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Abstract
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Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
Mitchell J. Weiss

Keywords
Cancer, Epidermis, Filaggrin, HIF, Hypoxia, MYC oncogene

Subject Categories
Cell Biology | Developmental Biology | Genetics

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/721

HYPOXIC REGULATION OF MYC AND EPIDERMAL BARRIER DEVELOPMENT

Waihay J. Wong

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2012

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ACKNOWLEDGEMENT

I am indebted to Theresa Richardson, Amar Majmundar, Bo Qiu, Michael Nakazawa, and Guoliang Qing for their generous contribution to the ideas and experiments described in this dissertation. They, and other members of the laboratory past and present, have contributed greatly to my education and welfare. Special thanks are due to Brian Keith for his inspired teaching, sound advice, and infinite forbearance with my attempts at humour during the four years when we were neighbours in the laboratory.

I am deeply grateful to my dissertation committee—Mitch Weiss, who chaired our meetings, George Cotsarelis, Steve McMahon, and Andrei Thomas-Tikhonenko—for their thoughtful supervision and good advice. In the Department of Dermatology, John Stanley, John Seykora, Aimee Payne, and Sarah Millar taught me all about the skin, provided histopathological diagnoses, and shared critical reagents. Thanks also to Gerd Blobel, Frank Lee, Robert Eisenman, Bert Vogelstein, Martin Eilers, Tatyana Svitkina, Alan Flake, and Irwin McLean for sharing techniques, reagents, data, and advice.

My family and friends have sustained this work through their love and patience. They deserve more thanks than I can express in words. Finally, to my mentor Celeste Simon, whose characteristic insight, deft guidance, and spirited support have buoyed and steered me through the uncharted currents of scientific research. Thank you.

ABSTRACT

HYPOXIC REGULATION OF MYC AND EPIDERMAL BARRIER DEVELOPMENT

Waihay J. Wong
M. Celeste Simon

Low O_2 tension, or hypoxia, activates a complex transcriptional program via hypoxia-inducible factors (HIFs) to facilitate adaptation to low-O_2 conditions. This work describes two instances of HIF activity in normal tissue development and disease progression. First, HIF is partly responsible for MYC inhibition in hypoxic human colon carcinoma cells. Hypoxic MYC down-regulation requires the E3 ubiquitin ligases FBXW7 and DDB1, as well as cytosolic cathepsins. Reduced MYC protein correlated with hypoxic inhibition of RNA polymerase III-dependent MYC target genes, suggesting that MYC suppression under hypoxia occurs independently of its binding partner MAX. MYC overexpression in hypoxic cells induced cell death in a *NOXA*- and *PUMA*-dependent manner. Therefore, MYC degradation can be an adaptive strategy to promote hypoxic cell survival. Second, murine genetic models revealed an unexpected role for HIFs in the low-O_2 environment of the developing epidermis. Mice lacking HIF1α and HIF2α in the epidermis exhibited defective keratinocyte differentiation, impaired epidermal barrier development, and reduced expression of the major cornified envelope gene filaggrin (*Flg*). Filaggrin expression in hypoxic keratinocytes requires HIF1α and HIF2α, suggesting that one mechanism by which HIF controls epidermal development lies in *Flg* regulation. Collectively, these data demonstrate that HIF-mediated responses to low O_2 tension play important roles in cell survival and differentiation.

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Chapter One

Hypoxia-inducible factors and the response to O_2 deprivation

SUMMARY

Oxygen (O_2) is an essential nutrient that serves as a key substrate in cellular metabolism and bioenergetics. Organisms encounter insufficient O_2 availability, or hypoxia, in a variety of physiological and pathological states. In order to cope with this stress, evolutionarily conserved responses are engaged. In mammals, the primary transcriptional response to hypoxic stress is mediated by the hypoxia-inducible factors (HIFs). HIF activity influences critical aspects of normal tissue development and disease progression. HIFs are intricately responsive to regulation by prolyl hydroxylase domain-containing enzymes (PHDs); other controllers of HIF activity include sirtuins, cytoplasmic proteases, and cellular metabolites. While highly homologous, HIF isoforms possess distinct and opposing functions. Recent discoveries have uncovered new roles for HIFs in the control of metabolism, inflammation, tumorigenesis, and physiological responses. Insights from basic HIF biology are being translated into pharmaceutical strategies targeting the HIF pathway.

INTRODUCTION

Aerobic organisms require oxygen (O_2) to produce energy. For this reason, O_2 deprivation (hypoxia) creates significant stress in living cells. During hypoxic conditions, cells adapt via numerous mechanisms in order to match O_2 supply with metabolic, bioenergetic, and redox demands. Cells temporarily arrest in the cell cycle, reduce energy consumption, and secrete survival and proangiogenic factors. These events are coordinated by various cellular pathways, including the unfolded protein response (Buchberger et al., 2010), mTOR signaling (Sengupta et al., 2010), and gene regulation by hypoxia-inducible factors (HIFs). Initially identified as a regulator of erythropoietin (EPO) production (Semenza and Wang, 1992), HIFs are recognized as key modulators of the transcriptional response to hypoxic stress. Besides their adaptive function in cellular stress responses, recent work has revealed new and important roles for HIFs in both physiological and pathological processes. In this chapter, I shall discuss recent insights into HIF expression, activation, and function in pathological responses and physiological development. I shall also summarize emerging data on the crosstalk between HIFs and other metabolic regulators, and provide a survey of recent pharmacologic strategies to modulate HIF activity in the treatment of diseases.

HYPOXIA INDUCIBLE FACTORS

HIFs are obligate heterodimers consisting of an O_2-labile α subunit and a stable β subunit. Mammals possess three isoforms of HIFα, of which HIF1α and HIF2α (also known as EPAS1) are structurally similar and the best characterized (**Fig. 1**). HIF3α (or

IPAS) exists as multiple splice variants, some of which inhibit HIF1α and HIF2α activity in a dominant-negative fashion (Kaelin and Ratcliffe, 2008). HIF1α is expressed ubiquitously in all cells, whereas HIF2α and HIF3α are selectively expressed in certain tissues, including vascular endothelial cells, type II pneumocytes, renal interstitial cells, liver parenchymal cells, and cells of the myeloid lineage (Bertout et al., 2008). In addition to their unique expression patterns, emerging data suggest that HIF1α and HIF2α possess distinct and even opposing roles in normal physiology and disease (Keith et al., 2012).

HIF1/2α heterodimerizes with the stable HIF1β subunit, or ARNT, through HLH and PAS domain interactions (**Fig. 1**). HIF heterodimers recognize and bind to hypoxia response elements (HREs) in the genome, which are similar to Enhancer box (E-box) motifs and have the consensus sequence G/ACGTG. Genome-wide chromatin immunoprecipitation experiments indicate that the correlation between HRE occupancy and hypoxic gene induction ranges from high for HIF1α-upregulated genes, to low for both HIF2α-induced genes and HIF-repressed genes (Mole et al., 2009; Xia et al., 2009). In these latter cases, flanking sequences and additional regulatory elements appear to further specify HIF binding and target gene regulation. Recent examples of additional modulators of HIF-dependent gene regulation include the forkhead transcription factor FOXA2 and the chromatin modifier Reptin (Lee et al., 2010; Qi et al., 2010).

HIF hydroxylation is regulated by O_2 availability. In well-oxygenated environments, HIF1/2α subunits are hydroxylated at conserved proline residues (**Fig. 1**). These proline modifications are mediated by prolyl hydroxylases (PHDs), whose activities are regulated by O_2 availability (Kaelin and Ratcliffe, 2008). Hydroxylated HIF1/2α is, in turn, recognized and marked for proteosomal destruction by the E3 ubiquitin ligase

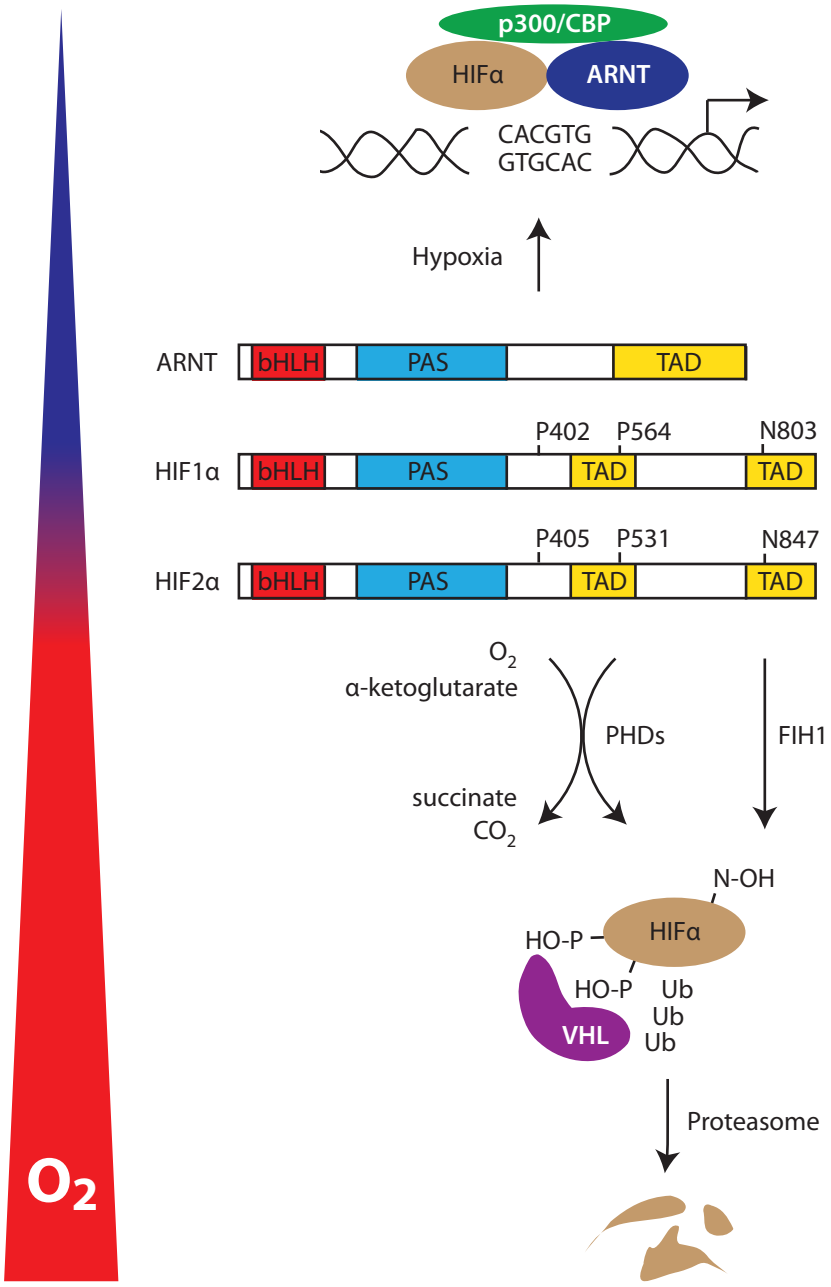

Figure 1. Regulation of hypoxia-inducible factor α-subunit (HIFα) by oxygen (O_2). In the presence of O_2 and α-ketoglutarate, HIF prolyl hydroxylase domain-containing enzymes (PHDs) and Factor Inhibiting HIF (FIH1) hydroxylate HIFα. Proline hydroxylation targets HIFα for VHL-mediated ubiquitylation and subsequent proteasomal degradation. Asparagine hydroxylation inhibits binding of the transcriptional co-activator p300/CBP. In hypoxic conditions, HIF is stabilized due to reduced PHD and FIH activity. Stabilized HIFα translocates into the nucleus, binds HIF1β subunit ARNT and coactivator p300/CBP, and activates target gene expression. Adapted from Patel and Simon, 2008.

von Hippel-Lindau protein (pVHL) complex (Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001). In the setting of hypoxic stress, PHD activity is diminished, and stabilized HIF1/2α proteins can induce the transcription of many genes with adaptive functions.

HIFα subunits are also substrates for an asparaginyl hydroxylase: factor inhibiting HIF1α (FIH1) (Lando et al., 2002). This enzyme is O_2-dependent and thus represents another component of the oxygen-sensing machinery. Hydroxylation by FIH1 disrupts a critical interaction between HIF1/2α and co-activators p300/CBP, impairing HIF transcriptional activity (Mahon et al., 2001; Webb et al., 2009). Therefore, O_2-sensitive hydroxylation by PHDs and FIH exert combinatorial control over HIF stability and activity.

PHD regulation by O_2 and metabolites. Because of their dependence on O_2 as a direct substrate, PHDs have been proposed to be 'oxygen sensors' linking cellular O_2 concentration to HIF-driven molecular responses. This hypothesis is supported by measurements of the apparent K_M of PHD enzymes *in vitro* (Kaelin and Ratcliffe, 2008). K_M values for O_2 were considerably higher than intracellular pO_2, suggesting that PHDs may function below saturation kinetics *in vivo*. While the enzymatic properties of PHDs *in vitro* may not necessarily reflect their properties *in vivo,* these measurements imply that O_2 availability can influence PHD activity across the entire physiological range of O_2 levels.

Cellular metabolites can also influence PHD activity. PHDs utilize the tricarboxylic acid (TCA) cycle intermediate 2-oxoglutarate (α-ketoglutarate) as a substrate. Furthermore, PHDs can be inhibited by other TCA cycle intermediates, for example fumarate and succinate (Kaelin and Ratcliffe, 2008; Klimova and Chandel, 2008;

Sudarshan et al., 2009). In the next section, I shall describe how several types of cancer have evolved to exploit this mechanism to stimulate HIF activity via the accumulation of specific metabolites.

Mitochondria as O_2 sensors and PHD regulators. Considerable evidence indicates that mitochondria also participate in O_2 sensing. Genetic and pharmacological approaches have been employed to inhibit components of the electron transport chain (ETC) in mitochondria. These studies have shown that in moderate hypoxia (1.5% O_2), mitochondria stimulate the production of cellular reactive oxygen species (ROS) which inhibit PHD activity and HIFα degradation (Kaelin, 2005; Klimova and Chandel, 2008). These oxygen radicals emanate specifically from complex III of the ETC (Klimova and Chandel, 2008). Moreover, Waypa and colleagues were able to visualize redox changes within specific cellular compartments using a novel redox-sensitive fluorescent protein (RoGFP) (Waypa et al., 2010). Hypoxia-induced oxidants were observed in the inner membrane space of mitochondria as well as in the cytosol, where ROS could influence PHD activity (Waypa et al., 2010). These findings suggest a mechanism in which mitochondria sense O_2 deprivation and produce ROS to regulate PHD activity. However, at extreme levels of O_2 deprivation (anoxia; 0% O_2), HIFα can be stabilized in the absence of functional mitochondria. This result suggests that in addition to mitochondrial ROS, other factors will inhibit PHD activity in severe O_2 deprivation (Kaelin, 2005; Klimova and Chandel, 2008).

Despite these results, the significance of mitochondria as O_2 sensors remains controversial and the proposed mechanisms incomplete. For instance, what triggers mitochondria to release ROS in response to low intracellular pO_2? It is also unclear

whether ROS release modulates PHD function directly or indirectly. One suggestion is that mitochondria signal to PHDs indirectly through their property as O_2 sinks and not via ROS production (Wenger, 2006). However, cytochrome b mutant cells, which generate mitochondrial ROS but do not consume O_2, stabilized HIF1α under hypoxic conditions (Klimova and Chandel, 2008). This finding indicates that mitochondrial ROS production, but not O_2 consumption, is important for HIFα stabilization. Therefore, there are likely multiple O_2 sensors—PHDs, FIH1, mitochondria, and others—that collectively regulate HIFα turnover and activity in hypoxic conditions.

Effect of intermittent hypoxia on HIFα stability. Intermittent hypoxia (IH) occurs when tissue pO_2 cycles between normal and hypoxic levels. This pattern of O_2 deprivation is clinically relevant, especially during recurrent sleep apnea in which transient pauses in breathing lead to chronic IH during sleep (Basner, 2007). Patients with this disease have a higher likelihood of developing hypertension, atherosclerosis, myocardial infarction and stroke (Basner, 2007). Prabhakar and colleagues have detailed how HIFα subunits are differentially regulated by IH. HIF1α protein expression is induced during IH as a result of many changes, including NADPH oxidase-dependent ROS generation (Peng et al., 2006; Yuan et al., 2008). This source of ROS is distinct from the mitochondrial ROS which inhibits PHD activity during continuous hypoxia. Importantly, studies in *Hif1α*$^{+/-}$ mice demonstrated that HIF1α promotes the acute respiratory and cardiovascular consequences of IH. In contrast, HIF2α expression is repressed by IH through calpain-dependent mechanisms (Nanduri et al., 2009). HIF2α inhibition appears to contribute to IH-induced oxidative stress and cardiovascular

responses *in vivo*. These data indicate that HIF1α and HIF2α may play opposing roles in IH-associated disease.

