correlates with the reduction in plasma EPO levels, and is reduced even more substantially, by approximately 75% relative to wild type levels post-hypoxia (Figure 2.4E).

To determine whether this was caused by a direct effect on gene expression, we examined EPO mRNA changes induced in an EPO-expressing cell line, the Hep3B cell line (Figure 2.4F). Suppression of FIH by siRNA in these
cells did not, however, alter hypoxic induction of \textit{EPO} expression significantly (Figure 2.4F).

To determine whether loss of EPO inducibility in mutants occurs in other contexts, we assayed plasma EPO levels following induction of experimental anemia (Figure 2.4G). This assay employs phenylhydrazine to induce red blood cell lysis; following treatment, the hematocrits in experimental animals drop, and the resulting anemia causes an increase in \textit{EPO} expression and erythropoiesis. In these experiments, there were no significant changes in plasma EPO levels in mutants relative to wild type animals (Figure 2.4G) and no alteration in induction of renal \textit{EPO} expression (Figure 2.4H). This demonstrates that the alteration in \textit{EPO} expression that we see in FIH mutants is not related to direct effects on \textit{EPO} expression in \textit{EPO}-synthesizing tissues, but is correlated with altered respiration during hypoxia.

\textbf{Loss of FIH causes decreased body mass and adiposity}

Loss of FIH causes an increase in both food and water consumption in conjunction with the increased metabolic rate described above (Figures 2.5A and 2.5B). Physical activity is a central aspect of energy expenditure, and so was measured together with O$_2$ consumption. As shown in Figure 2.5C, a significantly higher O$_2$ consumption in mutants accompanied a surprising hypoactivity, especially during dark cycles.
Body weight represents to some extent a balance between energy intake and expenditure. Overall body mass in FIH nullizygous mice is lower at birth (Figure 2.5D) and throughout life (Figure 2.5E). Mutants also have a significantly reduced body length (Figures S2.4A and S2.4B). This is accompanied by a decrease in epididymal white adipose tissue (WAT) (Figure 2.5F). Mass ratios of other tissues, including liver, quadriceps, and kidney, are similar between genotypes (Table S2.4A). Adipocytes from FIH nullizygous mice are significantly smaller than those from control mice (Figures 2.5G-2.5I), demonstrating decreased adiposity.

To determine the effect of FIH deletion on glucose physiology, glucose and insulin tolerance tests (GTTs and ITTs) were carried out (Figures 2.5J and 2.5K). Loss of FIH had little effect on glucose clearance, but had a significant effect on insulin sensitivity (Figure 2.5K). Fasting blood glucose levels in mutants were lower (data not shown). Correlated with an enhanced insulin sensitivity was a significantly lower fed plasma insulin level in mutants (Figure 2.5L) and improved lipid homeostasis. Serum triglyceride (TG), high-density-lipoprotein (HDL) cholesterol, low-density-lipoprotein, very low-density-lipoprotein (LDL/VLDL) and total serum cholesterol levels were all significantly lower in mutant animals (Table S2.3).

Tissue-specific loss of FIH indicates that hypermetabolism is neuronally regulated under normal chow
We next wished to determine whether the metabolic phenotypes described above could be ascribed to action of a single tissue, and began with the nervous system. We found that FIH is expressed at similar levels in different brain regions, i.e., cortex, hypothalamus and brain stem (Figure S2.5A). Animals were created with a pan-neuronal loss of FIH by crossing into a background of nestin promoter-driven cre expression. Specific deletion of FIH was confirmed by immunoblotting and qPCR (Figure 2.6A and Figure S2.5B). These tissue specific deletion mutants are significantly smaller than controls (Figure 2.6B) and phenocopy the increased metabolic rate seen in global FIH null animals (Figure 2.6C).

To investigate whether loss of FIH in neurons affects glucose homeostasis, GTTs and ITTs were performed: neuronal FIH null mutants exhibited improved insulin sensitivity, although glucose tolerance was unchanged (Figures 2.6D and 2.6E). There was no significant difference in fasting blood glucose levels in 2-month old animals (Figure S2.5C), but fasting plasma insulin levels in mutants were lower (Figure S2.5D), which correlates with an enhanced insulin sensitivity.

In contrast, hepatic loss of FIH in an albumin-cre background had no effect on overall body weight and metabolic rate (Figures S2.6A, S2.6B and S2.6C); loss of FIH in liver did not affect glucose homeostasis (Figures S2.6D, S2.6E and S2.6F).
Expression of HIF-1α target genes, including \textit{Pgk}, \textit{Vegf}, \textit{Glut-1}, \textit{Bnip3} and \textit{Car9} is not significantly induced in the brains of FIH nullizygous mice (Figure S2.5E), similar to results seen in other tissues examined in the mutants. This indicates that the neuronal FIH mutant phenotype is not directly related to a large and tissue-specific alteration in gene expression of HIF target genes in the central nervous system.

**FIH KO mice are protected from high fat diet-induced weight gain and hepatic steatosis**

To determine whether global deletion of FIH influences the weight gain, insulin resistance and hepatic steatosis seen when mice are administered a high fat diet (HFD), we fed FIH null mice and wild type littermates for four months with a 60% fat diet. As can be seen in Figure 2.7A and Figure S2.7A, this caused weight gain in both wild type and mutant mice; however, mutants gained significantly less weight relative to wild type animals. This divergence is not seen over time when animals are fed normal chow (Figure 2.7B). These results demonstrate that there is a selective capacity for coping with high fat diet in these animals.

We next measured individual tissue weights of liver, epididymal WAT, quadriceps, and kidney, and found that under a HFD, there were significant changes in tissue/body mass ratios of liver and quadriceps (Table S2.4B). FIH global deletion mice also have higher metabolic rates under HFD (Figure 2.7C); this difference is similar to that seen with a normal diet. There was still no
difference between WT and FIH nullizygous GTTs (Figure 2.7D and Figure S2.7C). ITTs of HFD animals demonstrate that the increase in insulin sensitivity evident in animals fed normal chow is maintained in mutants on a high fat diet (Figure 2.7E and Figure S2.7D). These were correlated with significantly lower levels of fasting plasma glucose (Figure 2.7F) and fed plasma insulin (Figure 2.7G) levels in mutants. In addition, although HFD increased serum cholesterol levels, FIH null mutants still showed significantly lower serum LDL/VLDL cholesterol levels (Table S2.3).

To examine gluconeogenic processes in the mutants, we measured expression levels of three genes involved in glucose homeostasis, glucokinase (GCK), phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase); these showed no significant changes in mRNA levels (Figure S2.7E). However, adiponectin, peroxisome proliferator-activated receptor γ (PPARγ), and c-Cbl-associated protein (CAP) mRNA levels in WAT were significantly induced in mutants (Figure 2.7H).

One of the other sequelae of a high fat diet is hepatic steatosis, or fatty liver. Overall liver/body mass ratio was significantly decreased in FIH nullizygous mice (Figure 2.7I, Figure S2.7B). As can be seen in Figure 2.7J, liver sections show loss of FIH causes a decrease in lipid droplets; quantification demonstrated that hepatic triglyceride content of FIH nullizygous mice was 60% lower (Figure 2.7K).
Reduced lipogenesis and increased \( \beta \)-oxidation are possible causes of reduced steatosis: we found no changes in mRNA levels of \( \beta \)-oxidation enzymes, but decreases in lipogenic enzymes (e.g., Scd1). Interestingly, we also detected significantly lower hepatic PPAR\( \gamma \) expression in FIH nullizygous livers from high fat diet fed mice (Figure 2.7L); this is the opposite of the trend in PPAR\( \gamma \) expression in WAT (Figure 2.7H), and indicate that metabolic changes in PPAR\( \gamma \) expression may help explain how FIH loss inhibits high fat diet-induced hepatic steatosis.

Finally, to examine whether a tissue-specific deletion of FIH in neurons also protects mutants from HFD-induced weight gain, we fed 4-week old FIH NKO mice and wild type littermates a HFD for 10 weeks. At 8-week and 10-week time points FIH NKO mice fed with HFD had gained significantly less weight (Figure 2.7M and 2.7N), consistent with a significant role for neuronal FIH expression in regulating HFD-induced weight gain.

**DISCUSSION**

Together, these data indicate that FIH, the asparaginyl hydroxylase, exerts a wide-ranging control over metabolism. This is surprising from many perspectives, particularly in reference to phenotypes of other mutations in the HIF pathway. Global and conditional mutations of genes regulating HIF are generally deleterious, causing embryonic lethality or significant changes in vascularization or erythropoiesis. The data presented here demonstrate that the two hydroxylase regulated HIF control pathways play very different roles *in vivo*. 
The FIH enzyme also has a higher affinity for oxygen than that of the PHD enzymes [21, 22]. This may indicate that FIH could suppress HIF at different oxygen concentrations than those where the PHDs are most active. We find little evidence for this in vitro, where alterations in gene expression caused by FIH are for the most part small at both normoxia and hypoxia. It is clear from our observations, however, that some genes, e.g., Car9, are highly dependent on FIH control when VHL is absent; it is intriguing to speculate that further investigation of genes that show a requirement for both elements of hydroxylase control may unveil a functional relationship amongst such targets.

We have found that cellular metabolism is altered by deletion of FIH. Curiously, this alteration in metabolism, with an increase in intracellular ATP levels in MEFs, and a repression of AMP kinase activation in developing embryos, is not accompanied by the acidosis and increased glycolysis thought to typify increased HIF activation in animals. Analysis of whole body energy expenditure demonstrated that there is an acceleration of metabolic rate in FIH mutants, but there is no evidence of metabolic increases in glycolytic rate. Thus the altered metabolism of these mutants is not simply a change in HIF activation leading to increases in glycolysis and suppression of oxidative metabolism. UCP1 and PGC-1α expression in BAT were significantly elevated in FIH KO mice, indicating increased energy expenditure in FIH KO mice may be due to increased thermogenesis in BAT. Deletion of PHD1 in mice results in reduced O2 consumption by attenuating glucose oxidation in skeletal muscle; this was
dependent on HIF function [8, 23]. As discussed above, different affinities of PHD1 and FIH for oxygen may represent a possible explanation for why they exhibit differential effects on energy expenditure, but further investigation is needed to understand this phenomenon.

Our data also connect the function of the FIH oxygen sensor with glucose and lipid metabolism in vivo. We have found that FIH null animals are smaller in size and weigh less when fed normal chow. They exhibit increased insulin sensitivity, which is correlated with significantly decreased plasma insulin levels. In contrast, there is no difference in glucose tolerance. FIH null mutants also show improved lipid homeostasis, with large reductions in serum triglyceride and cholesterol levels. Mice with tissue specific deletions of FIH in brain and liver demonstrate that deletion of FIH in neurons also leads to decreased body weight, increased energy expenditure and improved insulin sensitivity, and thus phenocopy global FIH null animals. Hepatic deletion animals did not have any of these metabolic phenotypes, arguing for a predominant role for FIH in the nervous system; clearly, further investigation of this is needed to isolate neuronal FIH pathways of metabolic control.

Deletion of FIH protects against high fat diet-induced weight gain and hepatic steatosis. The former may be explained by increased energy expenditure and improved insulin sensitivity in FIH global null animals. The latter is correlated with decreased expression of lipogenic genes in liver and increased adiponectin expression in white adipose tissue. Interestingly, FIH neuronal null mice are also
protected against HFD-induced weight gain. These findings implicate potential pharmaceutical application of FIH inhibitors, targeting insulin resistance and diet-induced obesity, although also indicate that these may need to be able to cross the blood/brain barrier.

The findings described here indicate a specific role for FIH in the process of respiratory control: maintenance of a set point for normoxic respiration. In the absence of FIH, tidal volumes at normoxia are equivalent to those seen in severely hypoxic wild type animals. This increased tidal volume is not constitutive: when breathing 30% oxygen, FIH mutant mice decrease tidal volumes to levels not significantly different from those of wild type mice. This indicates that loss of FIH has shifted oxygen-mediated control of respiration to a lower response point.

The regulation of chronic ventilatory adaptation has been shown to be a function of the HIF pathway in mice heterozygous for HIF-1α [24, 25]. Increased ventilation can be related to increases in metabolic rate; these would typically involve metabolically driven acidosis. In these mice, we see instead a respiratory alkalosis, which would indicate that some degree of hyperventilation is occurring. Hyperventilation could involve defective functioning of the carotid bodies, and the suppression of the hyperventilatory phenotype by hyperoxia in these mice could indicate an altered carotid body set point in the FIH null mice. The nestin gene, whose promoter drives cre recombinase expression in our neuronal FIH null mice, is expressed in carotid bodies [26]. Thus preservation of the respiratory
changes in neuronal FIH deletion could indicate that carotid body FIH is a key factor in regulating respiration. Further investigation is underway to determine this in isolated carotid bodies, with an ultimate goal of defining the mechanism of signaling controlled by FIH hydroxylation.