Differential regulation of HIFα subunits. The example of IH demonstrated that HIF1α and HIF2α can be regulated through distinct mechanisms. Selective control of HIF1/2α expression occurs at the transcriptional, translational, and posttranslational levels. For example, HIF1α, but not HIF2α, is degraded under hypoxia conditions in an HSP70/CHIP-dependent fashion (Luo et al., 2009). *HIF1A* translation is selectively inhibited by mTORC1 activity in renal carcinoma cell lines (Toschi et al, 2008). On the other hand, mRNA translation of *EPAS1* (encoding HIF2α) is uniquely responsive to iron content through an iron-response element in its 5' UTR (Sanchez et al., 2007). At the transcriptional level, NFκB signaling in activated macrophages directly induces *Hif1α* expression (Rius et al., 2008) (**Fig. 2**). Macrophages lacking IKKβ cannot stabilize HIF1α after hypoxic or microbial challenge and exhibit decreased HIF target gene expression. In contrast, hypoxic induction of HIF2α does not require IKKβ. However, activation of NFκB alone is insufficient for HIF1α stabilization, indicating that maximal HIF1α accumulation depends on both transcriptional regulation by NFκB and post-translational regulation by hypoxia (Rius et al., 2008). These observations underscore the complexity of the HIF response, and suggest that HIFα subunits may require distinct forms of regulation because they mediate non-overlapping biological effects.

HIF AND CANCER

There is ample evidence that solid tumors frequently encounter hypoxic stress. Rapidly proliferating cancer cells may outgrow their vascular network, limiting O_2 diffusion

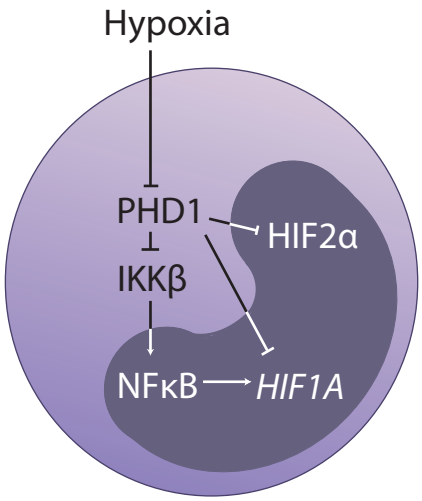

Figure 2. NFκB-dependent regulation of HIF in macrophages. In addition to direct HIF stabilization, hypoxic inhibition of PHDs results in IKK-mediated degradation of the NFκB inhibitor IκB. Consequently, activated NFκB directly transactivates *HIF1A*.

within the tumor. Hypoxic stress can also be caused by perfusion defects as a result of abnormal tumor blood vessel structure and function. Not surprisingly, therefore, solid tumors often exhibit high levels of HIFα accumulation (reviewed in Bertout et al., 2008). It should be noted that HIFα expression in cancer cells is also increased via hypoxia-independent mechanisms (**Table 1**). Genetic alterations such as *VHL* mutation in renal cell carcinoma, mutations in the Wnt/β-catenin signaling pathway in colon carcinoma, and other oncogenic events have been reported to result in HIFα stabilization (reviewed in Kaelin, 2008). Collectively, these findings indicate that HIFα expression and the downstream activation of the hypoxic stress response are widespread in many cancers.

Work from many laboratories has revealed that HIF-regulated gene responses play key roles in various aspects of cancer development, including proliferation (*MYC*), angiogenesis (*VEGF*, *PDGF*), apoptosis/autophagy (*NDRG2*, *BNIP3*), metabolism (*PDK1*, *LDHA*), DNA damage response (*GADD45A*), microRNAs (*MIR210*), extracellular matrix remodeling (*LOX*, *MMP1*), cell migration and invasion (*CXCR4*, *SDF1*) (Bertout et al., 2008; Huang et al., 2009; Kaelin, 2008) (**Fig. 3**). The importance of HIF activity in cancer is demonstrated by the fact that increased HIFα expression correlates with poor clinical prognosis in many cancer types (reviewed in Semenza, 2007).

HIF regulation by cancer cell metabolism. Inactivating homozygous mutations in the TCA cycle genes fumarate hydratase (*FH*) and succinate dehyrogenase (*SDH*) lead to elevated HIF1α expression in cells and human tumors (King et al., 2006). Mice bearing kidney specific inactivation of *Fh1* were generated and develop renal cysts marked by HIF activation (reviewed in Kaelin and Ratcliffe, 2008). HIF1α appears to be induced because of increased cellular levels of fumarate and succinate, which can inhibit PHD activity

Table 1 – Regulators of HIF activity

HIF regulators	
VHL	↓ HIFα stability (see text)
PHD1/2/3	↓ HIFα stability (see text)
FIH1	↓ HIFα transcriptional activity (see text)
Siah1a/2	↑ HIF1α stability; promotes degradation of PHD1/3 in hypoxia (Simon, 2004)
RSUME	↑ HIF1α stability; Enhances SUMOylation (Carbia-Nagashima et al., 2007)
SENP1	↑ HIF1α stability; Removes SUMO moieties (Cheng et al., 2007)
HSP90	↑ HIF1α stability (Isaacs et al., 2002)
COMMD1	↓ HIF1α stability and disrupts HIFα/β dimerization (van de Sluis et al., 2010, 2009)
HSP70/CHIP	↓ HIF1α stability but not HIF2α stability (Luo et al., 2009)
CITED2	↓ HIF1α activity (Bakker et al., 2007)
Metabolites/Related	
2-oxoglutarate	↓ HIFα stability as PHD co-factor (see text)
Ascorbate	↓ HIFα stability as PHD co-factor (Kaelin and Ratcliffe, 2008)
Iron (Fe^{2+})	↓ HIFα stability as PHD co-factor (Kaelin and Ratcliffe, 2008)
IRP	↓ *HIF2α* mRNA translation in response to high intracellular iron (see text)
NO	Modulates HIFα expression (Kaelin and Ratcliffe, 2008)
Intermittent Hypoxia	↑ HIF1α stability but ↓ HIF2α stability (See text)
Redox	
ROS	↑ HIFα stability (See text); observed in inflammatory cells (Shatrov et al., 2003)
Sirt1	↓ HIF1α and ↑ HIF2α, transcriptional activity (Dioum et al., 2009)
Sirt6	Binds to and ↓ HIF1α stability/activity (Zhong et al., 2010)
microRNAs	
miR-107	microRNA leads to ↓ ARNT expression (Yamakuchi et al., 2010)
miR-17-92	miRNA cluster; microRNAs lead to ↓ HIF1α expression (Taguchi et al., 2008)
Oncogenes/Tumor Suppressors	
PI3K/Akt	↑ HIF1α expression (Brugarolas and Kaelin Jr., 2004; Mottet et al., 2003)
mTORC1	↑ *HIF1α* mRNA translation (Brugarolas and Kaelin Jr., 2004; Bernardi et al., 2006)
GSK3β	↓ HIF1α stability (Mottet et al., 2003)
p53	↓ HIF1α/ARNT expression (Blagosklonny et al., 1998; Yamakuchi et al., 2010; Sano et al., 2007; Schmid et al., 2004; Ravi et al., 2000)
β-catenin	Binds to HIF1α; ↑ HIF1α transcriptional activity (Kaidi et al., 2007)
Ras	↑ HIF1α expression by ROS generation (Gerald et al., 2004)
ERβ	↓ HIF1α stability (See text)
SDH/FH	Mutations in these genes lead to ↑ HIF1α stability (See text)
Inflammation	
NFκB	↑ HIF1α transcription (See text)
p44/42 MAPK	↑ HIF1α expression downstream of LPS (Frede et al., 2006)
IFN-γ	↑ HIF1α and HIF2α(?) expression (See text)
IL-4	↑ HIF1α and HIF2α(?) expression (See text)

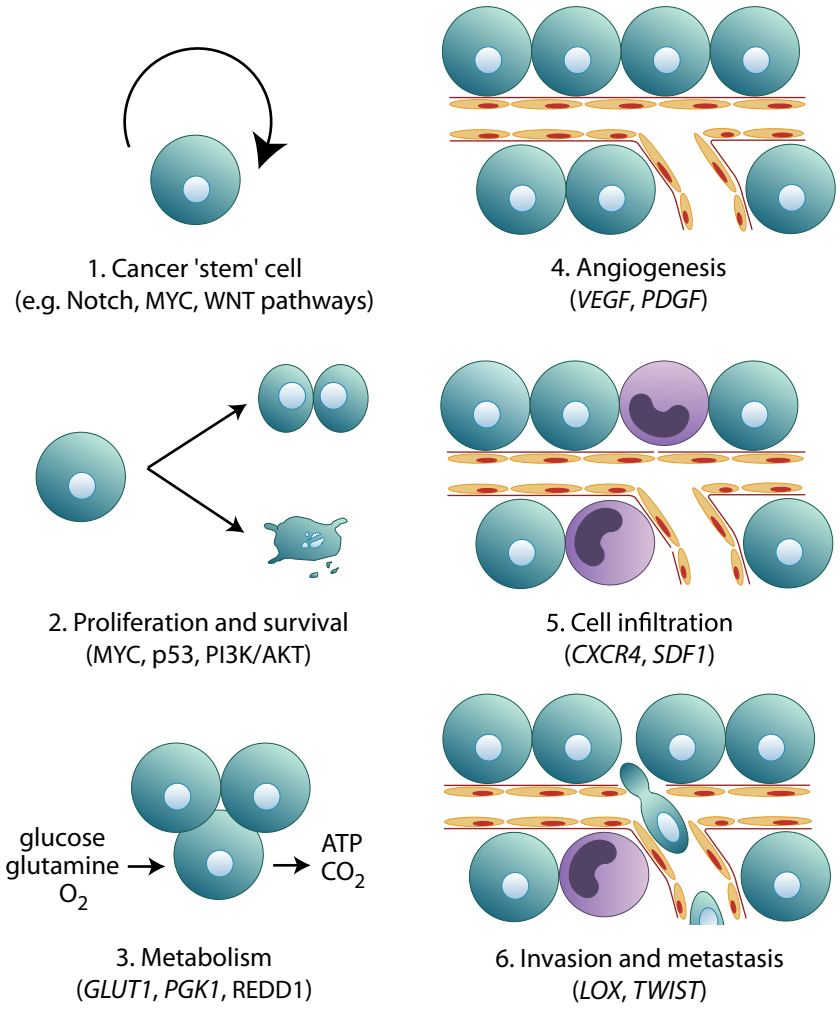

Figure 3. Effects of HIF on multiple steps of cancer development. HIF is stabilized by hypoxia and other nonhypoxic stimuli in many cancers. HIF activity in cancer has been associated with 1. putative cancer 'stem' cell maintenance and increased expression of genes involved in 2. proliferation and survival, 3. cell metabolism, 4. angiogenesis, 5. recruitment of infiltrating cells such as tumor-associated macrophages and bone marrow-derived cells, and 6. tumor cell invasion and metastasis. Examples of HIF-regulated genes and oncogenic pathways are given in parentheses.

directly or indirectly through promotion of cellular ROS (Kaelin and Ratcliffe, 2008; Klimova and Chandel, 2008; Sudarshan et al., 2009). These findings clearly demonstrate the extent to which metabolism influences HIFα expression in cancer. While it is assumed that HIF1α activation promotes oncogenesis in these settings, HIFs can be tumor suppressive in some contexts. The *Fh1* mutant mice will be a useful tool for testing this premise directly.

More recently, heterozygous mutations in isocitrate dehydrogenase 1/2 (*IDH1/2*) have been linked to cancer. Mutant IDH1 proteins are defective in their ability to oxidize isocitrate into α-ketoglutarate (αKG) (Dang et al., 2009; Zhao et al., 2009). Xiong and colleagues reported that because of a dominant negative effect, cells expressing mutant IDH1 have reduced levels of αKG, a PHD substrate. As a result, HIF1α is indirectly stabilized (Zhao et al., 2009). HIF1α expression is also elevated in human gliomas with mutant *IDH1*. Therefore, they proposed that similar to mutations in *FH* and *SDH*, *IDH1* mutations promote cancer by indirectly activating HIF1α. However, multiple studies have since shown that *IDH1/2* wild type and mutant cancers exhibit comparable intracellular levels of α*KG*, indicating that a single wild type allele of *IDH1/2* may be sufficient for αKG generation *in vivo* (Dang et al., 2009; Gross et al., 2010; Ward et al., 2010). Consequently, comparable levels of substrate would be available for PHD activity in both wild type and mutant cancers. In other words, *IDH1* mutations may stimulate oncogenic HIF responses, but not necessarily through decreased αKG levels.

Moreover, mutant IDH1 has an unexpected reverse activity which reduces αKG to generate 2-hydroxyglutarate (2HG), a metabolite associated with brain tumors (Aghili et al., 2009). Elevated 2HG is also observed in *IDH1/2* mutant glioblastoma and acute

myeloid leukemia (Dang et al., 2009; Gross et al., 2010; Ward et al., 2010). It is suggested that this metabolite, synthesized by mutant IDH1/2, promotes cancers of the brain and blood. However, the molecular and cellular functions of 2HG remain poorly understood. One attractive hypothesis is that 2HG inhibits PHD activity and, in turn, promotes oncogenic HIF responses in *IDH1/2* mutant tumors. 2HG may promote cancer through HIF-independent mechanisms as well. Further investigation is required to test the role of 2HG in modulating HIF activity and, more generally, tumor cell behavior.

Overall, these studies on *IDH1/2* mutations underscore the notion that metabolic enzymes can be oncogenic as well as tumor suppressive (e.g. FH and SDH). They also challenge the concept that mutations in metabolic genes induce cancer solely through aberrant HIF responses.

HIFα subunits and tumorigenesis. Surprisingly, HIF1α expression correlates with lower cancer stage or decreased patient mortality in certain cancers; examples include non-small cell lung cancer, head and neck squamous cell carcinoma, and neuroblastoma (Bertout et al., 2008). HIF2α expression in these malignancies, on the other hand, is a negative prognostic factor. This difference between HIF1α and HIF2α expression suggests that HIFα subunits may contribute differently towards tumorigenesis in certain cancers.

The distinct roles of HIF1α and HIF2α in tumorigenesis have been studied most thoroughly in *VHL*-deficient clear cell renal cell carcinoma (ccRCC) (Keith et al., 2012). *VHL*-deficient ccRCC cluster into tumors which express either both HIF1α and HIF2α or HIF2α only (Gordan et al., 2008). Overexpression and knockdown studies of HIF1α and HIF2α in *VHL*-deficient ccRCC cell lines indicate that HIF2α, but not HIF1α, is necessary for tumor growth (Kaelin, 2008). One possible explanation for this effect is that HIF1α

antagonizes MYC function, whereas HIF2α promotes MYC activity (Gordan et al., 2007). Microarray profiling of ccRCC specimens revealed that compared to tumors expressing both HIFα isoforms, tumors exclusively expressing HIF2α up-regulate MYC target genes, proliferate faster, and are relatively resistant to replication stress (Gordan et al., 2008). Because solid tumors are frequently hypoxic, HIF1/2α effects on MYC activity may be important in a large subset of cancers, especially in MYC-driven malignancies.

HIF in MYC-driven cancers. MYC was first identified as an oncogene in Burkitts lymphoma, where a balanced translocation between chromosomes 8 and 14 results in the fusion of the immunoglobulin heavy chain locus with the *MYC* gene (Dalla-Favera et al., 1982). Stabilizing mutations in *MYC* have also been identified in Burkitts lymphoma and other cancers (Salghetti et al., 1999). Furthermore, MYC is a downstream target of many signaling pathways that are involved in cancer development and progression, such as Wnt and Notch. As a result of these changes, high MYC activity is common in many cancer types, including multiple myeloma, colon adenocarcinoma, and breast cancer (Vita and Henriksson, 2006; Wolfer et al., 2010).

MYC is a transcription factor with key regulatory functions in cell growth, differentiation, apoptosis, and metabolism. MYC and its binding partner MAX bind DNA E-boxes (CACGTG) and activate target gene transcription by RNA Pol II (Dang, 2012). MYC-MAX heterodimers also act as transcriptional repressors by binding and inactivating the transcription factors MIZ1 and SP1 (Herkert and Eilers, 2010). As a result, transcription of MIZ1/SP1 target genes such as *CDKN1A* and *CDKN2B* are inhibited (Seoane et al., 2001; Staller et al., 2001). MYC also induces the expression of Pol III-transcribed genes in a MAX-independent manner (Gomez-Roman et al., 2003; Steiger et

al., 2008). Because of its many transcriptional targets, elevated MYC activity causes many changes in cancer cells, including increased proliferation (e.g. *CCND2*, *CDK4*), genomic instability, and metabolic changes (e.g. *LDHA*, *GLS1*).

MYC is normally kept under tight regulation transcriptionally, translationally, and posttranslationally. Hypoxic modulation of MYC activity largely occurs via HIF-dependent effects on MYC protein interactions (**Fig. 4**). For example, HIF2α enhances MYC transcriptional activity in ccRCC by increasing MYC-MAX heterodimerization (Gordan et al., 2007). Conversely, HIF1α competes with MYC for binding to MIZ1 and SP1, thus antagonizing MYC-dependent gene repression (Koshiji et al., 2004; Koshiji et al., 2005). HIF1α also transcriptionally activates the MYC antagonist *MXI1*, which disrupts MYC-MAX interaction and hence represses MYC-dependent transcription (Corn et al., 2005; Zhang et al., 2007). These findings are consistent with ccRCC presentation: HIF2α expressing tumors are more proliferative and resistant to replicative stress, whereas tumors that express HIF1α appear to experience tumor suppressive effects (Gordan et al., 2008). However, MYC inhibition may be advantageous in other contexts. For example, MYC downregulation is correlated with increased cell motility and tumor metastasis (Liu et al., 2012). In the next chapter, I shall illustrate how cells survive hypoxic stress by promoting MYC degradation under low O_2 conditions.

HIF and p53. Another mechanism by which HIFs exert opposing effects on tumor behavior lies in the hypoxic regulation of the tumor suppressor protein p53. HIF1α binds to p53, resulting in p53 stabilization and hypoxia induced cell death (An et al., 1998; Moeller et al., 2005). This interaction between HIF1α and p53 is probably a late evolutionary development in higher organisms, as HIF1α indirectly inhibits the p53

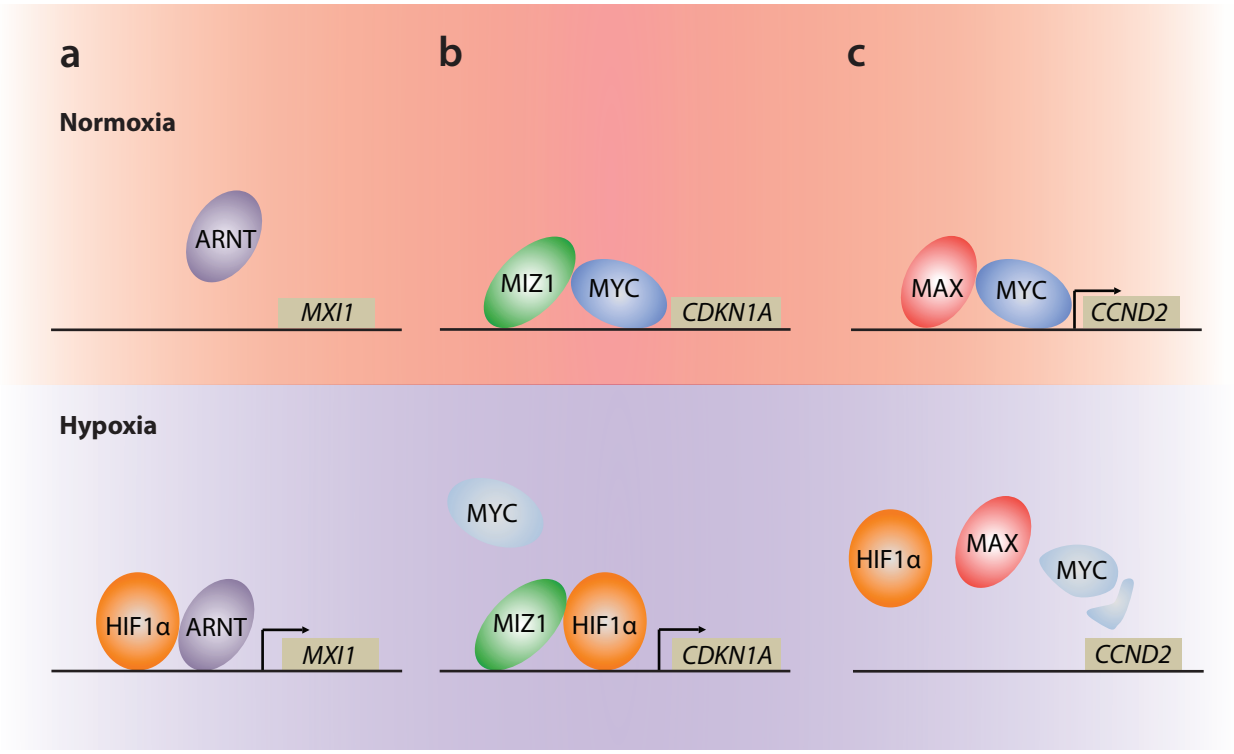

Figure 4. Effects of HIF1α on MYC transcriptional activity. (**a**) HIF1α directly induces MXI1 transcription. (**b**) HIF1α competes with MYC for binding to MIZ1 at the promoters of MYC-repressed genes. (**c**) HIF1α has been reported to disrupt MYC-MAX heterodimers.

homolog CEP-1 in *C. elegans* after radiation-induced DNA damage (Sendoel et al., 2010). In contrast, recent experiments have shown that HIF2α indirectly suppresses p53 activity and thereby promotes radioresistance and chemoresistance in tumor cells (Bertout et al., 2009; Roberts et al., 2009). These findings indicate that certain cancers, including but not limited to ccRCC, may differ in their tumor behavior and drug response according to the expression of HIF1α and/or HIF2α. Whether selective pressures exist in renal cell carcinomas for the loss of HIF1α and gain of HIF2α expression and whether HIFα expression patterns influence renal cancer progression remain subjects for further study.

HIFα: oncogenes or tumor supressors? It has been suggested that in non-*VHL* malignancies, HIF2α has greater oncogenic capacity than HIF1α. A recent report demonstrated that shRNA-mediated inhibition of HIF2α, but not HIF1α, in multiple human cancer cell lines reduced cell proliferation *in vitro* and subcutaneous xenograft growth in mice (Franovic et al., 2009). However, functional rescue experiments using exogenous HIF2α were lacking. *In vivo* models of lung tumorigenesis suggest a more complex role for HIF2α in cancer. Constitutively stabilized HIF2α increases lung tumour burden, tumour vascularity, and local invasion in *Kras* mutant mice (Kim et al., 2009). Intriguingly, a lung-specific deletion of HIF2α in the same *Kras* mutant model similarly enhances lung tumorigenesis (Mazumdar et al., 2010a). A clue to this paradox may lie in the observation that HIF2α gain of function and HIF2α loss of function promote tumorigenesis via two unrelated mechanisms (Kim et al., 2009; Mazumdar et al., 2010a). Assuming that various HIF2α targets have different activation thresholds—due to HRE sequence conservation, co-regulation by other transcription factors, composition of the transcription machinery, etc.—then according to the extent of HIF2α stabilization, either

tumor suppressive (e.g. AKT inhibition) or tumor promoting effects (e.g. angiogenesis, epithelial to mesenchymal transition) may ensue.

Compared to HIF1α and HIF2α, the biology of HIF3α in relation to tumorigenesis remains largely unstudied. HIF3α is down-regulated in ccRCC specimens, consistent with its known function as a dominant negative inhibitor of HIF1α and HIF2α (Maynard et al., 2007). In summary, HIF1α, HIF2α, and HIF3α have varying effects on cancer development because of their context-dependent functions and distinct modes of action.

HIF and metastasis. Epithelial to mesenchymal transition (EMT) is a key feature of invasive cells and can be characterized by the loss of epithelial cell-cell contact and the acquisition of mesenchymal features and motility. Hypoxia and HIF influence the expression of many EMT regulators to promote metastasis. Studies by Maxwell and colleagues revealed that HIF1α expression in renal cell carcinoma is sufficient to induce the loss of E-cadherin and an increase in invasion (Esteban et al., 2006). HIF1α directly regulates *TWIST1* transcription and increases tumor cell invasiveness and metastasis in head and neck squamous cell cancer (Yang et al., 2008). In prostate cancer, HIF1α promotes SNAIL1 nuclear localization in a VEGF-dependent manner (Mak et al., 2010). This finding is clinically relevant and implicates HIF1α expression in prostate cancer progression. Low grade prostate tumors repress HIF1α via estrogen receptor β (ERβ) activity, whereas high grade tumors down-regulate ERβ, resulting in increased HIF1α expression, SNAIL1 nuclear localization, and metastasis (Mak et al., 2010). HIF1α also induces lysyl oxidase (*LOX*), which is an extracellular matrix remodeling enzyme as well as an upstream regulator of SNAIL1. As Giaccia and colleagues demonstrated, inhibition of LOX reduces tumor cell invasion, adhesion, and metastasis in an orthotopic breast

cancer model (Erler et al., 2006). Recent work from the same group indicates that LOX secreted by the primary tumor remodels distant premetastatic sites to recruit tumor and stromal cells (Erler et al., 2009). The hypoxic tumor microenvironment therefore promotes metastasis via the activation of multiple HIF-responsive genes that together regulate all stages of cancer spread, including invasion, intravasation, and distant extravasation.

HIF and tumor angiogenesis. HIF exerts similar effects on endothelial cells in both tumor and nonmalignant tissues to mediate angiogenesis (**Fig. 5**). However, unlike 'normal' blood vessels, tumor-associated vasculature is leaky, tortuous, and noncontiguous (Jain, 2005). Tumor-associated endothelium interacts with tumor cells as well as nonmalignant stromal cells, such as fibroblasts and infiltrating bone marrow derived cells. These cell types differ widely in their responses to hypoxic stress and therefore may contribute differently to tumor angiogenesis. For example, HIF activity in glioblastoma promotes tumor angiogenesis, as HIF1α inhibition in glioblastoma cells reduces vascular remodeling and normalizes tumor vasculature (Du et al., 2008). Paradoxically, HIF1α depletion in these cells also increases perivascular invasion, because of the direct effect of decreased VEGF levels on glioblastoma cell migration (Du et al., 2008). The development of tumor vasculature also appears to require myeloid-derived VEGF specifically. Deletion of the HIF target gene *Vegf* in myeloid cells increases murine mammary tumor growth, tumor oxygenation, and tumor sensitivity to chemotherapy, most likely due to 'normalization' of tumor vessels (Stockmann et al., 2008). In contrast, haploinsufficiency of the HIF regulator *Phd2* in nonmalignant tissues allowed the 'normalization' of xenograft tumor vasculature, improved oxygenation, and reduced

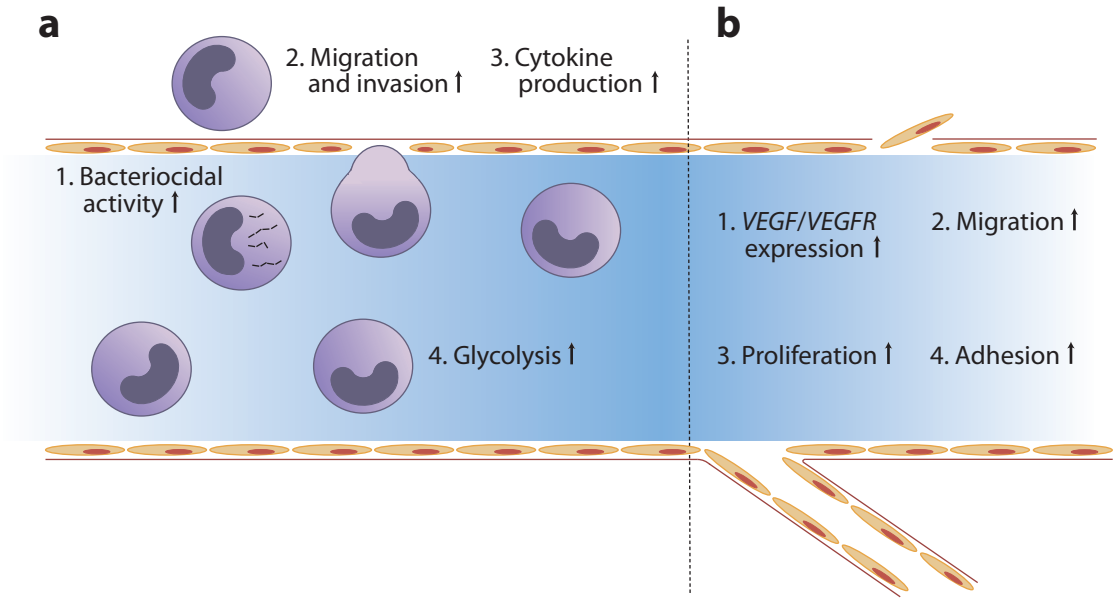

Figure 5. Vascular responses to HIF. (**a**) HIF activity is involved in many aspects of macrophage behavior via the induction of genes involved in 1. bacterial killing (*NOS2*, *CRAMP*), 2. migration and invasion (*CXCR4*, *FN1*, *MCSFR*), 3. cytokine production (*IL1β*, *IL6*, *IL12*, *TNFα*), and 4. metabolism (*GLUT1*, *PGK1*). (**b**) HIF1α stabilization in endothelial cells increase 1. *VEGF* expression, 2. migration, and 3. proliferation, whereas HIF2α stabilization promotes 4. endothelial cell adhesion to the extracellular matrix.

metastasis (Mazzone et al., 2009). However, the dependence of these effects on HIF stabilization remains uncertain. These studies illustrate that the tumor vasculature responds to distinct and perhaps opposing HIF activities in different cell types. Therefore, selective manipulation of the hypoxic stress response in distinct tumor subcompartments may be more effective than systemic HIF inhibition as an antitumor strategy.

HIF and tumor-associated macrophages. Studies in many cancer types have shown that macrophage infiltration correlates with unfavorable clinical prognosis (Lewis and Pollard, 2006). Macrophages are recruited to tumor areas primarily by the production of chemoattractants by hypoxic tumor and stromal cells, such as the HIF target genes *CSF1* and *VEGF* (Murdoch et al., 2004). Recent work has also shown that apoptotic cells, such as those in hypoxic regions of a tumor, produce soluble factors such as TGF-β to attract monocytes and macrophages (Herr et al., 2009). Once recruited, tumor associated macrophages (TAMs) exhibit a highly dynamic immune phenotype which promotes tumor growth, angiogenesis, metastasis, and tumor immunosuppression. Furthermore, as Harris and colleagues noted, TAMs exhibit elevated HIF1α and HIF2α expression due to the hypoxic tumor microenvironment (Murdoch et al., 2004).

HIF2α expression in breast and cervical cancer TAMs is correlated with unfavorable prognoses, suggesting a functional relevance for HIF2α in this setting (Kawanaka et al., 2008; Leek et al., 2002). Conditional HIF2α deletion in the myeloid lineage in mice has revealed key roles for HIF2α and TAMs in tumorigenesis. In mouse models of hepatocellular carcinoma and colitis-associated colon carcinoma, mice lacking HIF2α in their myeloid cells exhibit decreased recruitment of TAMs into tumor areas

(Imtiyaz et al., 2010). This finding correlates with reduced tumor mitotic index, lower tumor grade, and a downward trend in the number and size of colitis-induced colon carcinomas (Imtiyaz et al., 2010). It will be of interest to explore whether HIF1α expression in TAMs is functionally relevant to tumor progression in similar *in vivo* models, and if so, whether HIF1α and HIF2α complement each other in this context. Recent work suggests that the absence of HIF1α in macrophages has no effect on tumor spheroid infiltration, tumor cell proliferation, or tumor invasiveness *in vitro*, but reduces cell death in tumor spheroid cultures (Werno et al., 2010). Therefore, HIF1α and HIF2α may regulate distinct aspects of macrophage function.