As discussed above, our results in global FIH null and neuron-specific FIH null animals reveal the role of this gene in regulation of metabolism. A key question is whether this is related to regulation of HIF function, or to altered activity of other putative FIH targets. Loss of VHL in hepatocytes results in a severe HIF-dependent steatosis, and deletion of HIF-1β in liver results in increased hepatic gluconeogenesis and lipogenic gene expression but reduced hepatic lipid storage [27, 28]. These data indicate that HIF activation increases steatosis in the liver. However, we see reduction in steatosis following FIH deletion, which may indicate that the metabolic changes we are observing are not directly related to increased HIF activity.

Besides HIF, FIH has been shown to hydroxylate a range of other substrates in vitro; these include proteins containing ankyrin repeat domains (ARD), such as the intracellular domain of Notch receptors [10, 11], p105, IκBα [12], SOCS (suppression of cytokine signaling) box protein 4 (ASB4) [29], MYPT1 [30], Tankyrase-2, Rabankyrin-5 and RNase L [13]. This range of substrates has complicated the determination of direct FIH action, as the intersecting functional roles between these hydroxylation events are potentially large. As a further complication, one postulated role for the association of FIH
with an ARD-domain-containing substrate has been that they act to sequester FIH from HIF, and thus potentiate HIF activation [31] [32]. Our studies provide useful genetic mouse models for further investigating the physiological outcome of FIH hydroxylation on these substrates.

In conclusion, our data clearly show that the oxygen sensor FIH regulates respiration, energy balance, and lipid metabolism. They also demonstrate a novel role for neuronal FIH in regulation of body mass, energy expenditure and insulin sensitivity. Thus, FIH is a ready target for broad-spectrum pharmacological inhibition of hydroxylase activation, and in particular FIH-specific inhibitors [33, 34]. Our data indicate that such inhibitors, especially those able to inhibit FIH function in neurons, might have significant therapeutic potential in reducing body weight and increasing insulin sensitivity.

EXPERIMENTAL PROCEDURES

Generation of FIH knockout (KO) mice

The targeting vector was constructed by inserting three fragments of the FIH gene from murine Sv129 embryonic stem (ES) cells into pFlox\(^{\Delta TK}\) (a kind gift from Stephen M. Hedrick, University of California at San Diego, La Jolla, CA) and electroporated into Sv129 ES cells. To select ES clones with homologous recombination, genomic DNA from neomycin-resistant ES clones was digested with SpeI and subjected to Southern blotting using both outer and internal probes. Totally two clones were picked from total 1800 clones and injected into
C57BL/6J blastocysts. Chimeras were bred with wild-type C57BL/6J mice. Germ-line transmission was confirmed by PCR. The offsprings containing the modified FIH allele were subsequently mated with Ella-Cre transgenic mice (in C57BL/6J background, a kind gift from Cornelis Murre, University of California at San Diego, La Jolla, CA) to generate FIH$^{Δ2/+}$ and FIH$^{flox/+}$ mice. FIH$^{Δ2/+}$ mice were bred to get FIH KO mice (FIH$^{Δ2/Δ2}$). FIH$^{flox/+}$ mice were bred to obtain FIH$^{df}$ mice. Genotyping of these mice was done by PCR using genomic DNA from tail biopsies. Primers p1 (5′-GGGAGATGCTACGTCAGGAGAGAGT-3′), p2 (5′-TCAATGAAGTAATTCCAAGGCGCTAA-3′) and p3 (5′-TAAGTTCCACATAGGCACGGTCAAA-3′) were used.

**Animal experiments**

All animal experiments were performed according to the animal protocol approved by the Institutional Animal Care and Use Committee. Mice were maintained on a standard rodent chow in a pathogen-free animal facility with 12hr light and dark cycles. Age-matched wild-type (WT) and FIH KO mice used in all the procedures were from heterozygote crosses. They were backcrossed to C57BL/6J for five generations. Albumin-Cre and Nestin-Cre mice were obtained from Jackson Labs.

**Immunoblotting**

Cells or tissue samples were lysed in RIPA buffer. Total 15µg whole cell extracts were loaded on a 3-8% Tris-Acetate (HIF-1α) or 10% Bis-Tris gels (FIH,
VHL and tubulin) from Invitrogen (NuPAGE®). Immunoblotting analysis was performed using the standard methods. The primary antibodies used in this study are: rabbit polyclonal anti-HIF1AN (FIH) (1:2,000, #ab36814, Abcam), rabbit polyclonal anti-HIF-1α antibody (1:1,000, #100-449, Novus Biologicals), VHL (1:250, #556347, BD Pharmingen), anti-AMPK α (1:1,000, #2532, Cell Signaling Technology), anti-phospho AMPK α (Thr172) (1:1,000, #2531, Cell Signaling Technology), anti-α tubulin (1:10,000, (clone: DM1A) 05-829, Millipore), anti-Hsp90 α/β (1:10,000, (H-114) SC-7947, Santa Cruz Biotechnology). The secondary antibodies used are: donkey anti-rabbit IgG-HRP (1:5,000, #NA934V, GE Healthcare) and donkey anti-mouse IgG-HRP (1:5,000, #sc-2314, Santa Cruz Biotechnology).

**Quantitative PCR analysis of gene expression and gene deletion**

Total RNA was isolated from tissues and cells by using TRIzol regents (Invitrogen) and RNeasy kit (Qiagen), respectively. 1µg RNA was used for reverse transcription using SuperScript III First-Strand Synthesis System (Invitrogen). cDNAs were amplified in a SYBR Green or TaqMan Universal Master Mix (Applied Biosystems). Quantitative PCR (qPCR) was performed on ABI Prism 7700 sequence detection system. PCR conditions are: 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. Relative amount of mRNA was calculated after normalization to β-actin. Sequences for the primers and probes of mouse genes used in this study were shown as follows.
mVEGF total forward, 5′-ATCCGCATGATCTGCATGG-3′; mVEGF reverse, 5′-AGTCCCATGAAGTGATCAAGTCTCA-3′; mVEGF probe, 5′-(6-FAM)-TGCCACGTCAGAGGCAACATCAC-(BHQ)-3′.
mPGK forward, 5′-CTGTGGTACTGAGCAAGCAAGAAGTCA-3′; mPGK reverse, 5′-CAGGACCATTCCAAACAAATCTG-3′; mPGK probe, 5′-(6-FAM)-TAGCTCGACCCACAGCTCGCATAT-(TAMRA)-3′.
mGlut-1 forward, 5′-GGGCATGTGCTTCCAGTATGT-3′; mGlut-1 reverse, 5′-ACGAGGAACCGTGAAGAT-3′; mGlut-1 probe, 5′-(6-FAM)-CAACTGTGCGGCCTACGTTCC-(BHQ)-3′.
mCar9 forward, 5′-CCTCTCCCGGGAAGCTCATCTC-3′; mCar9 reverse, 5′-TGTCTGAGCCTGGGTGATCTC-3′.
mBnip3 forward, 5′-GCTCCAGACACCAACAGAGAT-3′; mBnip3 reverse, 5′-TGAGAGTAGCTGTGCATTCTC-3′.
mUCP1 forward, 5′-GCCTTCAGATCCCAAGTGAA-3′; mUCP1 reverse, 5′-TAAGCCGGCTGAGATCTTGT-3′.
mUCP2 forward, 5′-GCGTTCTGGGTACCATCCTA-3′; mUCP2 reverse, 5′-GCTCTGAGCCCTTGGTGTAG-3′.
mUCP3 forward, 5′-ATGAGGTCTTGCCTCCATTCA-3′; mUCP3 reverse, 5′-GGCGTGATCAGCTGGTTGAAT-3′.
mPGC-1α forward, 5′-GGAGCCGTGACCAACTGACA-3′; mPGC-1α reverse, 5′-TGGTTTGCTGACATTTGCTC-3′.
mEPO forward, 5′-AATGGAGGTGGAAGCAGAGGCAT-3′; mEPO reverse, 5′-CGAAGCAGTGAAGTGAGCTACGTA-3′.
mAdp forward, 5'-GTTGCAAGCTCTCCTGTTCC-3'; mAdp reverse, 5'-TCTCCAGGAGTGCCATCTCT-3'.
mFAS forward, 5'-CCTTAGAGGCAGTGAGGAC-3'; mFAS reverse, 5'-TTGCTGCACTTCTTGAGACAC-3'.
mACC1 forward, 5'-GTGCAGTGAAACTTGCCA-3'; mACC1 reverse, 5'-TGTGCTGACAGGAAGATTGAC-3'.
mScd1 forward, 5'-TTCTTACAGACCACCACCA-3'; mScd1 reverse, 5'-GCAGGAGGGAACAGATGTA-3'.
mAcox1 forward, 5'-TTATGCGCAGACAGAGATGG-3'; mAcox1 reverse, 5'-GGCATGTAACCACGTAGACT-3'.
mAcad1 forward, 5'-AGGTTTTCAAGATCGCAATGG-3'; mAcox1 reverse, 5'-CTCCTTGGCTGCACACTTA-3'.
mCPT1a forward, 5'-TCCATGCATACCAAAGTGGA-3'; mCPT1a reverse, 5'-TGGTAGAGGGAACAGATGGA-3'.
mCAP forward, 5'-TGGACCCTACCAAAATCTGC-3'; mCAP reverse, 5'-TATAGCCCTTGGCAGCAAGT-3'.
mPPARγ forward, 5'-GTCATCTACGACATGAAT-3'; mPPARγ reverse, 5'-TTCGGATGGCCACCTCTTT-3'.
mGCK forward, 5'-CCGAAGGAGATCCTTAACA-3'; mGCK reverse, 5'-AGCTTGTACAGGAGCACCAC-3'.
mPEPCK forward, 5'-AGCCTTTGGACACTGAATGG-3'; mPEPCK reverse, 5'-TGGCCTTGCAGGGTTAGT-3'. 
mG6P forward, 5’-AGCAGTTCCCTGTCACCTGT-3’; mG6P reverse, 5’-CAAGGTAGATCCGGGACAGA-3’.
mβ-actin forward, 5’-AGGCCCAGAGCAAGAGAGG-3’; mβ-actin reverse, 5’-TACATGGCTGGGTTGTTGAA-3’.

Sequences for the primers of human genes used in this study were as follows.
hEPO forward, 5’-TCACTGTCCCAGACACCAAA-3’; hEPO reverse, 5’-CACTGACGGCTTTATCCACA-3’.
hFIH forward, 5’-AGAATGAGGAGCCTGTGGTG-3’; hFIH reverse, 5’-TCTTGTGTATCCTGCCCTGA-3’.
hβ-actin forward, 5’-TCGTGCGTGACATTAAGGAG-3’; 5’-AGGAAGGAAGGCTGGAAGAG-3’.

For checking deletion efficiency of FIH, genomic DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen). Relative copies of the FIH gene were determined by comparing FIH DNA levels to HIF-1α DNA levels. qPCR analysis was done on ABI Prism 7700 sequence detection system. Primers used in this study are listed below.
mFIH forward, 5’-GTGCCAGCACCACCTATA-3’; mFIH reverse, 5’-CGCGCTGCTGTATAGCTT-3’.

HIF-1α deletion forward, 5’-GGTGCTGGTGTTGAAAATGTAG-3’; HIF-1α deletion reverse, 5’-ATGGGTCTAGAGAGATAGCTCCACA-3’; HIF-1α deletion probe, 5’-[6-FAM]-CCTGTTGGTGCAGCAAGCATT-[BHQ1a-Q]-3’.
Cell culture, immortalization of fibroblasts, growth curve assay and RNAi knockdown

Cells were cultured in DMEM (#11965-092, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U ml\(^{-1}\) penicillin and 100µg ml\(^{-1}\) streptomycin. For glucose deprivation, DMEM without glucose (#11966-025, Invitrogen) was used.

Mouse embryonic fibroblasts (MEFs) were isolated from E12.5 embryos and immortalized by stable transfection with SV40 large T antigen. FIH\(^{df}\), VHL\(^{df}\), VHL\(^{df}\)/FIH\(^{df}\), VHL\(^{df}\)/FIH\(^{df}\)/HIF-1\(\alpha^{df}\), FIH\(^{df}\)/HIF-1\(\alpha^{df}\) MEFs were transiently infected with adenovirus expressing β-galactosidase or Cre recombinase to obtain WT and null MEFs.

For the growth curve assay, MEFs were plated in 12-well plates (Falcon) in triplicates at a density of 1X10\(^5\) cells per well. Following overnight seeding, cells were exposed to 21% O\(_2\) and 1% O\(_2\) for up to 72 hr. Cells were harvested every 24 hr by trypsinization. Cell counts were performed on Beckman Coulter Counter.

Human FIH siRNA duplexes (predesigned siRNA from Qiagen) were transfected to Hep3B cells by HiPerFect reagent (Qiagen). Four different siRNA duplexes were used for knockdown experiment. Knockdown efficiency was examined by quantitative PCR using the primers as described above.

Measurement of free ATP in MEFs
Free ATP in primary MEFs was measured using ATP Bioluminescence Assay Kit CLS II (#11699695001, Roche) in accordance with the protocol provided by the manufacturer.

**Transfection and luciferase assay**

WT and FIH null MEFs were transfected by Lipofectamine 2000 (Invitrogen) with pGL3 and pGL3-HRE plasmids. pRSV-β gal was also cotransfected into cells for normalization. One day after transfection, cells were incubated in normoxic or hypoxic conditions for 18 hr. Cells were then lysed for luciferase assay as described before [16].

Gal-HIF1α-C-TAD reporter assays: 30,000 wildtype or FIH null MEFs were plated per well (24-well plate), in triplicate, and transfected the following day using Fugene6 (Roche) as per manufacturer’s instructions with 100 ng p-G5E1b-LUC, 20 mg RL-TK, 100 ng pGAL DBD-hHIF1α-C-TAD 737-826 and 20ng pcDNA3.1 FIH or pEF-BOS vector control [35]. Cells were then treated overnight for 16 hours with hypoxia (< 0.4% O₂) and then analysed for relative luciferase activity with the Promega dual luciferase kit. Data are representative of three independent experiments, mean + SEM, n = 3.

**Mass Spectrometry (MS)**

HIF-C-TAD purification: FIHΔF MEFs were transfected with pEF-IRES-puro-HIS6-myc-mouse HIF-2α 774-874 wild-type, as used previously [36]. The polyclonal stable transfectants were then diluted and grown in MEF-conditioned
media to enable selection of monoclonal cell-lines overexpressing the HIF-C-TAD. A clone displaying relatively moderate expression (‘C2’) was selected to ensure that FIH-mediated hydroxylation would not be saturated. Cells from this line were grown to 75% confluency and infected with adenovirus-cre recombinase at 100 pfu/cell to engineer FIH deletion. Deletion was confirmed with qualitative PCR and the efficiency calculated via qPCR was found to be > 99%. Accordingly, western blotting with anti-FIH polyclonal antibody [35] demonstrated no detectable FIH protein, and HIS6-myc-mHIF-2α 774-874 protein was not changed by the viral treatment or the FIH deletion. To purify the HIF-C-TAD plus/minus FIH, 30 X 175 cm² flasks of each of the FIH null and untreated control cell lines were grown to 95% confluency, and purified by a single affinity purification step [36]. Purified proteins were separated by SDS/PAGE, and after verification by western blotting the comissie blue stained HIF-C-TAD protein excised, subjected to in-gel tryptic digestion and analyzed by MS.

**DNA microarray**

Total RNA for microarray analysis was extracted from WT, FIH null, VHL null and VHL/FIH null MEFs under normoxic condition. GeneChip® Mouse Genome 430 2.0 Array (Affymetrix Inc.) was used in GeneChip® Microarray Core (UCSD). Duplicates were performed for each sample. Gene expression data were analyzed by using GeneChip® Operating Software (GCOS).

**Metabolic cage studies**
Energy expenditure of FIH KO mice and their WT littermates were measured by using the Columbus Instruments Oxymax System. Mice were put inside individual enclosed chambers. O$_2$ and CO$_2$ gases in and out of the chamber were monitored using Paramax O$_2$ sensor and a CO$_2$ sensor and analyzed to obtain metabolic parameters (VO$_2$, VCO$_2$, RER and heat production).

Food, water intake and physical activity of mice were measured using the comprehensive lab animal monitoring system (CLAMS, Columbus Instruments) on three consecutive days in Animal Care Program phenotyping core (UCSD).

**Hypoxia exposure**

For acute hypoxia exposure, age-matched FIH KO mice and their wild-type littermates were exposed to 9% O$_2$ for 14 hr in hypoxia chamber (Biospherix). During the exposure, mice were housed in the cages with free access to food and water. Immediately after hypoxia exposure, tissue and plasma samples were collected, fast frozen in liquid N$_2$ and store at -80°C before analysis.

**PHZ-induced anemia**

Phenylhydrazine (PHZ)(Sigma-Aldrich, 60mg/kg body weight) dissolved in sterile phosphate-buffered saline was injected intraperitoneally into 2-month old mice to induce hemolysis. 18 hr after administration, tissue and plasma samples were collected for analysis.

**EPO ELISA**
Plasma EPO levels were determined by using the Mouse EPO ELISA kit (R&D Systems).

**Ventilation measurement**

Ventilation was measured in unrestrained 2-month old mice using barometric pressure plethysmography as described previously for rats [37], with modifications to the chamber volumes and flows appropriate for mice and a new analog-digital recording system and custom Matlab analysis routine.

**Measurement of mouse heart rate**

According to manufacture’s protocol, the measurement was done using MouseOx® Pulse Oximeter with CollarClip™ Sensor (Starr Life Sciences Corp).

**High fat feeding**

In high-fat feeding experiments, one-month old WT and FIH KO males were fed with high fat diet (60% fat content, Research Diets, Inc) or normal chow for 16-weeks.

**Tamoxifen-induced Cre expression**

Tamoxifen was used to induce Cre expression in VHL<sup>df</sup>/K14<sup>ER</sup>-Cre and VHL<sup>df</sup>/FIH<sup>df</sup>/K14<sup>ER</sup>-Cre mice. Tamoxifen (T5648, Sigma) was dissolved in sunflower oil. 200ul tamoxifen (10mg/mL) was injected into each 6-8 weeks old mouse by i.p. for 5 consecutive days.

**Metabolic measurements**
Blood glucose levels were measured using OneTouch Ultra 2 Blood Glucose Monitoring System.

Plasma insulin levels were measured using Ultrasensitive Insulin ELISA kit (Mercodia).

Hepatic and serum triglyceride levels were determined using Triglyceride Quantification Kit (#K622-100, BioVision).

Serum free fatty acid levels were measured using Fatty Acid Assay Kit (#K612-100, BioVision).

Serum cholesterol levels were measured using HDL and LDL/VLDL Cholesterol Quantification Kit (#K613-100, BioVision).

Total bilirubin levels in serum were measured by UCSD Animal Care Program Diagnostic Laboratory.

For GTTs, mice were fasted overnight (for 18 hr) with free access to water and intraperitoneally injected with D-glucose (2g/kg body weight). For ITTs, mice were fasted for 4 hr (from 11am to 3pm) and then injected with human insulin (Lilly Humulin-R Insulin U-100) intraperitoneally (0.75U/kg body weight for 2-month old mice on normal chow and 1.0U/kg body weight for mice fed with high fat diet for 12-weeks). Blood glucose levels were measured before injection and at different timepoints after injection as indicated in figures.

**Histology**
Paraffin-embedded tissue sections were stained with hematoxylin and eosin using a standard protocol. For Oil Red O staining, it was performed on frozen liver sections by UCSD histology core according to the standard procedure.

Quantification of adipocyte area was done on H&E stained sections using ImageTool software.

**Statistics**

Values in the figures are expressed as mean ± SEM. Student t’s test (unpaired, 2-tailed) and two-way ANOVA were used for statistical analysis.
Figure 2.1. Generation of FIH knockout mice

(A) Immunoblotting for FIH in adult mouse tissues. Various organs from a 2-month old wild type female were harvested and frozen at -80°C before...
lysis. Whole cell extracts were obtained and immunoblotted for FIH. Hsp90 was used for the loading control.

(B) Strategy of targeting the *FIH* gene. The genomic wild-type FIH locus around exon 1-4 (filled boxes) is shown (I). A neo cassette (open box) and a loxP sites (filled triangle) from the targeting vector (II) were introduced upstream and downstream of exon 2 by homologous recombination (III), respectively. Restriction sites (Spel) and positions of outer and internal probes for Southern blotting are indicated. After Cre-mediated excision, the FIH total knockout allele (*FIH*Δ2) (IV) and the FIH floxed allele (*FIH*flox) (V) were obtained. PCR primers used for genotyping (p1, p2, p3) are indicated (arrows).

(C) Immunoblot analysis of E12.5 whole embryo lysates of wild-type (WT), heterozygous (Het) and knockout (KO) using FIH and tubulin antibodies.

(D) MALDI-TOF-MS spectra of asparaginyl hydroxylation on HIF-1α in immortalized WT and FIH null MEFs under normoxia (21% O2).

(E) Luciferase assay using HRE (hypoxia response element) reporter in immortalized WT and FIH null MEFs under normoxia and hypoxia (1% O2) (n=3/each). Luciferase activity was measured 24hr following transfection.

(F) Luciferase assay using Gal-HIF1α-C-TAD reporter in immortalized WT and FIH null MEFs under normoxia and hypoxia. WT is the wild type
form of HIF1α-C-TAD. N803A is the mutant form in which asparagine 803 is mutated.

Values in graphs are expressed as mean ± standard error of the mean (SEM).

See also Figure S2.1.
Figure 2.2. Synergistic effects of FIH and VHL on regulating HIF activity in vitro
(A) Representative quantitative PCR (qPCR) analysis of mRNA levels for *Vegf*, *Pgk* and *Glut-1*. Immortalized WT and FIH null MEFs were treated under normoxia and hypoxia for 16 hours. Results were normalized to β-actin.

(B) Growth of WT and FIH null MEFs was examined under normoxia and hypoxia. Cells seeded at a low density were incubated in normoxic or hypoxic conditions and harvested for cell counts every 24hr. The average cell number of triplicates for each condition is shown.

(C) Immunoblotting for HIF-1α, FIH and VHL from whole cell lysates of immortalized WT, FIH null, VHL null and VHL/FIH double null MEFs. Cells were treated under normoxia or hypoxia for 16 hours before harvest. Tubulin was used as the loading control.

(D) and (E) Representative qPCR analysis of mRNA level for *Vegf* (D) and *Car9* (E) in WT, VHL null and VHL/FIH null MEFs (n=3/genotype).

(F) and (G) mRNA levels of both *Vegf* (F) and *Car9* (G) were restored by a triple deletion of VHL, FIH and HIF-1α. For (D-F), cells were cultured under normoxia. Primers specific for β-actin were used for normalization.

(H) Venn diagram showing the numbers of up and down-regulated genes in MEFs using a filter of >=2-fold change.

(I) VHL and FIH have synergistic effects on regulating cell growth. They were restored by deletion of HIF-1α. MEFs were seeded at a low density and incubated under normoxia or hypoxia for 48 hr. Cell numbers of each nullizygous MEFs were counted and normalized to relative wild-
type MEFs (n=3/condition).

Values in graphs are expressed as mean ± SEM. p values are from Student’s t-test.

See also Table S2.1.
Figure 2.3. Hypermetabolism in FIH KO mice

(A) Intracellular ATP levels in primary WT and FIH null MEFs under normoxia (n=3/group).
(B) Levels of phospho-AMPK and total AMPK were measured by immunoblotting with whole cell lysates from primary WT and FIH null MEFs treated with conditions as indicated.

(C) Immunoblotting analysis of whole cell lysates from E12.5 WT (n=3), FIH Het (n=5) and FIH KO (n=5) embryos using phospho-AMPK, total AMPK, FIH and tubulin antibodies.

(D) Resting whole-body $O_2$ consumption ($VO_2$) in 2-month old WT (n=7) and FIH KO (n=5) mice. Shown are the average value over 3 hr.

(E) Resting $CO_2$ production ($VCO_2$) in the same mice as in (D).

(F) RER (respiratory exchange ratio) were determined during the same period as in (D).

(G) Heat production was determined during the same measurement as in (D).

(H) Heart rates were measured in resting 2-month old WT (n=9) and FIH KO (n=5) mice under normoxic condition.

(I) *UCP1*, *UCP2* and *UCP3* mRNA levels in brown adipose tissue (BAT) from 5-month old mice under normal chow were measured by qPCR (n=6/genotype).

(J) *PGC-1α* mRNA levels in BAT from 5-month old mice under normal chow were measured by qPCR (n=6/genotype).

For gene expression analysis in (I) and (J), specific primers for $\beta$-actin were used for normalization.
Values shown in graphs are expressed as mean ± SEM. p values are from Student’s t-test.

See also Figures S2.2 and S2.3.
Figure 2.4. FIH KO mice are hyperventilatory

(A) Respiration frequencies of unrestrained WT (n=7) and FIH KO (n=8) mice under acute hypoxia (10% O\textsubscript{2}), room air (21% O\textsubscript{2}), and
hyperoxia (30% O₂) were measured using barometric pressure plethysmography.

(B) Tidal volume (VT) was measured in the same assay as in (A).

(C) Inspired minute ventilation (V̇) was determined in the same assay as in (A).

(D) Plasma EPO levels in WT and FIH KO mice following acute hypoxic exposure at 9% O₂ for 14 hours (n=15/genotype).

(E) Analysis of renal EPO mRNA levels in hypoxia-treated WT (n=7) and FIH KO (n=8) mice (9% O₂, 14 hr) by qPCR. Kidneys were collected immediately after hypoxia treatment, fast frozen in liquid N₂ and stored at -80°C before RNA isolation.

(F) Representative qPCR analysis of mRNA levels for EPO in Hep3B cells following control or hFIH siRNA treatment. Cells were cultured under normoxic or hypoxic conditions for 16 hr before harvest (n=3/condition).