HIF and cancer stem cells. Hypoxia can promote an undifferentiated state in certain populations of stem and progenitor cells (Keith and Simon, 2007; Yoshida et al., 2009). Similarly, hypoxia and HIFs may contribute to the maintenance of putative cancer 'stem' cells. HIF depletion in $CD133^+$ glioblastoma cells, which are enriched for cancer stem cells, reduces their tumorigenic and angiogenic potential *in vitro* and *in vivo* (Li et al., 2009a). Furthermore, HIF2α is selectively expressed in the $CD133^+$ subpopulation of glioblastoma cells whereas HIF1α expression is widespread among both tumorigenic and nontumorigenic cells, suggesting that HIF2α may fulfill a specific function in glioblastoma stem cells (Li et al., 2009a). In a separate study, a small subset of immature cells in human neuroblastoma specimens was found to express neural crest markers and HIF2α; upon HIF2α knockdown, these cells underwent early sympathetic differentiation (Pietras et al., 2009). However, the precise identity and function of these cells remain unclear. It is interesting to note that both $CD133^+$ glioblastoma cells and putative neuroblastoma progenitor cells express high levels of HIF2α while residing in

periendothelial niches (Calabrese et al., 2007; Pietras et al., 2009). Although the extent of O_2 saturation within these capillaries is unknown, these findings suggest that HIFα expression in certain cancer cell subpopulations may be controlled by both hypoxic and nonhypoxic stimuli, including metabolic aberrations in cancer.

HIF AND DEVELOPMENT

Mammalian development takes place in a hypoxic environment. In a process that is analogous to cancer cell growth, rapid proliferation in the embryo occurs in the presence of an incompletely formed cardiovascular system. As a result, the high O_2 consumption rate of dividing fetal cells and the low efficiency of O_2 delivery create widespread areas of O_2 deprivation (Simon and Keith, 2008). This state of low O_2 tension can be described as 'physiological hypoxia', as opposed to acute or pathological conditions wherein O_2 concentration falls below the normal range. 'Physiological hypoxia' also occurs in specific compartments in the adult organism, for example the renal medulla. HIF stabilization in these contexts not only allow developing cells to survive, but also provides an important developmental cue for embryogenesis and tissue homeostasis.

The functions of HIF during embryonic development have been well reviewed by Simon and Keith (2008) and Dunwoodie (2009). In this section, I shall provide several examples of tissue homeostasis and regeneration under HIF control. I shall describe recent data on O_2 and HIF-dependent effects on neuronal differentiation, muscle repair, and red blood cell production. I shall also review current work on skin oxygenation and HIF-dependent functions of the epidermis. In chapter three, I shall discuss in further

detail the effects of HIF1α and HIF2α expression on keratinocyte terminal differentiation and skin barrier formation.

HIF in neuronal progenitor cells. Multiple studies have demonstrated that hypoxic culture conditions promote an undifferentiated, proliferative state in neuronal stem and progenitor cells. For example, hypoxia stimulates the growth of undifferentiated glomus cells in the carotid body (López-Barneo et al., 2009). Similar pro-proliferative effects have also been observed in neuronal stem cells (NSC) of human and mouse origin (Morrison et al., 2000; Rodrigues et al., 2010; Santilli et al., 2010). Interestingly, the dentate gyrus naturally exhibits low O_2 tension. Targeted neuronal deletion of HIF1α led to fewer neuronal precursor cells in the subgranular zone of the dentate gyrus (Mazumdar et al., 2010b). This decrease was associated with reduced proliferation of neuronal stem and progenitor cells and decreased neurite outgrowth upon differentiation. These changes were blocked using a Wnt agonist, indicating that HIF1α regulates proliferation and differentiation of neuronal precursor cells via Wnt signaling (Mazumdar et al., 2010b). In another study, HIF1α-mediated effects on NSC proliferation were correlated with increased activity of the JNK2/cyclin D1 pathway (Chen et al., 2010). Cyclin D1 is also a downstream target of Wnt/β-catenin signaling (Tetsu and McCormick, 1999). These results suggest that HIF1α may modulate key signaling pathways, such as Wnt and JNK, to control neuronal proliferation and differentiation via the activity of critical cell cycle regulators, such as cyclin D1.

HIF in muscle differentiation. Muscle satellite cells comprise the postnatal reservoir of skeletal muscle stem cells. Satellite cells are located in perivascular niches, suggesting that they are well oxygenated (Christov et al., 2007). However, muscle damage

is often accompanied by vascular disruption and acute hypoxia. It has been well established that hypoxic conditions inhibit myogenic differentiation but stimulate myoblast proliferation (Di Carlo et al., 2004; Yun et al., 2005), suggesting that tissue hypoxia could promote a rapid response to wounding by generating a proliferative burst in adult muscle stem cells. Giaccia and colleagues demonstrated that this inhibitory effect on myoblast differentiation is HIF- and Notch-independent (Yun et al., 2005). However, another study found that in hypoxic myoblasts, HIF1α bound and stabilized the intracellular domain of Notch (NICD), such that NICD activity at Notch-responsive promoters was enhanced (Gustafsson et al., 2005). Emerging data suggest that hypoxic regulation of myoblast differentiation occurs at multiple levels. For example, although HIF1α represses myoblast differentiation by inhibiting MYOD expression, this effect is masked by HIF-independent repression via the PI3K/AKT pathway (Majmundar et al., 2012). In fact, PI3K/mTORC2/AKT signaling may be a common pathway by which HIF-dependent and HIF-independent mechanisms control myoblast differentiation (Ren et al., 2010). Intriguingly, Majmundar et al. (2012) could not rescue hypoxic suppression of myogenesis with Notch inhibitor treatment, suggesting that hypoxic effects on myoblast differentiation may be Notch-independent. Because of these apparently contradictory findings, it will be important to determine the degree to which these various pathways (HIF, Notch, PI3K/AKT) contribute to myogenic differentiation.

Erythropoiesis: a systemic response to hypoxia. Organs involved in erythropoiesis can respond to systemic hypoxia to increase red blood cell numbers and the O_2-carrying capacity of blood (Fandrey, 2004). Erythropoetin (EPO) is the central stimulant of this process (Lee, 2008). Abundant data suggest that the PHD2/pVHL/HIF2α

axis controls EPO levels and, therefore, adult erythropoiesis. Genetic studies of familial polycythemia (abnormally elevated hemoglobin or red blood cell count) identified mutations in *VHL* that impaired HIF1α degradation (Lee, 2008). Subsequent genetic and biochemical analyses have identified inactivating mutations in *EGLN1* (encoding PHD2) and activating lesions in *EPAS1* (encoding HIF2α) (Furlow et al., 2009; Lee, 2008). Mouse models bearing mutations in these genes have also been generated and exhibit abnormal erythropoiesis similar to human patients (Gruber et al., 2007; Hickey et al., 2007).

Furthermore, studies comparing Tibetan highlanders and the closely related lowland Han Chinese have provided an evolutionary link between the PHD2/pVHL/HIF2α axis and erythropoiesis (Beall et al., 2010; Simonson et al., 2010; Yi et al., 2010). The authors compared the frequencies of single-nucleotide polymorphism (SNP) alleles between these groups, and noted significant divergence in allelic frequency of SNPs located in or near the *EGLN1* and *EPAS1* genes. These findings correlated with lower hemoglobin and erythrocyte levels in the blood of Tibetan subjects, suggesting that *EGLN1* and *EPAS1* alleles common amongst Tibetan highlanders reduced the rate of erythropoiesis. It has been proposed that the divergence in *EPAS1* and *EGLN1* occurred through natural selection, whereby Tibetan *EPAS1* and *EGLN1* alleles facilitate survival in the high altitude. For instance, Tibetans are less likely to developing chronic mountain sickness, which is marked by elevated hemoglobin and a high rate of erythropoiesis. These reports highlight the evolutionary selection of HIF-mediated adaptations to low O_2 tension.

Cutaneous pO_2 regulates erythropoiesis. Although kidney and liver are the main EPO-producing organs, recent work has identified bone and skin as crucial sites for

regulating EPO production in response to hypoxia (Boutin et al., 2008; Rankin et al., 2012; Weidemann et al., 2009). Specifically, epidermal HIF1α expression is required for hypoxic induction of nitric oxide (NO) production in the skin (Boutin et al., 2008). NO promotes cutaneous vasodilation, which reduces renal blood flow and hence upregulates EPO synthesis in the kidney (Boutin et al., 2008). Therefore, the PHD/pVHL/HIF axis coordinates a systemic response to low O_2 levels whereby HIF responds to hypoxia at the skin, produces EPO in the kidney, and regulates hematopoiesis in the bone marrow. Furthermore, these data indicate that HIF1α and HIF2α are biologically active in the epidermis.

O_2 and skin development. The epidermis consists of keratinocytes at various differentiation stages that are organized spatially to form a stratified squamous epithelium (**Fig. 6**). This thin layer of cells, which forms the first line of defense against the exterior environment, is constantly regenerated once every four weeks (Fuchs, 2008). This enormous regenerative potential had been ascribed to the existence of interfollicular stem cells; however, recent lineage tracing experiments suggest that instead of isolated cells dedicated to self- renewal, all basal keratinocytes are functionally equivalent and stochastically commit to differentiate at a given probability (Clayton et al., 2007). As these cells differentiate, they move superficially and progress through several morphological and transcriptional stages, forming the spinous layer, granular layer, and eventually the cornified envelope. Specialized progenitor cell pools exist for other components of the skin, including hair follicles, sebaceous glands, and dermal connective tissue (Blanpain and Fuchs, 2009). While these stem cells are normally restricted to differentiation along their specified sublineage, some degree of plasticity may exist during

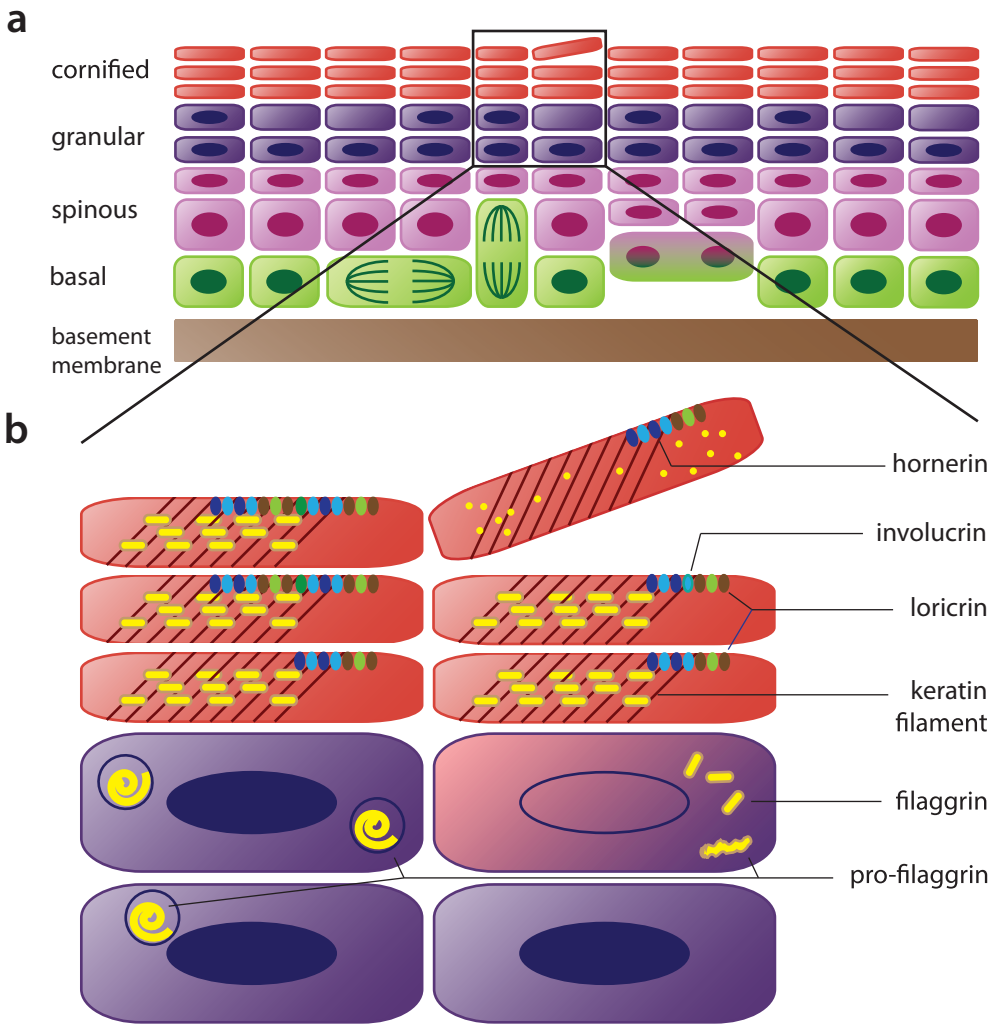

Figure 6. Key events and proteins in epidermal development. (**a**) The epidermis is maintained by mitotic keratinocytes of the basal layer. Three types of cell division are shown from left to right: symmetric division into two undifferentiated daughter cells, assymetric division into one basal cell and one spinous cell, and symmetric division into two differentiated daughter cells. As basal cells differentiate, they detach from the basal lamina and migrate superficially, forming the spinous layer, granular layer, and the cornified envelope. Dead corneocytes eventually slough off the skin surface. (**b**) Cornification begins in the granular layer, where cornified envelope proteins are synthesized. For example, profilaggrin is synthesized, packaged into keratohyalin granules, and proteolytically cleaved to yield FLG monomers. FLG monomers bind and organize keratin intermediate filaments into regular arrays, leading to cell flattening. In mature corneocytes, filaggrin is degraded into moisturizing peptides. Other cornified envelope proteins, including loricrin (LOR), involucrin (IVL), and hornerin (HNRN), are crosslinked by transglutaminase (TG1) to plasma membrane and extracellular lipids to produce a rigid structure.

periods of sudden and rapid growth. For example, bulge cells of the hair follicle do not participate in epidermal homeostasis, but migrate to the epidermis after wounding and give rise to functional epidermal keratinocytes (Ito et al., 2005).

The epidermis has long been recognized as a low O_2 environment. Initial measurements using O_2 electrodes, as well as recent radiological methods, have estimated that epidermal pO_2 lies between 0.5% and 5% O_2 (Evans and Naylor, 1967; Evans et al., 2006; Stewart et al., 1982; Varghese et al., 1986). This state of O_2 deprivation is exacerbated in wounds due to disrupted vasculature, as well as increased metabolic demand from proliferating keratinocytes and infiltrating cells. Acute hypoxia in this setting has been suggested to promote revascularization and re-epithelialization (Elson et al., 2000), whereas chronic hypoxia in a murine ischemic wound model delayed healing (Biswas et al., 2010). In comparison, few studies have evaluated the significance of low O_2 tension in epidermal homeostasis. Long-term hypoxic culture (7—14 days) reduced keratinocyte cell size and suppressed the expression of differentiation markers KRT1, KRT10, IVL, and LOR in keratinocyte cell lines (Ngo et al., 2007). Based on these findings, the authors argued that hypoxic conditions suppress keratinocyte terminal differentiation. Another rudimentary study showed that acute hypoxic treatment (12—48 hr) led to reversible morphological changes in suprabasal keratinocytes in organotypic culture and a temporary reduction in basal keratinocyte porliferatio (Straseski et al., 2009). However, the molecular mechanisms and functional importance of these O_2-dependent changes remain unaddressed.

Roles of HIF in the skin. Genetic models have indicated that epidermal HIF activity is involved in a wide range of functions. As discussed above, HIF1α expression in

the epidermis is important for cutaneous O_2 sensing (Boutin et al., 2008). Interestingly, HIF2α performs a similar function in *VHL* mutant epidermis, suggesting that while HIF1α and HIF2α may be functionally redundant, their relative abundance in the epidermis differs with *VHL* status (Boutin et al., 2008). HIF1α is also involved in epidermal immunological surveillance via the production of cathelicidin in keratinocytes *in vivo* (Peyssonnaux et al., 2008). Whereas wild type keratinocyte cultures controlled bacterial infection effectively, keratinocytes in which HIF1α was silenced did not mount a significant antibacterial response. Conversely, HIF1α overexpression in the epidermis enhanced dermal inflammatory cell infiltration at baseline and after cutaneous TPA challenge (Scortegagna et al., 2008). In this model, HIF1α overexpression correlated with increased IκB phosphorylation and NFκB activity. While hypoxic treatment inhibits PHD1 activity and thereby IKK hydroxylation in macrophages (Rius et al., 2008), HIF1α overexpression in keratinocytes activated NFκB directly via ERK1/2-dependent phosphorylation (Scortegagna et al., 2008). In a separate study, epidermal HIF1α overexpression promoted dermal hypervascularization, consistent with a well-characterized link between HIF1α and *VEGF* expression (Elson et al., 2001). To summarize, HIF1α activity in the epidermis is important in EPO regulation, innate immunity, wound healing, and angiogenesis.