(G) Plasma EPO levels in WT (n=6) and FIH KO (n=5) mice after overnight PHZ treatment.

(H) qPCR analysis of renal EPO expression in PHZ-treated mice (same as those in (G)).

Results in (E), (F) and (H) were normalized to β-actin.

Values shown in graphs are expressed as mean ± SEM. p values are from Student’s t-test.

See also Table S2.2.
Figure 2.5. Global loss of FIH causes decreased body mass, decreased adiposity and increased insulin sensitivity
(A) Food intake was measured in WT and KO mice (n=8/genotype) over 3 day/night cycles as monitored by CLAMS.

(B) Water intake was measured in the same assay as in (A).

(C) Physical activity was measured in the same assay as in (A) and (B). p=0.005 by two-way ANOVA.

(D) Body weights of newborn WT and KO mice. n=17-18 for females. n=11-14 for males, respectively.

(E) FIH KO mice are significantly smaller than their WT littermates. Growth curves show the average weights of WT (n=34) and KO (n=22) males. *p<0.05.

(F) Epididymal white fat pads weight normalized with body weight was measured from 2-month old and 5-month old WT and KO male mice. **p<0.01.

(G) Representative images of H&E stained epididymal WAT sections from 5-month old WT and KO mice fed with normal chow (NC). The scale bar represents 100µm.

(H) Epididymal adipocyte areas were measured from 5-month old WT and KO mice. n=3/genotype.

(I) Counts of adipocytes in different sizes from the same mice used in (H).

(J) Glucose tolerance test (GTT) was measured for 18hr-fasting 2-month old WT and KO mice fed with NC (2g D-glucose/kg body weight). n=10/genotype.
(K) Insulin tolerance test (ITT) was performed on 4hr-fasting 2-month old WT and KO mice fed with NC (0.75U/kg body weight). n=10-11/genotype. *p<0.05, **p<0.01.

(L) Fed insulin levels were measured on 5-month old WT and KO mice. n=9/genotype.

Values shown in graphs are expressed as mean ± SEM. p values are from Student’s t-test (except (C)).

See also Figure S2.4, Table S2.3, and Table S2.4A.
Figure 2.6. Neuron-specific loss of FIH indicates that hypermetabolism is neuronally regulated under normal chow

(A) Immunoblot analysis of whole cell lysates of brain and liver samples from FIH neuron-specific knockout mice (FIH NKO) and WT mice using FIH and tubulin antibodies. The upper bands in brain samples are non-specific.

(B) Growth curves of FIH NKO (n=17) and their WT (n=15) littermates under normal chow. ***p<0.001.
(C) Resting whole-body \( \text{VO}_2 \), \( \text{VCO}_2 \), RER and heat production in 2-month old WT (n=7) and FIH NKO (n=5) mice. Shown are the average value over 3 hr.

(D) GTTs were measured for 18hr-fasting 2-month old WT (n=6) and FIH NKO (n=6) mice (2g D-glucose/kg body weight).

(E) ITTs were performed on 4hr-fasting 2-month old WT (n=7) and FIH NKO (n=6) mice (0.75 U/kg body weight). *p<0.05, **p<0.01.

Values shown in graphs are expressed as mean ± SEM. p values are from Student’s \( t \)-test.

See also Figure S2.5 and Figure S2.6.
Figure 2.7. FIH KO mice are protected from high fat diet-induced weight gain and hepatic steatosis.
(A) Accumulated weight gain of WT and KO mice fed with high fat diet (HFD) for 12-weeks. n=23 for WT mice. n=12 for FIH KO mice. **p<0.01. ***p<0.001.

(B) Accumulated weight gain of WT and KO mice fed with NC for 12-weeks. n=16 for WT mice. n=12 for FIH KO mice.

(C) Resting whole-body VO\textsubscript{2}, VCO\textsubscript{2}, RER and heat production in WT (n=6) and KO (n=8) fed with HFD for 12-weeks. Shown are the average value over 3 hr.

(D) GTTs were measured for 18hr-fasting WT (n=9) and KO (n=7) mice fed with HFD for 12-weeks (2g D-glucose/kg body weight).

(E) ITTs were performed on 4hr-fasting WT (n=7) and KO (n=5) mice fed with HFD for 12-weeks (1.0U/kg body weight). *p<0.05, **p<0.01.

(F) Fasting blood glucose levels were measured on 18 hr-fasting WT (n=20) and KO (n=16) mice fed with HFD for 12-weeks.

(G) Fed insulin levels were measured on WT and KO mice fed with HFD for 16-weeks. n=6/genotype.

(H) Expression levels of genes encoding PPAR\textgamma, Adiponectin and CAP were increased in epididymal WAT of KO mice fed with HFD. n=6/genotype. **p<0.01. ***p<0.001.

(I) Liver weight normalized with body weight from WT and KO mice fed with HFD. n=11-19/genotype.
(J) (Top) Representative images of H&E stained liver sections from WT and KO mice fed with HFD. (Bottom) Representative images of Oil Red O-stained liver sections. The scale bar represents 100µm.

(K) Hepatic triglyceride contents were measured in WT and FIH KO livers under HFD. n=6/genotype. **p<0.01.

(L) Expression levels of genes encoding lipogenic enzymes (FAS, Acc1 and Scd1), those involved in β-oxidation (Acox1, Acad1 and CPT1a) and PPARγ in livers of WT and KO mice fed with HFD. n=6/genotype. *p<0.05. **p<0.01.

WAT and liver samples used in (H) to (L) were from mice fed with HFD for 16-weeks.

(M) Accumulated weight gain of FIH neuronal KO (NKO) mice (n=6) and their WT littermates (n=11) fed with HFD for 10-weeks. **p<0.01.

(N) Accumulated weight gain of FIH NKO (n=17) and their WT littermates (n=13) fed with NC for 10-weeks.

Values shown in graphs are expressed as mean ± SEM. p values are from Student’s t-test.

See also Figure S2.7, Table S2.3, and Table S2.4B.
Figure S2.1. Validation of gene targeting and PCR analysis of FIH knockout mice

(A) Southern blot analysis of WT and two targeted ES cell lines (#1 and #2). The fragments from WT and targeted alleles were 10.2kb and 5.75kb using the outer probe, respectively (left). When the internal probe was used, the fragments from WT and targeted alleles were 10.2kb and 6.41kb, respectively (right).

(B) Genotyping of WT, FIH Het and FIH KO embryos by PCR analysis. The amplified products from two pairs of primers are shown.
Figure S2.2. Analysis of FIH/HIF-1α null MEFs

(A) Intracellular ATP levels in primary WT and FIH/HIF-1α null MEFs under normoxia. n=4 per genotype. Values in graphs are expressed as mean ± SEM. p value is from Student’s t-test.

(B) AMPK activation in FIH/HIF-1α MEFs. Levels of phospho-AMPK and total AMPK were measured by immunoblotting with whole cell lysates from primary WT and FIH/HIF-1α null MEFs treated with conditions as indicated.
Figure S2.3. Gene expression in WAT and SM

(A) Relative *UCP2* and *UCP3* mRNA levels in epididymal WAT from 5-month old mice under normal chow were measured by qPCR (n=6/genotype).

(B) Relative *PGC-1α* mRNA levels in epididymal WAT from 5-month old mice under normal chow were measured by qPCR (n=4/genotype).

(C) Relative *UCP2* and *UCP3* mRNA levels in skeletal muscle (quadriceps) from 5-month old mice under normal chow were measured by qPCR (n=6/genotype).
For gene expression analysis in (A), (B) and (C), Primers specific for β-actin were used for normalization. Values in graphs are expressed as mean ± SEM. p values are from Student’s t-test.
Figure S2.4. FIH KO mice have smaller body size

(A) Representative images of 2-month old WT and FIH KO mice.
(B) Body length (from mouth to anus) from 2-month old WT and FIH KO mice. n=8-10/genotype. ***p<0.001.
(C) Representative images of ears from 2-month old WT and FIH KO mice. There was no significant difference on vasculature in the ear. Values shown in graph are expressed as mean ± SEM. p value is from Student’s t-test.
Figure S2.5. Analysis of FIH neuron-specific knockout mice (FIH NKO)

(A) Relative FIH mRNA levels in brain cortex, hypothalamus and brain stem were determined by qPCR using samples from WT mice (n=3).
(B) FIH deletion efficiency was determined by qPCR using genomic DNA samples of brain cortex, hypothalamus and liver from FIH NKO mice.

(C) Fasting blood glucose levels were measured on 18 hr-fasting 2-month old FIH NKO mice (n=6) and their WT littermates (n=11).

(D) Fasting insulin levels were measured on 6hr-fasting 2-month old FIH NKO mice (n=6) and their WT littermates (n=7).

(E) Expression levels of *Pgk, Vegf, Glut-1, Bnip3 and Car9* in WT and global FIH KO brains were determined by qPCR using specific primers. n=3~4 per genotype. *p<0.05.

Values shown in graphs are expressed as mean ± SEM. p values are from Student's *t*-test.
Figure S2.6. Analysis of FIH liver-specific knockout mice (FIH LKO)

(A) Immunoblot analysis of whole cell lysates of liver samples from FIH LKO and WT mice using FIH and tubulin antibodies.
(B) Body weights were measured from 4-weeks old WT (n=15) and FIH LKO (n=8) mice.
(C) Resting whole-body VO$_2$, VCO$_2$, RER and heat production in 2-month old WT (n=3) and FIH LKO (n=3) mice. Shown are the average value over 3 hr.

(D) Fasting blood glucose levels were measured on 18 hr-fasting 2-month old FIH LKO mice (n=6) and their WT littermates (n=4).

(E) GTTs were measured for 18hr-fasting 5-month old WT (n=3) and FIH LKO (n=3) mice (2g D-glucose/kg body weight).

(F) ITTs were performed on 4hr-fasting 2~4 month old WT (n=4) and FIH LKO (n=5) mice (0.75 U/kg body weight).

Values shown in graphs are expressed as mean ± SEM. p values are from Student’s t-test.
Figure S2.7. HFD induced weigh gain, fat accumulation in liver and glucose and insulin tolerance tests in WT and FIH KO mice

(A) Representative images of WT and FIH KO mice fed with NC or HFD for 16-weeks as indicated.

(B) Representative images of livers from WT and FIH KO mice under NC or HFD for 16-weeks as indicated.
(C) GTTs in WT and FIH KO mice fed with NC or HFD for 16-weeks. Dosage of D-Glucose was 2g/kg body weight in all groups. n=5-7 for each group.

(D) ITTs in WT and FIH KO mice fed with NC or HFD for 16-weeks. Dosage of insulin used was 0.85 U/kg body weight in all groups. The difference of insulin sensitivity between genotypes remained the same after HFD. n=6-7 for each group.

(E) Relative mRNA levels of GCK, G6Pase and PEPCK in liver from WT and FIH KO mice under HFD for 16-weeks were determined by qPCR. β-actin was used as the loading control. n=6/genotype. Black column, WT mice. White column, FIH KO mice. Values shown in graphs are expressed as mean ± SEM. p values are from Student’s t-test.
Figure S2.8. Synergistic effects of FIH and VHL in vivo

(A) Photograph of one-month old WT, FIH\textsuperscript{df}/Alb-Cre\textsuperscript{+}, VHL\textsuperscript{df}/Alb-Cre\textsuperscript{+} and VHL\textsuperscript{df}/FIH\textsuperscript{df}/Alb-Cre\textsuperscript{+} mice.

(B) Body weights of one-month old WT (n=15), FIH\textsuperscript{df}/Alb-Cre\textsuperscript{+} (n=8), VHL\textsuperscript{df}/Alb-Cre\textsuperscript{+} (n=16) and VHL\textsuperscript{df}/FIH\textsuperscript{df}/Alb-Cre\textsuperscript{+} (n=10) mice.
(C) Left: total bilirubin levels in plasma from one-month old mice are shown (n=3-4/genotype). Right: representative photographs of microcapillary tubes containing blood samples from one-month old mice.

(D) Glut-1 mRNA levels in livers of WT, FIH$^{df}$/Alb-Cre$^+$, VHL$^{df}$/Alb-Cre$^+$ and VHL$^{df}$/FIH$^{df}$/Alb-Cre$^+$ mice (n=6/genotype).

(E) Photographs of paws and back skins from representative WT, VHL$^{df}$/K14$^{ER}$-Cre$^+$ and VHL$^{df}$/FIH$^{df}$/K14$^{ER}$-Cre$^+$ mice 10 days after tamoxifen injection.

Values are shown as mean ± SEM, p values are from Student’s t-test.
Table S2.1. The list of genes significantly induced by deletion of both VHL and FIH in immortalized MEFs

These genes were selected from microarray data and confirmed by qPCR.