HIF1α and HIF2α have also been implicated in skin diseases. Melanoma transformation by AKT requires HIF1α (Bedogni et al., 2005). Suppression of HIF1α activity by rapamycin treatment also reduced the growth of AKT-expressing melanoma xenografts (Bedogni et al., 2005). Upregulation of AKT, HIF1α, and HIF2α expression have also been reported in the epidermis of psoriatic skin (Rosenberger et al., 2007).

While it is known that AKT increases HIF1α expression (Zhong et al., 2000; Zundel et al., 2000), the function of elevated HIF1α and HIF2α in psoriatic disease remains unclear. Psoriatic patients treated with UV radiation are at increased risk for malignant melanoma (Stern et al., 1997). It is intriguing to speculate that elevated AKT and HIF pathway activity in irradiated melanocytes may play a role in melanoma development. These findings illustrate that HIF1α and HIF2α activity are involved in epidermal physiology as well as disease.

HIF-INDEPENDENT RESPONSES TO HYPOXIC STRESS

While HIFs play critical roles in the response to O_2 deprivation, hypoxic adaptation is an integration of multiple O_2 sensing pathways. Substantial evidence indicates that mTOR signaling and the unfolded protein response (UPR) play critical roles in hypoxic adaptations by modulating protein translation, cell metabolism, and cell fate (Wouters and Koritzinsky, 2008). Other transcriptional regulators, such as PGC1α, can also complement the HIF response in ischemic settings: PGC1α, independent of HIF, promotes VEGF expression and neo-angiogenesis in a model of hindlimb ischemia (Arany et al., 2008).

In addition, PHDs and FIH1 have non-HIFα substrates which may underlie some of their biological functions (Webb et al., 2009). For example, recent reports indicate that prolyl hydroxylases play significant HIF-independent roles in cancer. PHD2 suppresses growth of xenograft tumors in a HIF- and (surprisingly) hydroxylase-independent fashion (Chan et al., 2009). PHD1, on the other hand, promotes tumor growth through

HIF-independent regulation of cyclin D1 (Zhang et al., 2009). It is also important to note that all 2-oxoglutarate-dependent dioxygenases, including but not limited to PHDs, require O_2 for their enzymatic activity and therefore could potentially mediate HIF-independent responses to hypoxia. These observations emphasize that hypoxic adaptations are mediated by more than simply HIF activity. Hypoxia can influence many distinct pathways and control other response mechanisms besides PHD/pVHL/HIFα activity.

HIF-TARGETED THERAPIES

Many insights from HIF research are being translated into clinical applications, in particular drug discovery. The most advanced HIF pathway-targeted pharmaceuticals in terms of clinical development to date are PHD inhibitors. These compounds, FG-2216 and FG-4592, are being evaluated for treatment of anemia and are currently in Phase I and II clinical trials (ClinicalTrials.gov NCT00456053, NCT00761657, NCT00978198, and NCT00978198).

HIF activators. In addition to PHD inhibition, several strategies to promote HIF1α activity and angiogenesis are in development for use in treating ischemic disease. In models of hindlimb ischemia, adenoviral delivery of constituitively active HIF1α has shown efficacy when administered alone or in combination with bone-marrow derived angiogenic cells (Bosch-Marce et al., 2007; Rey et al., 2009). HIF1α adenoviral therapy has also shown benefit in limb ischemia models in aged and diabetic mice (Bosch-Marce et al., 2007; Sarkar et al., 2009). These findings are significant, given that two large patient

populations afflicted by atherosclerosis and associated ischemic diseases are diabetics and the elderly (Beckman et al., 2002).

Similar approaches with hybrid HIF1α/VP16 have been used in rabbit and diabetic rat models of limb ischemia and have progressed through Phase I and II clinical studies in patients with severe peripheral arterial disease (Kajiwara et al., 2009; Rajagopalan et al., 2007; Vincent et al., 2000). These interventions have also been applied to other ischemic injuries such as wound healing and myocardial infarction (Heinl-Green et al., 2005; Liu et al., 2008; Mace et al., 2007). In addition to gene therapy, PHD inhibitors have shown utility in wound healing in diabetic animals (Botusan et al., 2008). Overall, these findings indicate that HIF activating therapies are effective in animal models of ischemic disease and merit further clinical investigation.

HIF inhibitors. Transcription factors have historically been considered undruggable targets. However, interest in HIF inhibition as a therapeutic strategy remains high (Semenza, 2007). A high-throughput screen of FDA-approved drugs for anti-HIF activity revealed that digoxin and other cardiac glycosides inhibit *HIF1/2A* translation and subcutaneous xenograft growth (Zhang et al., 2008). Importantly, HIFα inhibition is independent of digoxin's known effect on the Na^+/K^+ ATPase, but necessary for its tumor suppressive effect (Zhang et al., 2008). Other HIF inhibitors identified in similar screens include the antiseptic dye acriflavine, and anthracyclines such as doxorubicin and daunorubicin (Lee et al., 2009a; Lee et al., 2009b). Screening for HIF inhibitors among approved agents means that identified compounds, although pharmacologically well studied and suitable for human use, can be presumed to exhibit HIF-independent effects stemming from their original therapeutic purposes. Therefore,

for these drugs to be used in targeted HIF therapy, it will be crucial to demonstrate that HIF repression is sufficient for their intended biological effects.

Another challenge in HIF targeting involves the overlapping but distinct biological roles of HIFα subunits. Compounds that promote the binding of IRP1 to the 5' UTR of *EPAS1* mRNA (encoding HIF2α) reduce hypoxic induction of HIF2α expression, but also repress HIF1α synthesis via an independent mechanism (Zimmer et al., 2008). An RNA antagonist of HIF1α, EZN-2968, reduces HIF1α protein and target gene expression *in vitro* and *in vivo*, but not that of HIF2α, and is being evaluated in a Phase I clinical trial (Greenberger et al., 2008; Patnaik et al., 2009). RNAi will likely play a key role in targeted HIF therapy once effective and selective delivery methods become available.

With the emerging view of the importance of HIF1α and HIF2α in disease, there is a largely unmet need for specific HIF inhibitors. While combined inhibition of HIFα isoforms will be appropriate in certain disease situations, HIF1α or HIF2α specific therapies may be preferable in other scenarios.

CONCLUSION

Hypoxic stress is characteristic of many pathological settings and developmental niches. HIFs direct critical adaptations to enable cells, tissues, and organisms to survive and thrive in these conditions. Recent work has revealed new mechanisms of HIF induction, including PHD-dependent and -independent modes of regulation, as well as novel consequences of HIF activity in development and disease. In some contexts, these

responses promote disease progression, while in others, HIFs promote disease recovery and tissue regeneration. Recent evidence has also highlighted both shared and disparate features between HIF1α- and HIF2α-mediated responses in cancer, tissue ischemia, and inflammatory disease. A deeper understanding of how HIFα isoforms are uniquely regulated, and how they can be selectively modulated, will be essential for translating our current knowledge of HIF biology to clinical use.

Chapter Two

MYC degradation under low O_2 tension promotes survival by evading hypoxia-induced cell death

SUMMARY

Cells encounter oxygen deprivation (hypoxia) in various physiological and pathological contexts. Adaptation to hypoxic stress in these cells occurs in part by suppressing MYC, a key regulator of cellular metabolism, proliferation, and survival. Hypoxia has been reported to inhibit MYC through multiple means, including disruption of MYC transcriptional complexes and decreased MYC protein abundance. In this chapter, I describe enhanced proteasomal degradation and cathepsin-mediated proteolysis as important mechanisms for hypoxic MYC inhibition in human colon carcinoma cells. MYC protein levels were similarly reduced in hypoxic primary keratinocytes. Increased MYC turnover at low O_2 tension was dependent on the E3 ubiquitin ligases FBXW7 and DDB1, as well as cytosolic cathepsins. Reduced MYC protein levels coincided with hypoxic inhibition of RNA polymerase III-dependent MYC target genes, which MYC regulates independently of its binding partner MAX. Finally, MYC overexpression in hypoxic cells promoted cell cycle progression, but also enhanced cell death via increased expression of the pro-apoptotic genes *NOXA* and *PUMA*. Collectively, these results indicate that hypoxic cells promote MYC degradation as an adaptive strategy to reduce proliferation, suppress biosynthetic processes, and promote cell survival under low O_2 stress.

INTRODUCTION

MYC is a proto-oncogene that is broadly expressed in proliferating tissues. Decades of study have revealed crucial roles for MYC in the promotion of cell division, ribosomal assembly, and anabolic metabolism in both normal and cancer cells (Dang, 2010). MYC family deregulation occurs in more than 40% of all cancers, including Burkitt's lymphoma, neuroblastoma, and multiple myeloma, and high levels of MYC activity are frequently a poor prognostic indicator (Vita and Henriksson, 2006; Wolfer et al., 2010). Multiple mechanisms contribute to MYC overexpression in tumors, such as chromosomal translocation, amplification, or stabilizing mutations. MYC activity is also regulated by growth factor signaling pathways, which are in turn influenced by micro-environmental factors such as nutrient or O_2 availability (Meyer and Penn, 2008).

One of the principal functions of MYC is to coordinate the expression of multiple proteins responsible for cell cycle progression. MYC activates the transcription of its targets—e.g. cyclin D2 (*CCND2*) and cyclin dependent kinase 4 (*CDK4*)—by binding to CACGTG (E-box) DNA sequences in association with its heterodimeric partner MAX (Meyer and Penn, 2008). MYC and MAX can also bind to and inactivate the transcription factors MIZ1 and SP1 at initiator elements, thus repressing transcription of CDK inhibitors *CDKN1A* and *CDKN2B* (Herkert and Eilers, 2010). MYC activity is negatively regulated by the MAD family of proteins, including MXD1 and MXI1, which competitively titrate MAX away from MYC (Meyer and Penn, 2008). However, MYC target gene transcription by RNA polymerase III (Pol III) does not require MAX, MXD1, or MXI1. MYC binds TFIIIB subunits TBP and BRF1 directly to enhance Pol III-dependent transcription of 5S ribosomal RNA (*RN5S*) and transfer RNA genes (Gomez-Roman et al.,

2003; Steiger et al., 2008). These effects on ribonucleic acid synthesis and cell cycle progression comprise a key mechanism whereby MYC coordinates cell growth and proliferation.

Elevated MYC activity can commit cells to bioenergetic and synthetic demands that exceed available nutrient supplies (Dang, 2010). In this context, cell death resulting from hyperactive MYC can be a tumor suppressive response to prevent unrestrained tumor growth. MYC-induced apoptosis involves a number of effector molecules, including the tumor suppressors ARF and p53. For example, MYC-dependent induction of *ARF* stabilizes p53 by inhibiting its negative regulator MDM2 (Weber et al., 1999; Zindy et al., 1998). Stabilized p53 in turn stimulates the expression of pro-apoptotic proteins NOXA and PUMA, resulting in activation of the downstream effector BAX (Nakano and Vousden, 2001; Oda et al., 2000). MYC can also induce cell death independently of p53, for example by directly regulating the expression of *NOXA* and other apoptotic genes (Boone et al., 2011; Nikiforov et al., 2007). Importantly, elevated MYC activity sensitizes cells to numerous apoptotic stimuli, including TNF-α death receptor signaling, DNA damage, and O_2 and nutrient stress (Brunelle et al., 2004; Evan et al., 1992; Klefstrom et al., 1994; Shim et al., 1998).

To circumvent MYC-induced cell death under conditions of nutrient and growth factor deprivation, some cells can reduce their metabolic and proliferative requirements by downregulating MYC activity. In particular, MYC protein expression and activity can be modulated by nutrient and growth factor-responsive signal transduction pathways. For example, inhibition of RAS signaling reduces MYC stability via changes in MYC phosphorylation and subsequent FBXW7-dependent ubiquitylation and proteolysis

(Sears et al., 1999). Similarly, activation of SIRT1, a sensor of cellular metabolic state, leads to MYC deacetylation and degradation (Yuan et al., 2009). MYC also undergoes cytoplasmic proteolysis by calpains during myoblast differentiation (Conacci-Sorrell et al., 2010). Therefore, MYC abundance and activity responds to fluctuations in nutrient and growth conditions, including changes in O_2 tension.

O_2 is often in limited supply in solid tumors because of defective and inadequate vascularization in the context of rapid cell division (Carmeliet and Jain, 2011). In its absence, cells are unable to generate ATP via oxidative phosphorylation and must undergo metabolic adaptations in order to survive. Many of these adaptations are mediated by the stabilization of hypoxia inducible factors HIF1α and HIF2α, which activate transcription of genes encoding angiogenic, hematopoietic, and metabolic effectors (Majmundar et al., 2010). HIF induction in hypoxic cells suppresses oxidative phosphorylation and promotes nonoxidative forms of ATP production, such as glycolysis (Denko, 2008). Concurrently, HIF-dependent angio-genesis improves O_2 delivery. Because these adaptive changes require time, hypoxic cells decrease energy consumption by reducing proliferation, mitochondrial metabolism, and DNA replication and repair, often by inhibiting MYC activity (Gordan et al., 2007; Gordan et al., 2008; Koshiji et al., 2004; Zhang et al., 2007).

Hypoxic inhibition of MYC largely occurs via HIF-dependent effects on MYC-interacting proteins. For example, HIF1α directly induces *MXI1* expression to inhibit MYC-dependent mitochondrial biogenesis and O_2 consumption (Corn et al., 2005; Zhang et al., 2007). At the protein level, HIF1α competes with MYC for binding to SP1 at the promoters of MYC target genes such as *MSH2*, *MSH6*, and *NBS1*, which encode DNA

repair proteins. MYC displacement from these promoters represses gene expression and causes genomic instability in tumor cells (Koshiji et al., 2004; Koshiji et al., 2005). This effect is exclusive to HIF1α, as HIF2α fails to bind SP1 in the same manner (To et al., 2006). HIF1α has also been reported to bind to MAX and disrupt MYC-MAX complexes, leading to reduced *CCND2* expression, induction of *CDKN1A*, and G1-phase arrest (Gordan et al., 2007). However, it is unclear whether hypoxia affects important MYC activities that operate independently of MYC-interacting proteins (e.g. MAX, MXI1, MIZ1, SP1), especially transcription of MYC target genes by Pol III. Furthermore, the diversity of MYC-dependent cellular processes suggests that the biological consequences of MYC suppression under low O_2 tension remain to be fully elucidated.

Here we investigate the mechanisms and consequences of MYC suppression in hypoxic adaptation. We show that hypoxic stress decreases MYC protein stability and transcriptional activity in normal and cancer cells. Hypoxia represses not only MYC target genes transcribed by RNA polymerase II (Pol II), such as *CCND2* and *MCM5*, but also Pol III-dependent MYC targets encoding ribosomal and transfer RNAs. MYC suppression is independent of changes in transcription and mRNA translation rates, but results from increased degradation under hypoxic conditions. Hypoxia-induced MYC degradation requires proteasomal activity via the E3 ligases FBXW7 and DDB1, as well as cathepsin-mediated proteolysis. Forced expression of a stabilized MYC variant in hypoxic cells increases *NOXA* expression and enhances hypoxia-induced cell death. Therefore, we propose that MYC suppression under hypoxia is an adaptive response that promotes cell survival in low O_2 conditions.