Table S2.2. Arterial blood pH, O₂ saturation and partial pressure of CO₂ and O₂ in 2-month old mice

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<td>1.04</td>
<td>4.55</td>
<td>18.83 / 4.14</td>
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<tr>
<td>Cdkn1a</td>
<td>cyclin-dependent kinase inhibitor 1A</td>
<td>NM_007869</td>
<td>0.82</td>
<td>1.33</td>
<td>4.01 / 3.46</td>
</tr>
<tr>
<td>Adm2</td>
<td>adrenomedullin 2</td>
<td>NM_182328</td>
<td>1.33</td>
<td>10.98</td>
<td>37.18 / 3.39</td>
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<tr>
<td>Fabp4</td>
<td>fatty acid binding protein 4, adipocyte</td>
<td>NM_024408</td>
<td>1.15</td>
<td>1.70</td>
<td>5.19 / 3.06</td>
</tr>
<tr>
<td>If202b</td>
<td>interferon activated-gene 202B</td>
<td>NM_008327</td>
<td>0.90</td>
<td>2.64</td>
<td>7.25 / 2.75</td>
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<tr>
<td>Ddit4</td>
<td>DNA-damage-inducible transcript 4</td>
<td>NM_029083</td>
<td>1.43</td>
<td>1.96</td>
<td>4.87 / 2.49</td>
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<td>Adam8</td>
<td>a disintegrin and metaproteinase domain 8</td>
<td>NM_007403</td>
<td>1.23</td>
<td>2.69</td>
<td>6.32 / 2.35</td>
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<td>Papss2</td>
<td>3-phosphoadenosine 5-phosphosulfate synthase 2</td>
<td>NM_011864</td>
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<td>1.70</td>
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<td>Galr2</td>
<td>galanin receptor 2</td>
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<td>0.88</td>
<td>4.74</td>
<td>9.56 / 2.02</td>
</tr>
<tr>
<td>Kdm3a</td>
<td>lysine (K)-specific demethylase 3A</td>
<td>NM_173001</td>
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<td>2.00</td>
<td>3.99 / 2.00</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th># of mice</th>
<th>pH*</th>
<th>PCO₂ ** (mmHg)</th>
<th>PO₂ (mmHg)</th>
<th>HCO₃⁻ (mmol/L)</th>
<th>SO₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5</td>
<td>7.372 ± 0.006</td>
<td>38.00 ± 0.548</td>
<td>78.00 ± 2.214</td>
<td>22.14 ± 0.534</td>
</tr>
<tr>
<td>FIH KO</td>
<td>7</td>
<td>7.416 ± 0.015</td>
<td>33.43 ± 1.043</td>
<td>82.43 ± 2.318</td>
<td>21.43 ± 0.442</td>
</tr>
</tbody>
</table>

Results are the mean ± SEM.

*p=0.0387, **p=0.0064 compared to the corresponding WT controls (Student’s t-test).
Table S2.3. FIH KO mice exhibit improved lipid homeostasis

<table>
<thead>
<tr>
<th></th>
<th>Normal chow</th>
<th></th>
<th>High fat diet</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>FIH KO</td>
<td>WT</td>
<td>FIH KO</td>
</tr>
<tr>
<td>Serum TG (mM)</td>
<td>1.43 ± 0.15</td>
<td>0.88 ± 0.14 *</td>
<td>0.90 ± 0.14</td>
<td>1.03 ± 0.14</td>
</tr>
<tr>
<td>Serum FFA (mM)</td>
<td>0.43 ± 0.06</td>
<td>0.35 ± 0.06</td>
<td>0.71 ± 0.07</td>
<td>0.56 ± 0.07</td>
</tr>
<tr>
<td>Serum HDL (mg/dL)</td>
<td>69.64 ± 3.98</td>
<td>55.06 ± 3.44 *</td>
<td>148.79 ± 13.96</td>
<td>136.80 ± 6.26</td>
</tr>
<tr>
<td>Serum LDL/VLDL (mg/dL)</td>
<td>13.39 ± 1.61</td>
<td>9.06 ± 0.04 *</td>
<td>34.59 ± 5.57</td>
<td>19.10 ± 3.46 *</td>
</tr>
<tr>
<td>Serum cholesterol (mg/dL)</td>
<td>83.03 ± 5.09</td>
<td>64.12 ± 4.23 *</td>
<td>183.38 ± 16.69</td>
<td>155.90 ± 3.91</td>
</tr>
</tbody>
</table>

Serum was collected from fed WT and FIH KO mice under normal chow or high fat diet for 16-weeks. Serum triglyceride (TG), free fatty acid (FFA), high-density-lipoprotein (HDL) cholesterol, low-density-lipoprotein and very low-density-lipoprotein (LDL/VLDL) cholesterol and total cholesterol levels were measured. Values are expressed ± SEM. n=6~10 per group. *p<0.05 for FIH KO mice compared to their WT littermates under the same diet. p values are from Student’s t-test.
Table S2.4. Tissue/body weight ratios from WT and FIH KO mice under NC or HFD for 16 weeks

(A) Tissue/body mass ratios from WT and FIH KO mice under NC for 16-weeks. n>5 per group.
(B) Tissue/body mass ratios from WT and FIH KO mice under HFD for 16-weeks. n>5 per group.

**p<0.01. ***p<0.001. ****p<0.0001.

Values shown are expressed as mean ± SEM. p values are from Student t's test.

<table>
<thead>
<tr>
<th>A</th>
<th><strong>BW (g)</strong></th>
<th>Liver</th>
<th>Epididymal WAT</th>
<th>Quadriceps</th>
<th>Kidney</th>
</tr>
</thead>
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<tr>
<td>WT-NC</td>
<td>32.19 ± 0.74</td>
<td>5.08 ± 0.14</td>
<td>2.73 ± 0.20</td>
<td>1.49 ± 0.03</td>
<td>1.29 ± 0.03</td>
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<tr>
<td>FIH KO-NC</td>
<td>26.13 ± 0.83</td>
<td>5.39 ± 0.22</td>
<td>1.71 ± 0.17</td>
<td>1.57 ± 0.04</td>
<td>1.26 ± 0.03</td>
</tr>
<tr>
<td>p values</td>
<td>&lt;0.0001****</td>
<td>0.2271</td>
<td>0.0008***</td>
<td>0.1545</td>
<td>0.4572</td>
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<table>
<thead>
<tr>
<th>B</th>
<th><strong>BW (g)</strong></th>
<th>Liver</th>
<th>Epididymal WAT</th>
<th>Quadriceps</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-HFD</td>
<td>46.34 ± 0.81</td>
<td>4.38 ± 0.22</td>
<td>4.87 ± 0.28</td>
<td>0.95 ± 0.02</td>
<td>0.90 ± 0.03</td>
</tr>
<tr>
<td>FIH KO-HFD</td>
<td>34.30 ± 0.99</td>
<td>3.29 ± 0.12</td>
<td>4.89 ± 0.53</td>
<td>1.15 ± 0.05</td>
<td>0.99 ± 0.12</td>
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<tr>
<td>p values</td>
<td>&lt;0.0001****</td>
<td>0.0017**</td>
<td>0.9593</td>
<td>0.0003***</td>
<td>0.2790</td>
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REFERENCES


Chapter 2 contains the material published in Cell Metabolism 2010.

HIF-1alpha is an essential regulator of metabolism. Cell Metabolism. 2010, 11(5):364-78. The dissertation author was the primary investigator and author of this paper.
Chapter 3

Endothelial HIF-1α is a key regulator of lung metastasis
ABSTRACT

Metastasis is the primary cause for mortality in human cancer. It is a complex multi-step process that can be affected by tumor microenvironment. Stabilization and activation of hypoxia inducible factor-1α (HIF-1α) in tumor cells by intratumoral hypoxia have been shown to promote tumor progression and metastasis. Here, we have found that conditional deletion of HIF-1α in the endothelium does not affect primary tumor growth, but leads to decreased number of lung metastasis in the Polyoma Middle T (PyMT) breast cancer mouse model. We also found that endothelial HIF-1α is involved in both tumor cell intravasation and extravasation tested by orthotopical implantation or intravenous injection of GFP-labeled Lewis Lung Carcinoma (LLC) cells, respectively. In vitro migration assays showed that deletion of HIF-1α in primary endothelial cells causes significantly less transendothelial migration of LLC cells under hypoxia. These results indicate that endothelial HIF-1α is a relevant contributor for endothelium permeability to tumor cells and facilitates the establishment of distant metastases.

INTRODUCTION

Distant metastasis is the primary cause of death for patients with breast cancer, the most common malignant disease amongst women worldwide, and responsible for one fifth of global cancer related deaths (Hood and Cheresh, 2002; Robinson et al., 2009; White and Muller, 2007). Intratumoral hypoxia is present in most human solid tumors, including breast cancer (Semenza, 2003).
Studies have suggested that intratumoral hypoxia is the principal stimulus for a cascade of events directly correlated with metastasis and a poor prognosis (Bertout et al., 2009; Liao et al., 2007; Semenza, 2003; Semenza, 2004). These events are orchestrated by the stabilization and activation of hypoxia inducible factor-1α (HIF-1α) (Maxwell et al., 2001), a basic helix-loop-helix transcription factor, which dimerizes with HIF-1β, and initiates expression of genes required for hypoxic glucose metabolism such as lactate dehydrogenase (LDH), phosphoglycerate kinase ( Pgk ) and glucose transporter-1 ( Glut-1 ), or angiogenesis, such as vascular endothelial growth factor ( Vegf ) (Schildl et al., 2002; Ryan et al., 2000; Harris et al., 2002; Pouyssegur et al., 2006).

Previous results have established the role of HIF-1α in tumor growth. Immortalized and transformed HIF-1α null mouse embryonic fibroblasts grew smaller tumors in xenograft models than their wild-type counterparts (Ryan et al., 2000). In addition, loss of HIF-1α in mammary epithelium led to delayed mammary tumor progression, tumor vascularization and significantly decreased lung metastasis, as a result of reduced motility of HIF-1α null tumor cells (Liao et al., 2007). HIF-1α has also been shown to promote tumor metastasis by induction of gene targets involved in epithelial-mesenchymal transition, tumor cell invasion and angiogenesis (Gordan and Simon, 2007). Several important mediators of cell signaling known to contribute to cell motility and metastasis are upregulated by HIF-1α, and these include chemokine receptors ( CXCR4 and CXCR6 ), matrix metalloproteinases (MMPs), lysyl oxidase (LOX) and TWIST
(Staller et al., 2003; Leufgen et al., 2005; Erler et al., 2006; Yang et al., 2008). These results provide strong evidence supporting that HIF-1α in tumor cells plays a key role in metastasis.

The process of metastasis is affected by both tumor cell motility and the interactions between tumor cells and the microenvironment, such as the tumor vasculature (Blouw et al., 2003). The endothelium is an extremely versatile organ at the crossroads between most of the signals required for cellular exchanges and transport that are underlying crucial steps of metastasis (Konstantopoulos and Thomas, 2009). Most of the information regarding the spread of invasive breast carcinoma cells involves signals perceived or released by the endothelium, and refer to changes in cell adhesion and systemic transport of tumor cells (Konstantopoulos and Thomas, 2009). Many of these signals are hypoxia dependent or HIF-1α regulated, but to date no clear mechanism has been characterized. It is therefore relevant to investigate the part taken by the endothelial cells in this foreseeable HIF-1α-dependent cross-talk.

*In vitro* experiments have shown that hypoxia-induced VEGF levels result in increased endothelial monolayer permeability (Lee et al., 2003; Hu et al., 2006), and that the expression levels of endothelial cell-specific receptors for VEGF (VEGFR-1 and VEGFR2) are also upregulated during hypoxia (Tang et al., 2004), establishing HIF-1α as a likely key regulator of endothelial permeability in the intratumoral microenvironment. When HIF-1α is deleted specifically in the endothelium, subcutaneous Lewis Lung Carcinoma (LLC)
tumors grew smaller with less vascularization and increased necrosis, as a direct consequence of down-regulation of VEGF levels (Tang et al., 2004). We have now expanded this study to specifically look into the role of endothelial HIF-1α in tumor progression and metastasis using the Polyoma Middle T (PyMT) breast cancer model, and found that HIF-1α expression in the endothelium is an important contributor for the establishment of metastasis, with an evident effect on the transendothelial migration of tumor cells. Our data shows that expression of HIF-1α in the endothelium is required to assist tumor cell movement through the vasculature during dispersion of tumor cells to secondary proliferation sites.

**MATERIAL AND METHODS**

**Animal experiments**

HIF-1α<sup>df</sup> mice were generated as previously described (Ryan et al., 1998). To obtain endothelial cell-specific deletion of HIF-1α in polyoma middle T (PyMT) tumor model, we crossed HIF-1α<sup>df</sup> females (exon2 of HIF-1α is flanked by two loxP sites) with HIF-1α<sup>df</sup>/Tie2Cre<sup>+</sup>/PyMT<sup>+</sup> males. Only virgin females were used in this study. Wild-type is HIF-1α<sup>df</sup>/PyMT<sup>+</sup>. Conditional knockout is HIF-1α<sup>df</sup>/Tie2Cre<sup>+</sup>/PyMT<sup>+</sup>. Starting at 4-weeks of age, mice were palpated once per week. Tumor onset age was when a 2 x 2 mm palpable solid mass appeared. Tumor size was measured in two dimensions using digital calipers. Mice were sacrificed when any tumor had reached 1 x 1 cm (end-point). HIF-1α<sup>df</sup> and HIF-1α<sup>df</sup>/Tie2Cre mice used were backcrossed to C57BL/6J for at least 8
generations. All animal studies were carried out according to the animal protocol approved by the Institutional Animal Care and Use Committee. Mice were maintained on a standard chow in a pathogen-free animal facility with 12hr light and dark cycles.