MATERIALS AND METHODS

Cell lines and cell culture. HCT116, DLD1, and REF52 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum at 37°C, 5% CO_2. HCT116-Dicer^{ex5}, HCT116 *FBXW7*-/-, and DLD1 *FBXW7*-/- cells were gifts of Dr Bert Vogelstein. REF52 rat fibroblasts were a gift of Dr Tatyana Svitkina. Primary keratinocytes were isolated from neonatal mice as described in (Lichti et al., 2008) and cultured in complete MCDB 153 media (Sigma) with 45 μM calcium chloride at 34°C, 8% CO_2. To generate HCT116-MYC^{HAM}, HCT116-MYC^{T58A}, REF52-MYC^{HAM}, and REF52-MYC^{T58A} cells, cells were transduced with retroviral pMSCV-*HA-MYC^{HAM}* or pMSCV-*HA-MYC^{T58A}* vector (Addgene) and selected with puromycin (Sigma). For hypoxia treatment, cells were cultured in an InVivo2 400 hypoxia workstation (Ruskinn) at 0.5% O_2. For proliferation studies, $3x10^4$ cells were seeded in duplicates in 60 mm tissue culture dishes and grown under normoxic or hypoxic conditions for 1-6 days. Cell number was counted using a hemacytometer.

Proteasome and protease inhibitors were purchased from Calbiochem and used at the following concentrations: MG132, 25 μM; lactacystin, 25 μM; cathepsin inhibitor I (CAT I), 10 μM; cathepsin inhibitor III (CAT III), 10 μM; calpastatin, 10 μM; chloroquine, 20 μM.

Plasmid construction. Total RNA from HCT116 cells was reverse transcribed as described below. Specific primers were used to amplify full length *MXD1*, *MXI1A*, and *MXI1C* cDNA with 5' and 3' restriction sites for HindIII and XbaI respectively (primer sequences available on request). PCR products were purified using QIAquick gel

extraction kit (Qiagen) and cloned into pcDNA3.1 vector (Invitrogen) for sequence analysis and transient expression.

Lentiviral transduction. To create stable cell lines in which HIF1α and HIF2α were silenced, HCT116 cells were transducted with lentivirus encoding scrambled, *HIF1A*, or *HIF2A* short hairpins (Openbiosystems) and selected with puromycin (Sigma).

Transient transfection. *MXD1*, *MXI1A*, *MXI1C*, *HIF1A-FLAG*, and *HIF2A-FLAG* were transiently expressed in HEK293T cells using Lipofectamine2000 (Invitrogen) and cell lysates prepared after 48 h. 6xHis-tagged and FLAG-tagged ubiquitin were overexpressed in HCT116 cells using Lipofectamine2000. For knockdown studies, siRNA targeting *CUL4A*, *CUL4B*, *DDB1*, *FBXW7*, *HIF1A*, *HIF2A*, *MYC*, *MXD1*, *MXI1*, *NOXA, PUMA, TRPC4AP*, *UBR5*, and scrambled control (Qiagen) or siRNA against *MIR34B*, *MIR34C*, and scrambled control (Dharmacon) were transfected into cells at 50 nM concentration using HiPerFect (Qiagen) or DharmaFECT 2 (Dharmacon) respectively. Hypoxic treatment was initiated 24 h after transfection and maintained for a further 24 h unless otherwise specified.

Flow cytometry. For cell cycle analysis, HCT116 cells were pulsed with 10 μM BrdU for 20 min after 24 h culture in appropriate conditions. Cells were stained with Alexa Fluor 488 anti-BrdU (Invitrogen) and propidium iodide for analysis in a FACScalibur flow cytometer (Becton Dickinson). Cell viability was measured using Annexin V-FITC apoptosis detection kit (BD Pharmingen) and analyzed by flow cytometry.

Quantitative RT-PCR. Total RNA was extracted with Trizol reagent (Invitrogen) and cDNA produced using High Capacity RNA-to-cDNA kit (Applied Biosystems).

Analysis of gene expression was performed in a 7900HT Sequencer (Applied Biosystems) using specific primers (sequences available on request). Expression levels were normalized to levels of *HPRT1*.

Chromatin immunoprecipitation (ChIP). ChIP was performed as described (Forsberg et al., 2000). Cells were crosslinked in 1% formaldehyde for 10 min. Precleared chromatin was immunoprecipitated with anti-MYC N262 antibody (Santa Cruz) and protein A agarose beads (Roche) for 16 h at 4°C. Immunoprecipitated and total DNA were quantified in a 7900HT Sequencer using specific primers (sequences available on request).

Immunoprecipitation (IP). Cells were lysed in 25 mM Tris pH 8.0, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 1% NP-40 containing Complete Mini Protease Inhibitor Cocktail (Roche), 100 mM N-ethylmaleimide (NEM; Sigma), and 200 μM deferoxamine (DFO; Sigma). Precleared lysates were immunoprecipitated in lysis buffer with anti-MYC N262 or anti-MAX H2 (Santa Cruz), or anti-FLAG M2 (Sigma) antibody.

Western blotting. 20-50 μg of total protein extract were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels. Antibodies used for western blotting include anti-FLAG M2-HRP (Sigma), anti-MYC C33, anti-MYC N262, anti-MYC 9E10, anti-phospho-T58/S62-MYC E203 (Epitomics), anti-MAX C17, anti-MAX H2, anti-HIF1α C-Term (Cayman), anti-HIF2α NB100-122 (Novus), anti-HA (Roche), anti-cyclin E M20, anti-β-actin (Sigma), and anti-ARNT H172 (all antibodies from Santa Cruz unless otherwise noted). Optical densities were quantified using ImageJ software (NIH).

Radiolabeling. HCT116 cells were starved of methionine and cysteine for 2 h. Labeling was carried out for 1 h in DMEM without methionine or cysteine supplemented

with 10% dialyzed fetal calf serum and 100 μCi/mL of [^{35}S]methionine/cysteine. As a negative control, cells were treated with 100 μg/mL cycloheximide (CHX; Calbiochem). 0.5—2 mg of total protein extract was used for immunoprecipitation with anti-MYC N262 antibody or rabbit pre-immune serum. Bound proteins were eluted with 2x sample buffer, separated by SDS-PAGE, and visualized by autoradiography. Phosphorescence was quantified using Storm 820 Molecular Imager (Molecular Devices) and ImageQuant software (GE).

Statistical analysis. Data are presented as mean+SEM. Differences between groups were analyzed for significance using Student's t test, with $p<0.05$ considered to be significant.

RESULTS

MYC transcriptional activity is reduced under hypoxia. To assess the extent of hypoxic effects on MYC dependent gene transcription, we cultured HCT116 colon carcinoma cells in hypoxia (0.5% O_2) for 24 h. Quantitative RT-PCR (qRT-PCR) analysis revealed that hypoxia-inducible genes *PGK1* (encoding phosphoglycerate kinase 1), *MXD1* (MAX dimerization protein 1), and *MXI1* (MAX interactor 1) were robustly induced (**Fig. 7a**). Hypoxia also inhibited the expression of MYC target genes involved in cell cycle progression (*CCND2*) and DNA replication (*MCM5,* minichromosome maintenance deficient 5; *MCM7*, minichromo-some maintenance deficient 7) (**Fig. 7a**). Conversely, expression of the MYC-repressed gene *CDKN1A* (p21 cyclin dependent kinase inhibitor

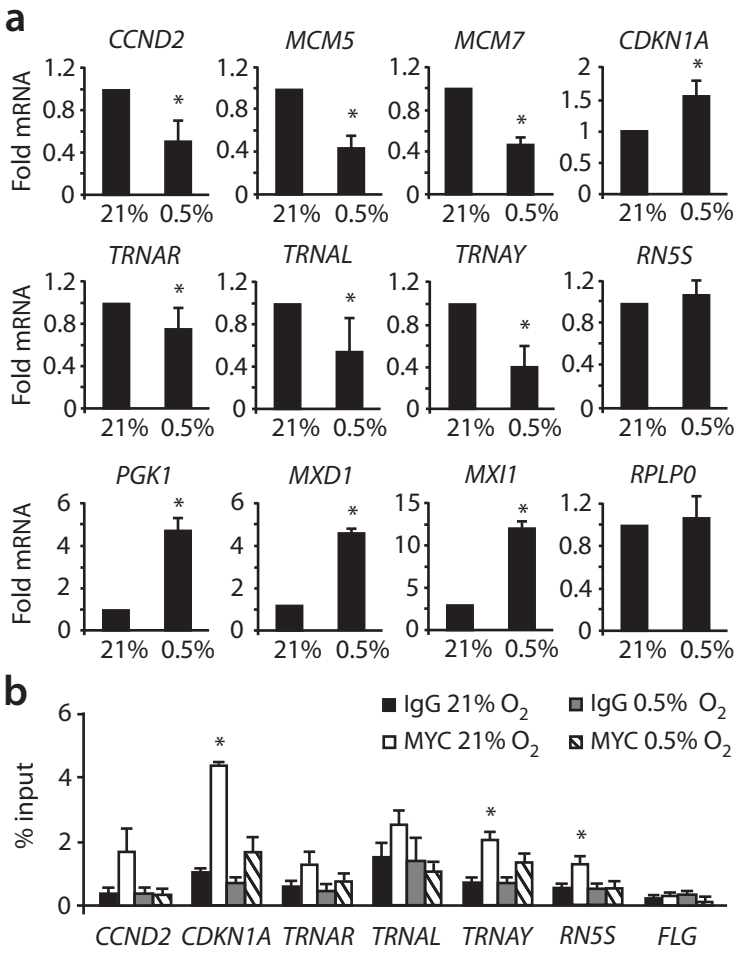

Figure 7. Hypoxia downregulates MYC-dependent transcription. (**a**) HCT116 cells were grown at 21% or 0.5% O_2 for 24 h and expression of MYC-induced (*CCND2*, *MCM5*, *MCM7*, *RN5S*, *TRNAR*, *TRNAL*, *TRNAY*) or suppressed genes (*CDKN1A*) determined using qRT-PCR. HIF target genes (*PGK1*, *MXD1*, *MXI1*) and *RPLP0* serve as positive and negative controls respectively. While *RN5S* expression was unchanged under hypoxia, lower levels of *TRNAR*, *TRNAL*, and *TRANY* were statistically significant. Mean results from three independent experiments are shown. *, $p<0.05$. (**b**) MYC occupancy at target gene promoters was determined by chromatin immunoprecipitation after 24 h growth at 21% or 0.5% O_2. Filaggrin (FLG) promoter does not contain MYC binding sites and is used as a negative control. Mean results from two independent experiments are shown. *, $p<0.05$.

1A) was increased, consistent with prior reports (Gordan et al., 2007; Koshiji et al., 2004) (**Fig. 7a**).

To determine whether MYC-induced genes transcribed by Pol III were also repressed by hypoxic treatment, we examined the expression of MYC target genes involved in protein translation. Transcript levels of *TRNAR* (transfer RNA arginine), *TRNAL* (transfer RNA leucine), and *TRNAY* (transfer RNA tyrosine) were also decreased under low O_2 tension (**Fig. 7a**). In contrast, expression of *RPLP0* (ribosomal protein large P0), which is not a MYC target gene, was unchanged by hypoxia (**Fig. 7a**). Chromatin immunoprecipitation assay demonstrated that the reduction in MYC-dependent gene transcription was accompanied by decreased MYC occupancy at target gene promoters (**Fig. 7b**). These results demonstrate that O_2 limitation not only antagonizes the expression of MYC target genes transcribed by Pol II, but also represses Pol III-dependent MYC target gene expression. In this way, hypoxic inhibition of growth, ribosome assembly, and protein synthesis are coordinated via MYC suppression.

Hypoxia reduces MYC protein expression. Suppression of MYC activity in hypoxic cells could be mediated by disruption of MYC-MAX complexes, induction of the negative regulator *MXI1*, or degradation of MYC protein (Corn et al., 2005; Gordan et al., 2007; Li et al., 2009b). Consistent with previous reports (Gordan et al., 2007; Qing et al., 2010), MYC association with its positive binding partner MAX decreased after 24 h of hypoxia (**Fig. 8a**). While the loss of MYC-MAX heterodimers was expected to inhibit Pol II-dependent transcription of MYC target genes, it could not account for the observed reduction in Pol III-dependent MYC target gene transcription. Western blot analysis showed that whereas MAX protein expression remained constant, MYC protein levels

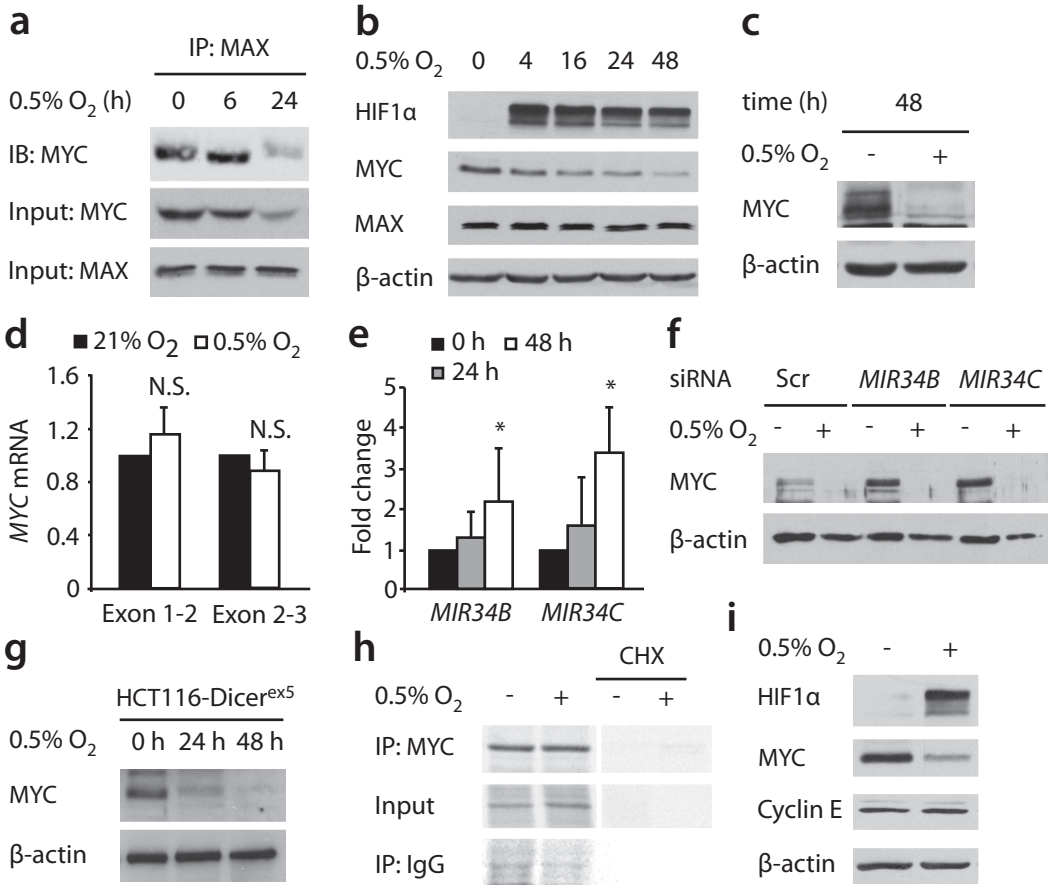

Figure 8. Hypoxic MYC downregulation is independent of transcriptional and translational changes. (**a**) MYC was co-immunoprecipitated with MAX from HCT116 cells grown at 21% or 0.5% O_2 for 6 or 24 h. (**b**) HCT116 cells were grown at 0.5% O_2 for the indicated times and lysates immunoblotted for MYC. (**c**) Primary human keratinocytes were grown at 21% or 0.5% O_2 for 48 h and lysates immunoblotted for MYC. (**d**) HCT116 cells were grown at 21% or 0.5% O_2 for 24 h and MYC transcript levels measured using qRT-PCR. Mean results from three independent experiments are shown. N.S., not significant. (**e**) HCT116 cells were grown at 0.5% O_2 for the indicated times and expression of *MIR34B* and *MIR34C* determined using qRT-PCR. Mean results from two independent experiments are shown. *, $p<0.05$. (**f**) HCT116 cells were transfected with scrambled control (Scr) or siRNA targeting *MIR34B* or *MIR34C* and lysates immunoblotted for MYC protein. (**g**) HCT116-Dicer^{ex5} cells were grown at 0.5% O_2 for the indicated times and cell lysates immunoblotted for MYC protein. (**h**) HCT116 cells were grown at 21% or 0.5% O_2 for 24 h and pulsed with [^{35}S]Cys/Met for 1 h. Newly translated MYC protein was immunoprecipitated and visualized by autoradiography. (**i**) Hypoxic MYC levels were contrasted with another short-lived protein, cyclin E, by western blotting.

were decreased in hypoxic HCT116 cells and primary keratinocytes (**Figs. 8b—c** and data not shown). Taken together, these results suggest that hypoxia reduces MYC protein abundance and thus represses MYC target gene transcription by both Pol II and Pol III. Furthermore, decreased MYC protein abundance may be a general response to hypoxia in both malignant and untransformed cells.