**Histology and immunohistochemistry**

Lung and tumor tissues were fixed in 10% phosphate-buffered formalin (Fisher Scientific) for 6hr before embedded in paraffin. Lung metastasis was determined by H&E staining as previously described (Liao et al., 2007). For immunohistochemistry, 5 μm tumor sections were rehydrated and stained according to routine procedures. Biotinylated anti-PCNA antibody (1:400 dilution, #555567, BD Pharmingen) was used to detect proliferating cells. Blood vessels in tumors were detected using rat anti-mouse CD34 antibody (1:100 dilution, #NB600-1071, Novus). Secondary antibody for CD34 staining is biotinylated goat anti-rat IgG (1:200 dilution, #sc-2041, Santa Cruz Biotechnology). Staining was revealed using Vectastain ABC kit (Vector Laboratories). Microvessel density was quantified using a Chaukley graticule eyepiece.

**Generation of LLC\(^{\text{GFP}}\) cells**

Lewis lung carcinoma cells (ATCC) were infected with a lentivirus expressing GFP (a kind gift from Dr. Cornelis Murre, UCSD) according to the standard protocol for retrovirus infection. Single clones from FACS-sorted GFP-positive cells were picked out. The LLC\(^{\text{GFP}}\) clone used in this study showed the
highest GFP expression confirmed by FACS. LLC\textsuperscript{GFP} cells were cultured in DMEM (#11965-092, Invitrogen) supplemented with 10% fetal bovine serum (#26140-079, Invitrogen), 100 U ml\(^{-1}\) penicillin and 100 µg ml\(^{-1}\) streptomycin.

**Bone marrow transplantation**

To generate endothelial cell-specific HIF-1\(\alpha\) conditional knockout mice, bone marrow transplantation was performed on 2-month old virgin HIF-1\(\alpha\)^df and HIF-1\(\alpha\)^df/Tie2Cre\(^+\) female mice.

Recipient mice were irradiated at a single dose of 1,000 Rad (10 Gy) (whole-body irradiation) and let recover overnight before bone marrow transplantation. Wild-type bone marrow was isolated from age-matched HIF-1\(\alpha\)^df mice. Total 10 \(\times\) 10\(^6\) fresh bone marrow per mouse was injected into irradiated HIF-1\(\alpha\)^df or HIF-1\(\alpha\)^df/Tie2Cre\(^+\) mice via tail vein to generate wild-type (WT) or endothelial cell null (EC null) mice. Bone marrow reconstituted mice were provided with water containing antibiotics and let recover for two months before experiments.

**Tumor cell intravasation experiment**

First, fat pads of #4 right mammary gland in 3-4 weeks old HIF-1\(\alpha\)^df and HIF-1\(\alpha\)^df/Tie2Cre\(^+\) female mice were cleared by surgery (Medina, 2000). Successful removal of ductal tree was confirmed by carmine whole-mount staining. Two months after surgery, bone marrow reconstitution was performed on these mice to obtain WT and EC null mice with cleared fads. After 2-month
recovery, 2.5 X 10^6 viable single cells of LLC\textsuperscript{GFP} in 50\textmu l sterile PBS were injected orthotopically into cleared fat pad four. At the end-point (3-weeks later), mice were sacrificed, and tumors were weighed, lungs removed and fixed for histological analysis of metastasis, and blood cells were collected upon lysis of red blood cells with ACK buffer. Relative copies of the \textit{GFP} gene in blood were determined by quantitative PCR to assess the presence of circulating tumor cells.

\textbf{Tumor cell extravasation experiment}

A total of single-celled 1 X 10^6 LLC\textsuperscript{GFP} cells in 200 \textmu l sterile PBS were injected intravenously into bone marrow-reconstituted WT and EC null mice. Lungs were collected from these mice 15 days post injection and fixed for histological analysis. The numbers of lung metastatic tumors were quantified by H&E staining on 10 \textmu m serial lung sections of the whole lungs and compared between wild type and EC null mice.

\textbf{Quantitative real-time PCR analysis}

Genomic DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen). Relative copies of the \textit{GFP} gene in blood from tumor cell intravasation experiment were determined by comparing \textit{GFP} DNA levels to \textit{HIF-1\alpha} DNA levels. Quantitative real-time PCR (qPCR) analysis was done on ABI Prism 7700 Sequence Detector (Applied Biosystems). Conditions for the PCR: one 10-min incubation at 95°C, followed by 40 cycles of 15s at 95°C and 1 min at 60°C. Primers used in this study are listed below.
GFP forward, 5'-GGAGCGCACCCTTTCTTCA-3'; GFP reverse, 5'-AGGTTGTGTCGCCCTCGAA-3'.

HIF-1α deletion forward, 5'-GGTGCTGGTGTCCAAAATGTAG-3'; HIF-1α deletion reverse, 5'-ATGGGTCTAGAGAGATAGCTCCACA-3'; HIF-1α deletion probe, 5'-[6-FAM]-CCTGTTGGTTGCGCAGCAAGCATT-[BHQ1a-Q]-3'.

For quantitative analysis of steady-state mRNA levels, total RNA was extracted from primary endothelial cells exposed to hypoxia at different time points, using Qiagen RNeasy mini kit. cDNA was synthesized from 1 µg of total RNA, using Superscript III (invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A dilution of 1:50 of obtained cDNA was used as template for qPCR analysis using specific primers for several HIF-1α target genes. Relative abundance of transcripts of interest was assessed by normalization to β-actin transcript levels. Primer pair and primer/probe sets used were as follows:

mβ-actin forward, 5'-AGGCCAGAGCAAGAGAGG-3'; mβ-actin reverse, 5'-TACATGGCTGGGTGTGGA-3'.

mVEGF total forward, 5'-ATCCGCATGATCTGCATGG-3'; mVEGF total reverse, 5'-AGTCCCATGAAGTGATCAAGTTCA-3'; mVEGF probe, 5'-[6-FAM]-TGCCCACGTCAGAGGCAACATCAC-[BHQ1a-Q]-3'.
mPGK forward, 5’-CAAATTGATGAGAATGCCAAGACT-3’; mPGK reverse, 5’-TTCTTGGCTGCTCTCAGTACCACA-3’; mPGK probe, 5’-[6-FAM]-TATACCTGCTGGCTGGATGGGCTTGGACT-[BHQ1a-Q]-3’.
mBNIP3 forward, 5’-GCAGGGCTCCTGGGTAGAA-3’; mBNIP3 reverse, 5’-GACGGAGGCTGGAACGCT-3’; mBNIP3 probe, 5’-[6-FAM]-TGCACTTCAGCAATGGCAATGGGA-[BHQ1a-Q]-3’.
miNOS forward, 5’-ACCCTAAGAGTCACCAAAATGGC-3’; miNOS reverse, 5’-TTGATCCTCACATACTGTGGACG-3’.

**Isolation of primary endothelial cells from mouse lungs**

Primary endothelial cells were isolated and cultured from lungs of HIF-1α-def mice with minor modifications of previously described methods (Tang et al., 2004; Dong et al., 1997). Briefly, the lungs were digested in 0.2 % collagenase A in HBSS enriched buffer (HBSS containing 2 mM CaCl₂, 2 mM MgSO₄ and buffered with 20 mM Hepes). The digest was filtered through a 70 µm nylon cell strainer and washed twice in HBSS, resuspended in PBS containing 0.1% BSA and incubated with anti-CD31 coated magnetic beads (Dynal, Invitrogen Inc., Carlsbad, CA) for 45 min. Cells and beads were plated in endothelial cell growth medium (ECGM) consisting of low glucose DMEM: F12 containing 1% penicillin/streptomycin, 1% non-essential aminoacids, 2 mM Sodium Pyruvate, buffered with 20 mM Hepes, 20% FBS (Omega Scientific, Tarzana, CA), 20 µg/ml heparin (Sigma, St. Louis MO) and 75 µg/ml endothelial mitogens
Culture purity was confirmed by VE-cadherin immunostaining (Santa Cruz Biotechnology, sc-6458) on fixed cells.

**Transendothelial cell migration assay**

After 12 to 14 days, primary endothelial cells from original isolates (P0) were infected with adenovirus expressing Cre recombinase (for HIF-1α deletion) or adenovirus expressing β-gal, for control WT cells. 5x10^5 endothelial cells were seeded into COSTAR transwells (6.5mm diameter, pore size 8 μm; Corning, NY) and fed daily until an even monolayer was formed. Transendothelial cell migration of tumor cells was examined by seeding 5x10^5 LLC cells onto the endothelial cell layer and the inserts incubated for 9 hr under 21% or 1% O_2. Inserts were stained with 0.1% crystal violet in 10% ethanol and mounted onto glass slides. Five fields per insert were photographed at 100X magnification and migrated cells were counted. At least three inserts per treatment were used.

**Statistical analysis**

Statistics analysis was done using unpaired Student’s t test. Values in the figures are expressed as mean ± SEM unless otherwise stated.

**RESULTS**

Tumor initiation and development are not affected by Tie2Cre-mediated deletion of HIF-1α in PyMT breast cancer model.
To study the effects of endothelial cell HIF-1α on tumor initiation and development, we generated PyMT mice carrying a double floxed allele of HIF-1α (HIF-1α<sup>df</sup>) and expressing Tie2-driven Cre expression (HIF-1α<sup>df</sup>/Tie2Cre/PyMT), which allows deletion in the endothelium. Only virgin female mice were used in this study. They were palpated once per week beginning at 4 weeks of age. Both HIF-1α<sup>df</sup>/ PyMT<sup>+</sup> mice and HIF-1α<sup>df</sup>/Tie2Cre<sup>+</sup>/PyMT<sup>+</sup> mice developed mammary gland tumors between 50 and 80 days of age. We did not see any significant difference of tumor onset age in these mice (Fig 3.1A), which suggest that HIF-1α in endothelium does not affect tumor initiation in PyMT model. Mice were sacrificed at the end point when any tumor had grown to a size of 1 x 1cm. There was no significant difference in the survival (Fig 3.1B). Although previous study found smaller xenografted tumors in mice with deletion of HIF-1α in endothelium (Tang et al., 2004), tumor burden at the end point was similar between two groups (Fig 3.1C). We also compared tumor burden at two time-points (16 and 18 weeks). Although a tendency of slower tumor growth in HIF-1α<sup>df</sup>/Tie2Cre<sup>+</sup>/PyMT<sup>+</sup>, particularly at 16 weeks of age, the differences were not found to be significant (Fig 3.1D). These results suggest that HIF-1α in endothelium does not have significant effects on tumor initiation and growth in this spontaneous breast cancer model.

**Significantly reduced pulmonary metastasis in HIF-1α<sup>df</sup>/Tie2Cre<sup>+</sup>/PyMT<sup>+</sup> with deletion of HIF-1α in endothelium.**
In the PyMT breast cancer model, the transition to malignancy of the primary tumor is accompanied by a significant frequency of distant pulmonary metastasis (Lin et al., 2003), which elects this as an evident model for the study of metastasis establishment. To examine the effect of HIF-1α deletion in endothelium on pulmonary metastasis, we counted the number of metastatic foci in end point mice. Although their average end point tumor burden was comparable to HIF-1α<sup>df</sup>/PyMT<sup>+</sup>, HIF-1α<sup>df</sup>/Tie2Cre<sup>+</sup>/PyMT<sup>+</sup> showed a significant decrease in pulmonary metastasis (Fig 3.2A). To detect and quantify every metastatic tumor, we sectioned the formalin-fixed and paraffin-embedded lungs at 100 µm intervals. These serial sections were stained with hematoxylin and eosin to visualize metastatic foci, which were counted under a stereomicroscope. Lungs from HIF-1α<sup>df</sup>/Tie2Cre<sup>+</sup>/PyMT<sup>+</sup> contained significantly fewer metastatic foci compared with those from HIF-1α<sup>df</sup>/PyMT<sup>+</sup> at the end point (p=0.0272) (Fig 3.2B). The mean foci count from HIF-1α<sup>df</sup>/PyMT<sup>+</sup> is 106.4 ± 30.66 (n=18) compared with the mean metastatic foci count of 31.71 ± 7.036 (n=17) from HIF-1α<sup>df</sup>/Tie2Cre<sup>+</sup>/PyMT<sup>+</sup>. Notably, 6 of 17 null mice had less than 10 pulmonary tumors in total while this only occurred in one HIF-1α<sup>df</sup>/PyMT<sup>+</sup> at the end point. In this model, mice start developing pulmonary metastasis when they are around 4-month of age. To determine if there is any difference in the early timepoint, we also examined pulmonary metastasis in 16-weeks old mice. HIF-1α<sup>df</sup>/Tie2Cre<sup>+</sup>/PyMT<sup>+</sup> developed much less foci in this early stage (Fig 3.2C). These results indicate that although HIF-1α in endothelium does not influence
tumor initiation and progression, it does promote pulmonary metastasis *in vivo*. Deletion of HIF-1α in mammary epithelial cells led to less pulmonary metastasis in this model (Liao D et al., 2007). Compared with that data, both the mean difference and the difference in variance in HIF-1α^df/Tie2Cre^+/PyMT^+ are significantly greater. These also raised the question about how deletion of HIF-1α in endothelium has such profound effects on formation of pulmonary metastasis.