Hypoxic MYC suppression is independent of transcription and translation. To determine which regulatory steps in MYC expression are O_2-responsive, we used qRT-PCR to measure *MYC* mRNA levels. *MYC* mRNA abundance was maintained after the same duration of hypoxic exposure that reduced MYC target gene expression (**Fig. 8c**). Because *MYC* mRNA translation is repressed by microRNA-34B/C (*MIR34B/C*) induction in response to DNA damage (Kress et al., 2011), we investigated the possibility that microRNAs regulate MYC expression during O_2 deprivation. Interestingly, hypoxia induced *MIR34B* and *MIR34C* expression (**Fig. 8d**). However, RNAi-mediated inhibition of *MIR34B* or *MIR34C* failed to prevent MYC suppression under hypoxia (**Fig. 8e**). MYC protein was also suppressed by hypoxia in both control and *DICER1* mutant HCT116 cells, which have reduced expression of most mature microRNAs (Cummins et al., 2006) (**Fig. 8f**).

To examine *MYC* mRNA translation under hypoxia directly, HCT116 cells were pulse-labeled with ^{35}S-cysteine/methionine to evaluate new protein synthesis. The amount of radiolabeled protein in anti-MYC immunoprecipitates was unchanged in normoxic (21% O_2) versus hypoxic (0.5% O_2) cells, demonstrating that hypoxia does not inhibit MYC protein synthesis (**Fig. 8g**). These results indicate that hypoxia decreases

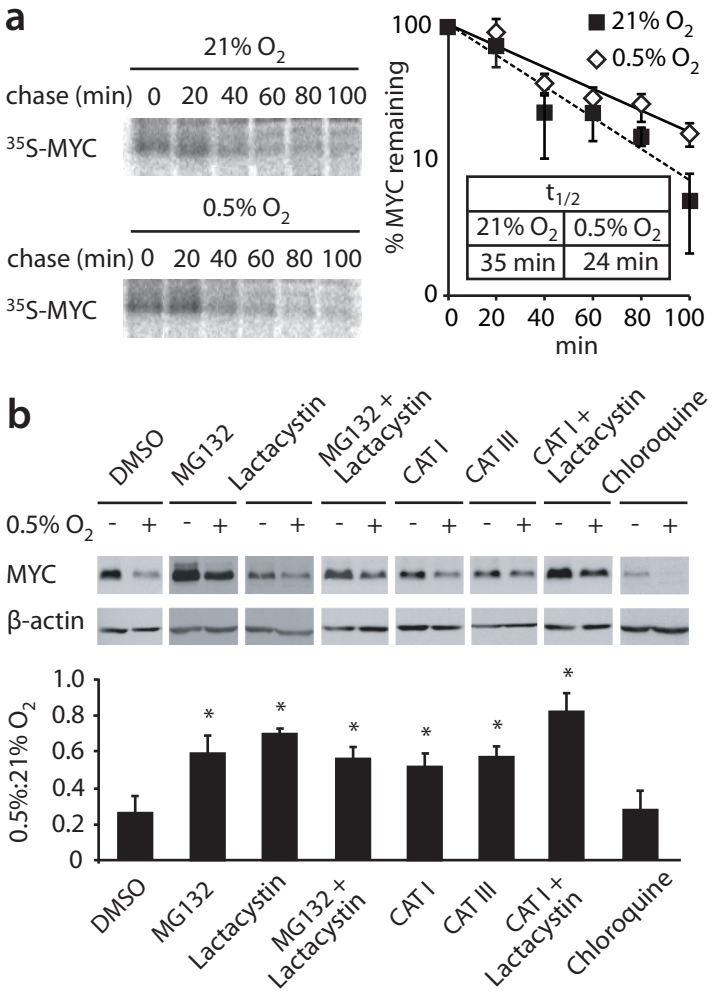

Figure 9. Hypoxia promotes MYC degradation via proteasomal- and protease-dependent mechanisms. (**a**) HCT116 cells grown at 21% or 0.5% O_2 for 24 h were pulsed with [35S]Cys/Met and lysates obtained during subsequent chase periods as indicated. MYC protein was immunoprecipitated and visualized by autoradiography. Average densitometric values and half life measurements from two independent experiments are shown. (**b**) HCT116 cells grown at 21% or 0.5% O_2 for 44 h were treated with various chemical inhibitors for 2 h and lysates immunoblotted for MYC protein (top panel) and quantified. Densitometric quantifications are presented as the mean of two independent experiments (bottom panel). *, $p<0.05$.

MYC protein abundance in a manner that is independent of transcriptional and translational effects, including regulation by microRNAs.

Hypoxia decreases MYC protein stability. We next measured the effect of O_2 deprivation on MYC protein turnover. MYC protein is highly unstable, with a reported half-life of 20 to 30 min (Hann and Eisenman, 1984; Waters et al., 1991). Pulse chase analysis indicated that hypoxic treatment reduced the half-life of MYC protein from 35 min to 24 min (**Fig. 9a**). To study the mechanism for increased MYC degradation under hypoxia, we used chemical compounds to inhibit different components of the protein degradation machinery. Treatment of hypoxic cells with proteasomal inhibitors MG132, lactacystin, or both partly restored MYC protein levels (**Fig. 9b**). Because previous studies suggested a role for cytoplasmic proteases in normoxic MYC turnover, non-proteasomal contributions to hypoxic MYC turnover were evaluated using cathepsin inhibitors I and III (inhibits cathepsins B, L, S) and chloroquine (inhibits lysosomal proteases). While chloroquine treatment had no effect on MYC expression under hypoxic conditions, cathepsin inhibition significantly restored hypoxic MYC levels (**Fig. 9b**). Furthermore, inhibition of either cathepsins (by CATIII) or calpains (by calpastatin) extended MYC half-life at both 21% and 0.5% O_2 (**Figs. 10a—b**). Importantly, CATIII or calpastatin treatment abolished the difference in stability between normoxic and hypoxic MYC protein (**Figs. 10a—b**).

Hypoxic induction of cathepsin expression. Interestingly, the requirement of cathepsin activity for hypoxic MYC degradation correlated with hypoxic gene induction of cathepsins B, D, K, and S (**Fig. 11a**). Cathepsins B, K, and S are cysteine cathepsins, whereas cathepsin D belongs to the family of aspartic cathepsins. Cathepsin B expression

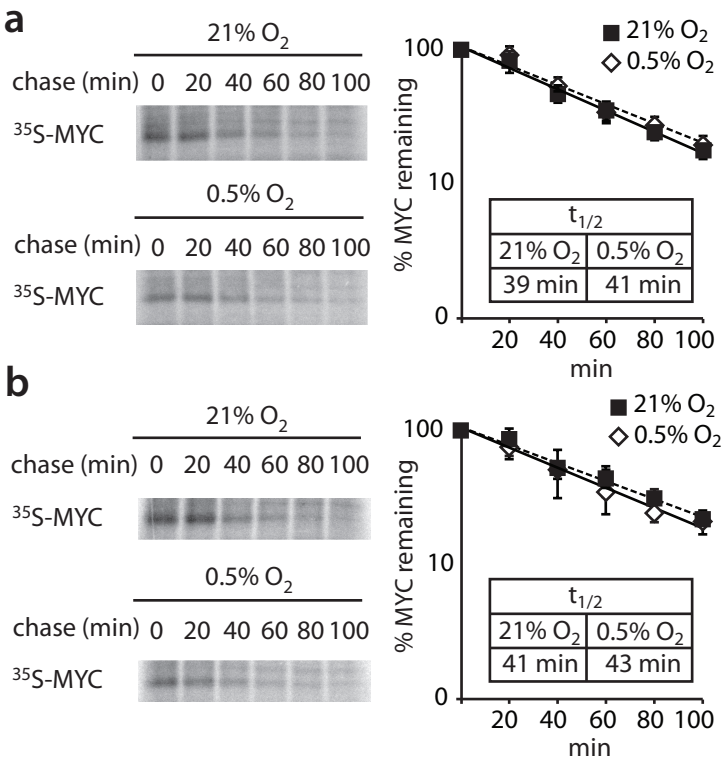

Figure 10. Hypoxic MYC degradation requires cathepsin and calpain activity. (**a**) HCT116 cells grown at 21% or 0.5% O_2 for 44 h were treated with various chemical inhibitors for 2 h and lysates immunoblotted for MYC protein (top panel) and quantified. Densitometric quantifications are presented as the mean of two independent experiments (bottom panel). *, $p<0.05$. (**b**) HCT116 cells grown at 21% or 0.5% O_2 for 24 h were pulsed with [^{35}S]Cys/Met in the presence of cathepsin inhibitor III (**a**) or calpastatin (**b**) and lysates obtained during subsequent chase periods as indicated in the presence of inhibitor. MYC protein was immunoprecipitated and visualized by autoradiography. Average densitometric values and half life measurements from two independent experiments are shown.

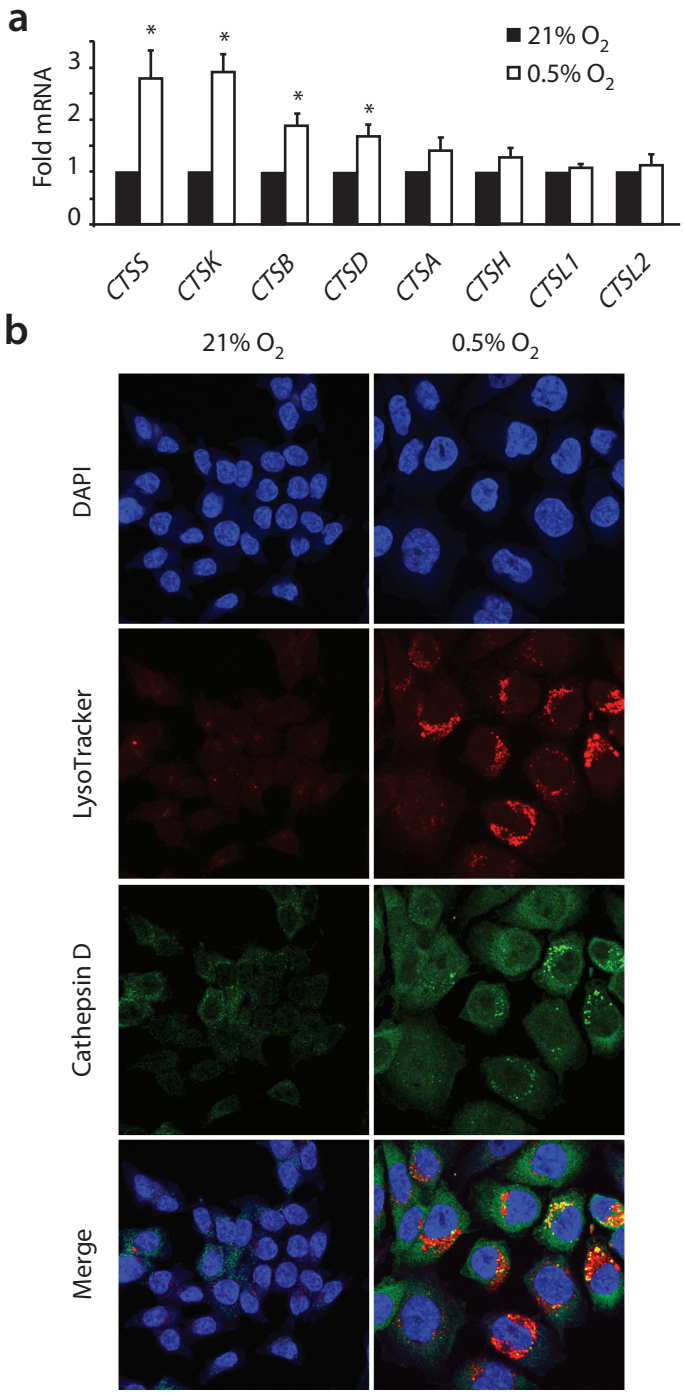

Figure 11. Hypoxia promotes cathepsin gene expression. (**a**) HCT116 cells were grown at 21% or 0.5% O_2 for 24 h and expression of cathepsin genes determined using qRT-PCR. Mean results from four independent experiments are shown. *, $p<0.05$. (**b**) HCT116 cells were grown at 21% or 0.5% O_2 for 24 h and expression of cathepsin D determined by immunofluorescence.

in hypoxic cells co-localized with the lysosomal marker LysoTracker (**Fig. 11b**). Taken together, these data suggest that low O_2 tension promotes MYC degradation via both proteasome-dependent and cathepsin-dependent mechanisms.

MXD1 and MXI1 do not regulate MYC protein abundance. Although O_2 limitation is known to induce the expression of MYC antagonists (Corn et al., 2005; Tsao et al., 2008), overexpression of MXD1 or MXI1 was insufficient to cause MYC protein downregulation in normoxic cells (**Fig. 12a**). In the converse experiment, in which *MXD1* or *MXI1* was silenced using RNAi (**Fig. 12b**), MYC protein levels were unaffected (data not shown). However, *MXI1* knockdown resulted in transcriptional de-repression of most MYC target genes (**Fig. 12c**). These observations are consistent with a role for MXD1 and MXI1 in regulating MYC activity, but not MYC protein abundance. Collectively, these experiments suggest that MYC protein is specifically targeted for proteasome-dependent and independent degradation in hypoxic conditions.

MYC phosphorylation at Thr58 and Ser62 are unchanged under hypoxia. We further examined the proteasomal regulation of MYC in response to hypoxia. MYC degradation via the ubiquitin-proteasome pathway is triggered by MYC phosphorylation by ERK and GSK-3 on amino acid residues Ser62 and Thr58 (Sears et al., 2000). These phosphorylation events promote MYC recognition by its E3 ubiquitin ligase FBXW7, leading to polyubiquitylation and subsequent degradation (Sears et al., 2000). Although MYC degradation is increased under hypoxia, we did not observe the expected increase in MYC phosphorylation on Ser62 and Thr58 (Corn et al., 2005; Li et al., 2009b) (**Figs. 13a—b**). Antibody specificity against phospho-T58/S62-MYC was verified using RNAi-mediated *MYC* knockdown and treatment of cell lysates with λ protein phosphatase (**Figs.**

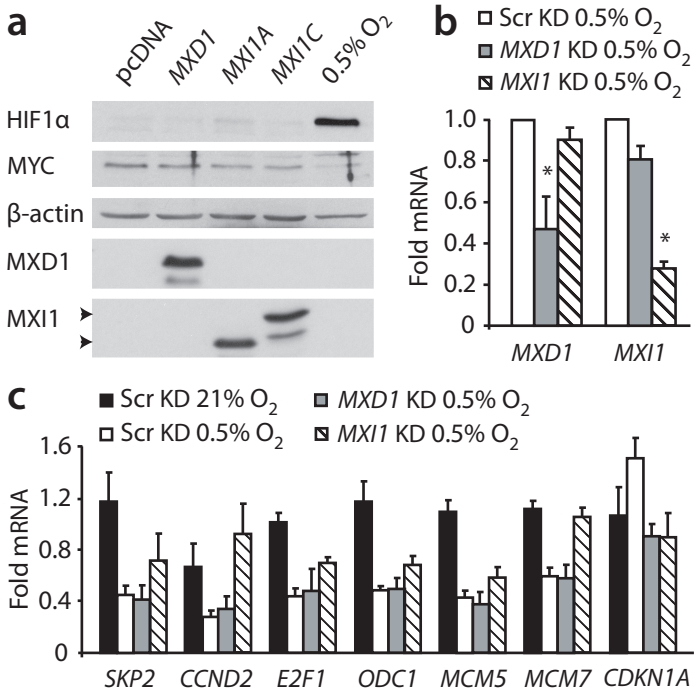

Figure 12. MAD proteins regulate hypoxic MYC activity, but not MYC protein levels under hypoxia. (**a**) HEK293T cells were transfected with empty vector or DNA encoding MXD1, MXI1A, or MXI1C, and lysates immunoblotted for endogenous MYC and overexpressed proteins. (b, c) HCT116 cells were transfected with scrambled control (Scr) or siRNA targeting *MXD1* or *MXI1* and qRT-PCR performed on RNA extract to evaluate knockdown efficiency (**b**) and MYC target gene expression (**c**). Mean values from two independent experiments are presented. *, $p<0.05$.