**Microvessel density in primary tumors is not affected by Tie2Cre-mediated deletion of HIF-1α.**

As previously described, HIF-1α in endothelium affects microvessel density in xenografted tumors (Tang et al., 2004). It is possible that difference in pulmonary metastasis was due to a direct effect of endothelial cell HIF-1α on the microvessel density of primary tumors. Blood vessels in endpoint primary tumor sections were stained using a CD34 antibody (Fig 3.3A and 3.3B). After quantification of microvessel density, we found no difference between HIF-1α^df/PyMT^+ and HIF-1α^df/Tie2Cre^+/PyMT^+ tumors at endpoint (Fig 3.3C). This indicates that tumor angiogenesis was not affected in HIF-1α^df/Tie2Cre^+/PyMT^+. It is not surprising considering that these primary mammary gland tumors developed in a blood vessel rich microenvironment instead of the subcutaneous areas ablated of local blood vessels. This result may explain why tumor burden was unchanged in HIF-1α^df/Tie2Cre^+/PyMT^+ and both genotypes had similar percentage of proliferating tumor cells at endpoint (data not shown).
Deletion of HIF-1α in endothelium affects both tumor cell intra- and extravasation.

Formation of metastasis is a complicated and multi-step process, that can be in simple terms summarized by (1) the detachment of cancer cells from the primary tumor, (2) the transendothelial migration of these cells into circulation (intravasation) and (3) out of the vasculature at distant organs (extravasation), and finally (4) the seeding and proliferation (growth) at the metastatic site. We used two experimental models to investigate if endothelial cell HIF-1α is involved: tumor cell intravasation and extravasation. Cre recombinase expression is present in both endothelial cells and hematopoietic cells in the Tie2-Cre transgenic mouse line. HIF-1α<sup>df</sup> (WT) and HIF-1α<sup>df</sup>/Tie2Cre<sup>+</sup> mice were lethally irradiated and received WT bone marrow to restore HIF-1α in bone marrow-derived myeloid lineage cells, thus generating WT and EC null mice with exclusive deletion of HIF-1α in endothelium (Fig 3.4A). We used these mice for the following studies.

To assess how endothelial cell HIF-1α mediates the escape of tumor cells from the primary tumor site, we injected GFP-labeled Lewis Lung Carcinoma cells (LLC<sup>GFP</sup>) orthotopically into the fourth cleared fat pads in the right sides of both WT and EC null female mice. LLC<sup>GFP</sup> cells used were from a single clone (Fig 3.4B). At the endpoint, primary tumor size was compared. Pulmonary metastasis was assessed by examination of fixed and H&E stained lung sections. As observed in the PyMT model described above, we found no
difference in tumor growth between the WT and the EC null mice (Fig 3.4C). However, the number of metastastic foci was significantly reduced in EC null mice (Fig 3.4D). The number of circulating tumor cells, one further quantifiable parameter of tumor cell detachment and intravasation, was assessed in these mice, by measuring relative copies of the GFP gene in blood. WT tumor-bearing show meaningfully higher levels of tumor cells than those detected in the EC null counterparts (Fig 3.4E).

To examine the importance of endothelial cell HIF-1α in mediating pulmonary colonization, both WT and EC null mice were inoculated with LLC<sup>GFP</sup> cells via tail vein (Fig 3.5A). Two weeks after tail vein injection, the lungs were inflated, fixed and sectioned for both fluorescence-based detection of GFP-labeled tumors (Fig 3.5B) and H&E staining (Fig 3.5C). We found that the EC null mice show a remarkable reduction in the occurrence of metastatic foci when compared to the WT (Fig 3.5D). These results indicate that HIF-1α in endothelium plays an important role in both tumor cell intra- and extravasation.

**Transendothelial migration of tumor cells is impaired in the absence of HIF-1α.**

Our data provides evidence supporting that endothelial HIF-1α has a role in facilitating tumor cell movement through the endothelial layer, both from the primary tumor site and into new target secondary sites. We used an in vitro system to verify and quantify this phenomenon (Fig 3.6C). Primary lung endothelial cells (ECs) were isolated from HIF-1α<sup>diff</sup> mice, and the cell culture
purity, estimated as > 98 % was confirmed by VE-cadherin staining on fixed cells (Fig 3.6A). Original isolates (passage zero) primary EC cells were infected with Cre-expressing adenovirus to generate a HIF-1α null endothelial cells, and cells to use as controls were infected with β-Gal-expressing adenovirus (WT). The average deletion of HIF-1α was 75%, and was found to result in a significant decrease in expression of several HIF-1α target genes (Fig 3.6B). WT and HIF-1α null endothelial cells (P1) were used to generate endothelial cell monolayers in transwell inserts. LLC<sup>GFP</sup> cells were seeded onto the confluent EC monolayer, and the system incubated for 9 hr of normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>), while allowed random migration. Inserts were stained with 0.1% crystal violet and mounted onto glass slides to count LLC<sup>GFP</sup> cells on the opposite side of the filter (i.e. migrated cells through the endothelial cell monolayer). We found that a significantly higher number of cells migrated through WT than through HIF-1α null ECs (Fig 3.6D). This data is in agreement with our previous knowledge that hypoxia stress promotes cancer cell motility, and verifies our most recent in vivo finding that the deletion of HIF-1α in ECs severely impairs tumor cell transendothelial movement.

**DISCUSSION**

As the main regulator of cellular responses to hypoxia, HIF-1α is an evident and relevant target for cancer therapy, as intratumoral hypoxia is a trademark of aggressive malignancies with potential for tumor spread and growth in secondary sites (Ryan et al., 2000; Semenza GL, 2003; Bertout et al., 2008;
Sullivan and Graham, 2007). HIF-1α directly induces endothelial cell proliferation, via hypoxia-dependent up-regulation of VEGF expression and subsequent angiogenesis, required for solid tumor growth (Ryan et al, 2000; Giordano and Johnson, 2001). The role of HIF-1α expression in tumors has been thoroughly evaluated. Overexpression of HIF-1α in tumors and metastasis is established as a poor prognostic marker in breast cancer (Schindl et al., 2002; Zhong et al., 1999). HIF-1α contribution at the cell-specific level has impacts on both primary tumor growth and metastasis, as HIF-1α expression is relevant in other cellular components of the tumor microenvironment such as endothelial cells (Tang et al., 2004; Liao et al., 2007). Previous studies have shown that endothelial cell-specific deletion of HIF-1α resulted in impaired growth and vascularization of subcutaneous LLC tumors (Tang et al., 2004). This finding indicates the important role of HIF-1α in endothelium in tumor growth and angiogenesis. However, data derived from xenograft model does not address the function of endothelial HIF-1α in mammary tumor initiation, progression and especially metastasis in vivo.

In this paper, we used the spontaneous breast cancer mouse model to investigate the role of endothelial HIF-1α in mammary tumor initiation, progression and metastasis. Based on previous findings, we anticipated that deletion of HIF-1α in endothelium would have an adverse effect on tumor angiogenesis, resulting smaller tumors with decreased vascularization. Suprisingly, unlike what was observed in subcutaneous LLC tumors, the onset
and growth of spontaneous mammary gland tumors were not affected by Tie2-Cre mediated deletion of HIF-1α. Specific deletion of HIF-1α in endothelium also did not influence the size of orthotopically-implanted tumors. Consistently with these observations, we found no changes in microvessel density or tumor cell proliferation in primary mammary gland tumors, indicating that there is no apparent adverse effect on tumor angiogenesis. This observation may be explained by the different microenvironments accommodating tumor cells in the two distinct models. Primary mammary gland tumors develop in a well-vascularized environment, whereas subcutaneous tumors grow in an area ablated of local blood vessels, where neoangiogenesis is key to tumor cell proliferation. It seems likely that the VEGF contributing for primary breast tumor angiogenesis is not of endothelial origin or is regulated independently of HIF-1α expression; tumor cell derived VEGF appears to be necessary and sufficient for tumor angiogenesis (Liao et al., 2007).

We found that, despite the absence of an evident effect in primary tumor development, endothelial HIF-1α has a significant impact on metastatic incidence as mice lacking endothelial HIF-1α display significantly less lung metastasis. We demonstrate that both tumor cell extravasation and intravasation processes are impaired in HIF-1α EC null mice, and verify that deletion of HIF-1α in endothelial cells leads to ~67% reduction in transendothelial migration of tumor cells under hypoxic conditions. The mechanism underlying this phenomenon is yet to be deciphered, but there are several possible explanations. Firstly, the structure of
endothelial cell layer is affected upon hypoxia exposure, due to excessive VEGF levels and disproportionate endothelial cell proliferation leading to the signature leaky tumor vasculature (Jain RK, 2005; Mazzone et al., 2009; De Bock et al., 2009). With a decrease of HIF-1α level in endothelium, VEGFR2 level is reduced and responses from the endothelium to VEGF pressure are limited (Tang et al., 2004). As a result, the permeability of tumor vasculature could be decreased. Secondly, endothelial cells have been recently described as sensors of oxygen imbalance (De Bock et al., 2009), and we propose that there is HIF-1α target signature unique to endothelial cells. We found a striking difference in levels of hypoxic induction of iNOS in endothelial cells when compared to the hypoxic transcript profile of LLC cells or even with other classic HIF-1α targets in endothelial cells. The dramatic decrease of iNOS induction upon HIF-1α deletion from endothelial cells is concomitant with the decrease in transendothelial migration of tumor cells under hypoxia. iNOS belongs to a group of enzymes that produce nitric oxide (NO), which is a regulator of vascular permeability and angiogenesis (Chiarugi et al., 1998). iNOS inhibits smooth muscle cell proliferation while promoting endothelial cell growth (Nussler and Billiar, 1993; Takaki et al., 2008; Shimokawa, 1999) which can result in a more relaxed and possibly more permeable vessel structure. In addition, in vivo experiments have shown that PyMT/iNOS−/− mice exhibited significantly reduced lung metastasis (Ellies et al., 2003). Therefore, decreased iNOS expression may contribute to less metastasis in HIF-1α EC null mice. Thirdly, functions such as adhesion and motility are altered as a result of hypoxia-induced changes in gene expression
(Konstantopoulos and Thomas, 2009). Tumor cell directional movement is mediated via chemokine ligand and receptor combinations. Specific chemokines such as CXCR4 and CXCR6 are overexpressed in most common human cancers, and the corresponding receptors are constitutively expressed in endothelial cells (Konstantopoulos and Thomas, 2009; Kakinuma and Hwang, 2006; Nieman et al., 1999; Niu et al., 2007). HIF-1α-dependent regulation of these receptors is manifestly affected during hypoxia and likely to intervene in the process of metastasis. Further investigation will focus on determining how HIF-1α targets are involved in the metastasis phenotype discussed here.

In summary, we show that HIF-1α in endothelium does not affect mammary tumor initiation and growth, but facilitates the formation of mammary tumor metastasis. As a key component of tumor microenvironment, changes in endothelium have a profound impact in metastasis formation. Our findings will shed light on the dissection of the mechanism of metastasis in breast cancer, and contribute to the potential development of anti-metastasis therapeutic drugs targeting HIF-1α-mediated hypoxic interaction between tumor and endothelial cells.

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Figure 3.1. Initiation and growth of mammary gland tumors in HIF-1α^{df}/Tie2Cre/PyMT mice

(A) Mean age at tumor onset, determined by the appearance of a 2 x 2 mm tumor, detectable by palpation starting at 4 weeks of age, in HIF-1α^{df}/PyMT^{+} (black squares, n=18) and HIF-1α^{df}/Tie2Cre^{+}/PyMT^{+} (black triangle, n=16). No significant differences were found between the two mouse genotypes.

(B) Mice were sacrificed when any tumor had grown to a size of 1 X 1cm. Age at endpoint tumor development was compared between the two genotypes, n=32 for HIF-1α^{df}/PyMT^{+} (black squares); n=25 for HIF-1α^{df}/Tie2Cre^{+}/PyMT^{+} (black triangle), and again results were comparable.
(C) Comparison in tumor burden between HIF-1α^{df}/PyMT^{+} (black column, n=33) and HIF-1α^{df}/Tie2Cre^{+}/PyMT^{+} (white column, n=19) mice at endpoint reveals similar.

(D) Both genotypes had similar tumor burden at 16-weeks (n=8 for HIF-1α^{df}/PyMT^{+}, n=12 for HIF-1α^{df}/Tie2Cre^{+}/PyMT^{+}) and 18-weeks (n=12 for HIF-1α^{df}/PyMT^{+}, n=12 for HIF-1α^{df}/Tie2Cre^{+}/PyMT^{+}) timepoints. Although tumor burden was bigger at later timepoint inside each group, there was no significant difference between two groups. Onset and growth of primary tumors are not affected by Tie2-specific deletion of HIF-1α. Values in the graphs are expressed as mean ± SEM. p values are from Student’s t-test.
Figure 3.2. HIF-1α<sup>df</sup>/Tie2Cre<sup>+</sup>/PyMT<sup>+</sup> mice showed significantly reduced pulmonary metastasis

(A) Representative H&E stained lung sections from HIF-1α<sup>df</sup>/PyMT<sup>+</sup> (left panel) and HIF-1α<sup>df</sup>/Tie2Cre<sup>+</sup>/PyMT<sup>+</sup> (right panel). Black arrows indicate the metastatic foci.

(B) Quantification of metastatic foci in lungs from both HIF-1α<sup>df</sup>/PyMT<sup>+</sup> and HIF-1α<sup>df</sup>/Tie2Cre<sup>+</sup>/PyMT<sup>+</sup> mice showed significantly less metastases in the mutant background, as evaluated by student’s t-tests. All the metastases foci in lungs were counted on H&E serial sections (HIF-1α<sup>df</sup>/PyMT<sup>+</sup> - black column, n=18 and HIF-1α<sup>df</sup>/Tie2Cre<sup>+</sup>/PyMT<sup>+</sup> - white column, n=17).
(C) Quantification of lung metastasis at 16-weeks showed significantly fewer
foci in HIF-1αdf/Tie2Cre+/PyMT+ (HIF-1αdf/PyMT+ - black column, n=6 and
HIF-1αdf/Tie2Cre+/PyMT+ - white column, n=9). One out of six lungs from
HIF-1αdf/PyMT+ mice did not contain metastasis, whereas 6 out of 9 lungs
from HIF-1αdf/Tie2Cre+/PyMT+ mice contained no metastasis. Values in
the graphs are expressed as mean ± SEM. p values were from Student’s
t-test.
Figure 3.3. Tumor angiogenesis was not affected by Tie2Cre-mediated deletion of HIF-1α

(A) CD34 stained representative section of primary tumors from HIF-1α<sup>df</sup>/PyMT<sup>+</sup> mice at endpoint.
(B) CD34 stained representative section of primary tumors from HIF-
$1\alpha^{df}$/Tie2Cre$^+$/PyMT$^+$ mice at endpoint. Scale bar represents 100µm.
CD34 staining was shown in brown color.

(C) Microvessel density was determined using a Chaukley graticule eyepiece.

There was no significant difference in microvessel density between the
two genotypes (n=8 per group). Values in this graph are expressed as
mean ± SEM. p value was from Student’s $t$-test.
Figure 3.4. Deletion of HIF-1α in endothelium impaired tumor cell intravasation

(A) Schematics of the timeline for tumor cell intravasation model.

(B) FACS analysis of the purity of the LLC\textsuperscript{GFP} clone used in this study.

(C) LLC\textsuperscript{GFP} cells were implanted into the cleared fat pad four of WT (black column, n=7) and EC null (white column, n=7) mice. Tumors were
collected 3 weeks post implantation and weighed. No significant difference was seen between two genotypes.

(D) Numbers of metastatic foci in lungs from the same mice in (C) were counted on H&E stained serial sections. There were significantly reduced metastatic foci in EC null mice.

(E) Blood from these mice were also collected and red blood cells were lysed. Genomic DNA from remaining cells was isolated for quantitative PCR using primers for GFP and HIF-1α (the loading control). The presence of circulating LLC$^{GFP}$ cells was determined by calculating relative copies of the $GFP$ gene. Blood from WT mice (black squares, n=6) contained higher levels of genomic $GFP$ than those from EC null mice (black triangles, n=7), indicating more elevated number of circulating tumor cells in WT mice. Values in the graphs are expressed as mean ± SEM. $p$ values were from Student’s $t$-test.
Figure 3.5. Loss of HIF-1α in endothelium prevents tumor cell extravasation

(A) Schematics of the timeline for tumor cell extravasation model.

(B) Representative images of whole lungs from WT (left) and EC null (right) mice 2 weeks post inoculation of LLC^{GFP} cells via tail vein. White arrows show the metastatic foci on the lung surface. Lungs from EC null mice had fewer foci on the surface.
(C) Representative H&E stained lung sections from WT (left) and EC null (right). Black arrows show the metastatic foci.

(D) Quantification of metastatic foci in lungs on H&E stained serial sections. There were significantly reduced metastatic foci in EC null mice. n=6 for WT (black column). n=9 for EC null (white column). Values in the graphs are expressed as mean ± SEM. p values were from Student’s t-test.
Figure 3.6. Deletion of endothelial HIF-1α prevents tumor cell transmigration

(A) EC primary cultures were incubated with goat anti-human VE-cadherin antibody followed by donkey anti-goat FITC secondary staining. Cells
were mounted in DAPI-containing medium for nuclear staining. Over 98% of the cells identified by nuclear DAPI staining are VE-cadherin positive (endothelial cells). Scale bar represents 100 µm.

(B) Original isolates (P0) of lung primary endothelial cells from HIF-1αdf mice were infected with adenovirus expressing β-gal (WT) or Cre recombinase (HIF-1α null); cells were treated for 9 hr of 21% O₂ or 1% O₂ to quantify changes in mRNA levels of HIF-1α targets (Bnip3, Vegf, Pgk and iNOS), measured by qPCR. HIF-1α deletion significantly affects relative abundance of several HIF-1α-regulated transcripts.

(C) Schematic representation of the migration assay.

(D) Quantification of tumor cell number migrated through WT and HIF-1α null endothelial cell monolayer during normoxia and hypoxia treatments show that significantly less LLC cells were found to transpose the HIF-1α null endothelial layer. Data is shown as average ± SEM (n ≥ 45), five fields per insert were counted, at least three inserts per treatment/cell type. Experiment was performed in triplicate. Statistical significance of differences observed was evaluated by unpaired student’s t tests, *p<0.05, ** p< 0.01, *** p<0.001.
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Chapter 3 is currently being prepared for submission for publication of the material. Zhang N, Price CB, Liao D, Johnson RS. Endothelial HIF-1α is a key regulator of lung metastasis. The dissertation author was the primary investigator and author of this paper.
Chapter 4

General Discussion
4.1. General discussion for CHAPTER 2

As shown the chapter 2, for the first time, the global and conditional null animals for FIH, an oxygen-dependent asparaginyl hydroxylase, were generated and well characterized. We found that FIH is an essential regulator in metabolism. Especially, FIH expression in the nervous system makes the major contribution. These findings are very exciting because they propose an interesting connection between oxygen sensing and control over metabolism and exhibit great therapeutic potential for treating diet-induced obesity.

In aerobic organisms, oxygen is the essential molecule for energy metabolism and it is indispensable for their performance and survival. In our living world, oxygen is usually in a sufficient supply. However, organisms always face challenge of fluctuating oxygen levels. For example, in the case of ischemic diseases, they are characterized for the imbalance in the demand and supply for oxygen. In addition, intratumoral hypoxia is widely present in solid tumors. Even in healthy humans, some tissues such as bone marrow are exposed to lower oxygen tensions [1-3]. To deal with the fluctuation in tissue oxygenation, various oxygen-sensing systems have been developed during evolution [4]. In the anatomical level, there are specific tissues for oxygen sensing, such as carotid body. In the molecular level, the existence of oxygen-dependent enzymes may contribute to a cascade of changes in response to changes in tissue oxygenation. These enzymes belong to a 2-oxoglutarate and Fe (II)-dependent dioxygenases (2OG oxygenase) superfamily, including PHDs and FIH.
In humans, 2OG oxygenases have about 60 members, which are involved in a wide range of oxidative reactions such as hydroxylation, desaturations and ring closures [5, 6]. They exhibit diverse functions [7-9]. Recent studies revealed several members such as PHD1 (prolyl hydroxylase 1 for HIF) and FTO (fat mass and obesity associated) are involved in energy metabolism, lipid metabolism, diabetes and obesity [10-12]. Global deletion of PHD1 in mice led to decreased whole-body oxygen consumption by attenuating glucose oxidation in skeletal muscle, which was in a HIF-dependent manner [3, 10]. FTO was first found by several independent genome-wide searches for genes susceptible to type II diabetes [13-16]. There is a strong correlation between polymorphisms in the human FTO gene and body mass index. It was identified as a 2OG oxygenase that demethylases the 3-methylthymine in single-stranded DNA [11]. FTO deficient mice are lean and resistant to high fat diet-induced weight gain due to increased energy expenditure and activation of the sympathetic nervous system [12].

Here, as shown in the chapter 2, our data revealed FIH, another member from 2OG oxygenase superfamily, has important roles in regulating metabolism. FIH deficient mice showed increased energy expenditure, enhanced glucose and lipid metabolism and resistance to high fat diet-induced weight gain and hepatic steatosis. Further, using conditional knockout mice for FIH, we found that its expression in the nervous system makes a major contribution to the metabolic phenotypes. Notably, inhibitors specific for FIH have significant therapeutic
potential in treating insulin resistance and diet-induced obesity, which a great number of Americans are fighting against.

There are still questions that remain to be answered: 1) Which substrate of FIH is involved in metabolism regulation? 2) Where in the nervous system FIH contributes to regulate metabolism? 3) Is there any correlation between the FIH gene and obesity and diabetes in humans?

First, besides HIF-1α, FIH has a wide range of ARD-containing proteins as its substrates. Further investigations are needed to determine which substrate(s) is linked to the metabolic regulation of FIH. Genetic approaches could be used to address this question. The global and conditional null mice for FIH could also be used as great systems to understand the physiological outcome of FIH hydroxylation on these substrates. Second, we revealed that FIH expression in the nervous system plays an important role in regulating metabolism. Our data also showed the activation of sympathetic nervous system in the FIH global null animals. Closer examination could be performed to reveal the underlying mechanism. Third, this question is inspired by the similar phenotypes between FIH and FTO deficient animals. Polymorphisms in the FTO gene are associated with obesity and diabetes in humans. It would be interesting to examine if there is any correlation between changes in the FIH gene and obesity in humans using genome-wide association studies.
4.2. General discussion for CHAPTER 3

In the chapter 3, the data revealed that deletion of HIF-1α expression in endothelial cells resulted in significantly reduced pulmonary metastasis formation although it didn’t affect tumor angiogenesis and overall growth suprisingly. The decreased metastasis could be explained by down-regulation of VEGF and iNOS in endothelial cells. These data add a new piece in the puzzle of how HIF signaling pathway in tumor microenvironment is involved in regulating breast cancer progression.

The novel aspect of this research project is that we focus on HIF-1α function in endothelial cells, but not tumor cells. Much work have been done to interpret the roles of tumor cells-derived HIF-1α in tumor progression, including the formation of metastasis [17, 18]. A great deal of data revealed that several important targets of HIF-1α contribute to the invasion and dissemination of tumor cells, such as E-Cadherin [19-21], LOX [22] and CXCR4/SDF-1 [23-27], etc. It is evident that changes in tumor cells play important roles in metastatic formation. However, tumor cells are not alone. They are surrounded by a variety of normal cells, including endothelial cells, stromal fibroblasts, macrophages, neutrophils and pericytes, etc [28] [29]. These normal non-malignant cells are recruited to the tumor site and contribute to both positive and negative signals to the tumor. They can produce a number of growth factors (e.g. EGF and CSF 1), cytokines (e.g. SDF-1), and matrix-degrading proteases (e.g. MMP) that facilitate the proliferation and invasion of tumor cells [29]. The crosstalk between tumor cells
and these non-malignant cells makes significant contributions to tumor growth, invasion and metastasis. Our lab is currently using the combination of genetically manipulated mouse models and breast cancer model to study how HIF-1α signaling pathway in these non-malignant cells is involved in regulating tumor progression.

We used the combination of HIF-1α<sup>df</sup>/Tie2-Cre and MMTV-PyMT to initiate the study shown in chapter 3. Finally, both <em>in vivo</em> mouse models and <em>in vitro</em> transmigration assays were carried out to first demonstrate the crucial roles of endothelial HIF-1α in regulating formation of pulmonary metastasis <em>in vivo</em>. Notably, compared to subcutaneous xenograft models, the breast cancer model and orthotopic transplantation model used in this research project recapitulate the tumor microenvironment of breast cancer more accurately.

In conclusion, within tumor microenvironment, HIF-1α mediated gene expression in endothelial cells could significantly contribute to metastatic spread of tumor cells. This study helps better understanding of the underlying mechanisms in breast cancer metastasis and proposes potential therapeutic approaches for treatment.
REFERENCES