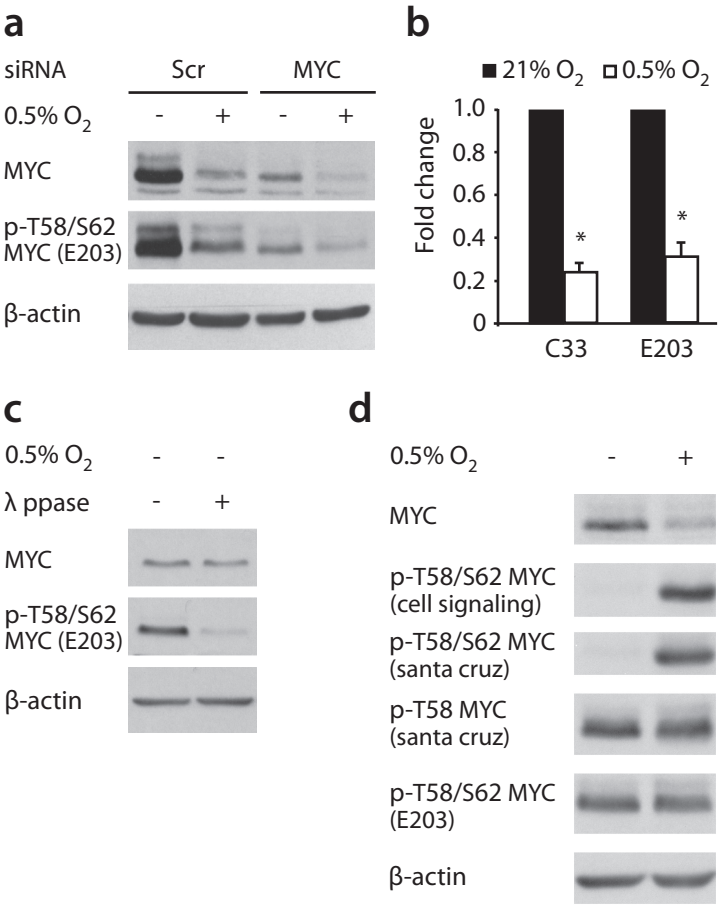

Figure 13. MYC phosphorylation at Thr58/Ser62 does not change under hypoxia. (**a**) HCT116 cells were transfected with scrambled control (Scr) or siRNA targeting MYC and lysates immunoblotted using total MYC (C33) or phospho-T58/S62-MYC (E203) antibodies. (**b**) Densitometric quantifications are presented as the mean of two independent experiments. *, $p<0.05$. (**c**) HCT116 cell lysates were treated with λ protein phosphatase and immunoblotted for total MYC or phospho-T58/S62-MYC. (**d**) HCT116 cells were grown at 21% and 0.5% O_2 and cell lyates immunoblotted for MYC and phospho-MYC using various antibodies. Only phospho-T58/S62-MYC (E203) exhibited specificity for phospho-MYC as shown in (**a**) and (**c**).

13a and **13c**). We also found that different commercially available antibodies exhibit varying degrees of specificity towards phospho-T58/S62-MYC (**Fig. 13d**).

MYC ubiquitylation is increased under hypoxic conditions. Despite the absence of changes in MYC phosphorylation, we found that hypoxic treatment increased MYC ubiquitylation. FLAG-tagged ubiquitin was transiently expressed in HCT116 cells and MYC protein immunoprecipitated from cell lysates. MYC was more highly ubiquitylated in hypoxic cells compared to normoxic control (**Fig. 14a**). These results indicate that hypoxia promotes MYC polyubiquitylation, and subsequently its proteasomal degradation, in a manner that is independent of changes in MYC phosphorylation on Thr58 and Ser62.

Hypoxic MYC degradation requires FBXW7. Multiple E3 ubiquitin ligases for MYC have been identified, including SKP2, FBXW7, HUWE1, BTRC, TRPC4AP, and DDB1 (Adhikary et al., 2005; Choi et al., 2010; Kim et al., 2003; Popov et al., 2010; von der Lehr et al., 2003; Yada et al., 2004). The activity of these E3 ligases towards MYC are regulated by distinct upstream signaling events. For example, MYC phosphorylation on Thr58 and Ser62 is a prerequisite for FBXW7-dependent ubiquitylation; however, MYC mutants lacking phosphorylation sites at amino acid residues 58 and 62 can still be ubiquitylated and degraded (Kim et al., 2003). To assess the role of FBXW7 in hypoxic MYC degradation, we made use of colorectal cancer cell lines in which *FBXW7* had been deleted (Rajagopalan et al., 2004). Similar to proteasomal inhibition by MG132, *FBXW7* deletion partially (but significantly) restored MYC levels in hypoxic colorectal cancer cells (**Figs. 14b—c**). Pulse chase analysis in *FBXW7* deleted cells indicated that MYC protein was stabilized in both normoxic and hypoxic conditions in the absence of *FBXW7*: MYC

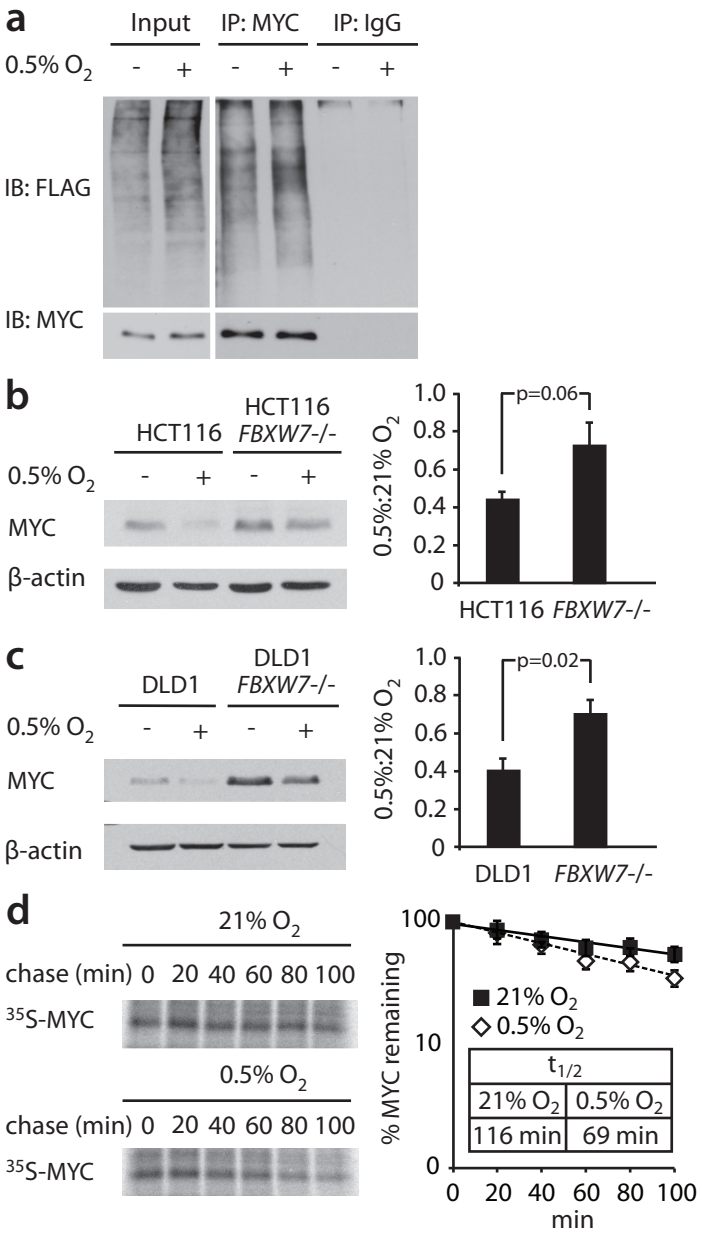

Figure 14. MYC downregulation under hypoxia requires FBXW7. (**a**) HCT116 cells were transfected with DNA encoding FLAG-tagged ubiquitin and the abundance of FLAG-tagged species in cell lysate, grown at 21% or 0.5% O_2 for 16 h, and anti-MYC immunoprecipitate determined. (**b**, **c**) Wild type and *FBXW7* deficient HCT116 (**b**) or DLD1 (**c**) cells were grown at 21% or 0.5% O_2 for 48 h and lysates immunoblotted for MYC protein (left panel). Densitometric quantifications are presented as the mean of at least two independent experiments (right panel). (**d**) *FBXW7* deficient HCT116 cells grown at 21% or 0.5% O_2 for 24 h were pulsed with [^{35}S]-Cys/Met and lysates obtained at the indicated times during the subsequent chase period. MYC protein was immunoprecipitated and visualized by autoradiography. Mean densitometric values and half life measurements from two independent experiments are shown.

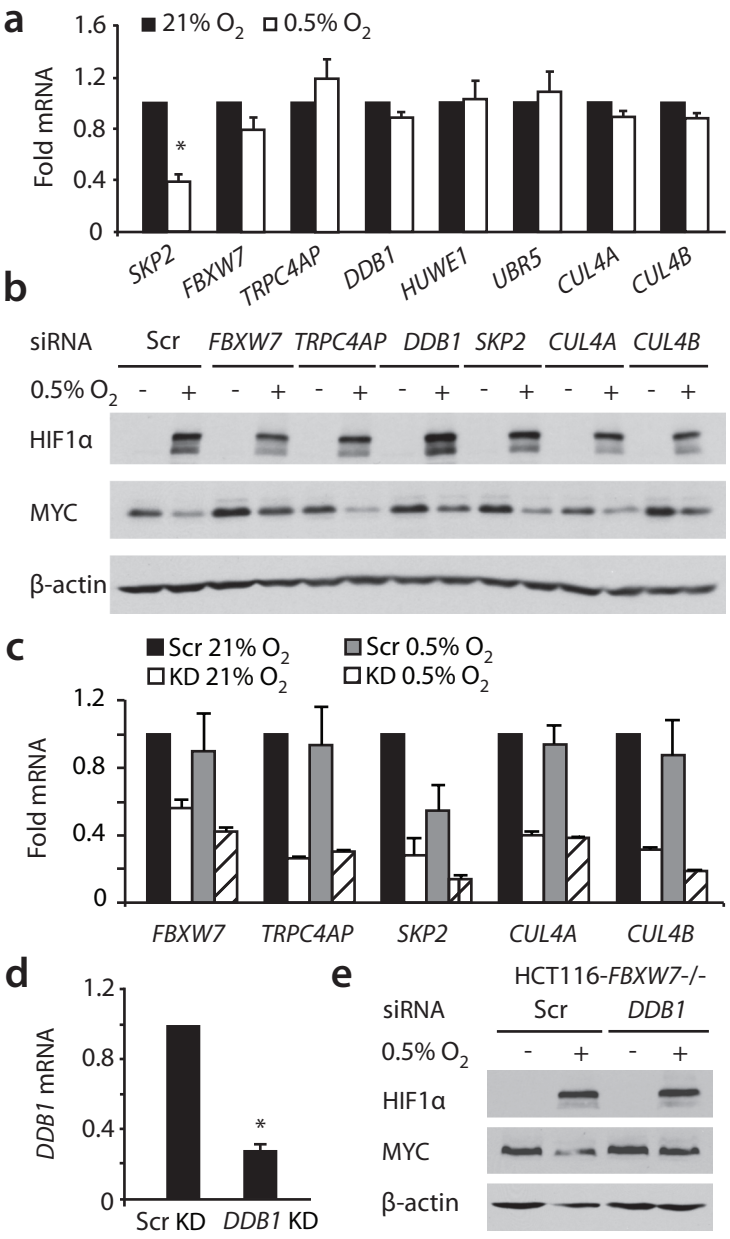

Figure 15. MYC downregulation under hypoxia requires DDB1. (**a**) HCT116 cells were grown at 21% or 0.5% O_2 for 24 h and the expression of known MYC E3 ubiquitin ligases determined using qRT-PCR. (**b**) HCT116 cells were transfected with scrambled control (Scr) or siRNA targeting various MYC E3 ligases, grown at 21% or 0.5% O_2 for 24 hr and lysates immunoblotted for MYC protein. (**c**) Knockdown efficiency of siRNA targeting various MYC E3 ligases were assessed using qRT-PCR. (**d**) HCT116 *FBXW7*-/- cells were transfected with scrambled control (Scr) or siRNA targeting *DDB1* and *DDB1* expression determined using qRT-PCR. (**e**) HCT116 *FBXW7*-/- cells transfected with scrambled control (Scr) or siRNA targeting *DDB1* were grown at 21% or 0.5% O_2 for 24 hr and lysates immunoblotted for MYC protein. Gene expression data are shown as the mean of two independent experiments. *, $p<0.05$.

half life at 21% O_2 was 116 min while that at 0.5% O_2 decreased to 69 min (**Fig. 14d**). Furthermore, the extent of MYC destabilization under hypoxia was abrogated in cells lacking *FBXW7* (31% reduction from normoxic levels in wild type cells; 21% reduction in *FBXW7*-/- cells) (**Figs. 9b** and **14d**). These results indicate that FBXW7 partially contributes to MYC inhibition under O_2 deprivation.

Hypoxic MYC degradation requires DDB1. Interestingly, the rise in MYC ubiquitylation under hypoxia was not accompanied by enhanced expression of *FBXW7* or other MYC E3 ubiquitin ligases examined (**Fig. 15a**). However, RNAi-mediated silencing of *DDB1* and its binding partner *CUL4B* restored MYC protein levels under hypoxic conditions (**Figs. 15b—c**). *DDB1* knockdown in HCT116 cells lacking *FBXW7* restored MYC protein abundance under hypoxia to higher levels than in cells lacking either *DDB1* or *FBXW7* alone (**Figs. 15d—e**). These results suggested that the E3 ligases FBXW7 and DDB1 are both responsible for hypoxic MYC downregulation. Furthermore, hypoxic enhancement of MYC ubiquitylation may occur via increases in their protein expression levels or conjugating activity.

HIFs are required for hypoxic MYC degradation. Previous reports have demonstrated that HIF proteins can regulate hypoxic MYC activity (Dang et al., 2008). To characterize the requirement for HIF in hypoxic MYC degradation, we employed RNAi-mediated knockdown to inhibit HIF1α and HIF2α alone or in combination (**Fig. 16a**). Absence of HIF1α or HIF2α alone had no effect on MYC degradation under hypoxia. When both HIF isoforms were inhibited simultaneously, MYC protein abundance was partially restored (**Figs. 16a—b**). Interestingly, although HIF1α and HIF2α are required for hypoxic MYC degradation, we were unable to detect direct interactions between MYC,

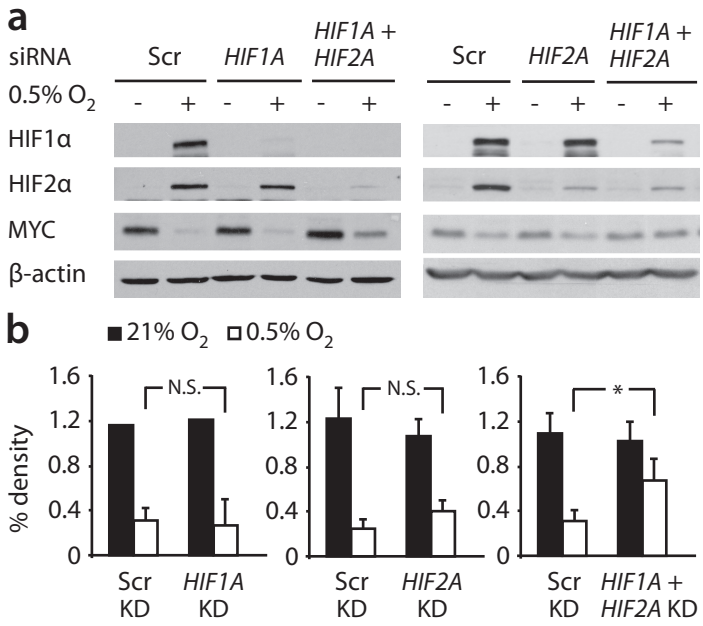

Figure 16. MYC downregulation under hypoxia requires HIF1α and HIF2α. (**a**) HCT116 cells were transfected with scrambled control (Scr) or siRNA targeting *HIF1A*, *HIF2A*, or both, grown at 21% or 0.5% O_2 for 24–48 h and lysates were immunoblotted for HIF and MYC proteins. (**b**) Densitometric quantifications are presented as the mean of three independent experiments. N.S., not significant. *, $p<0.05$.

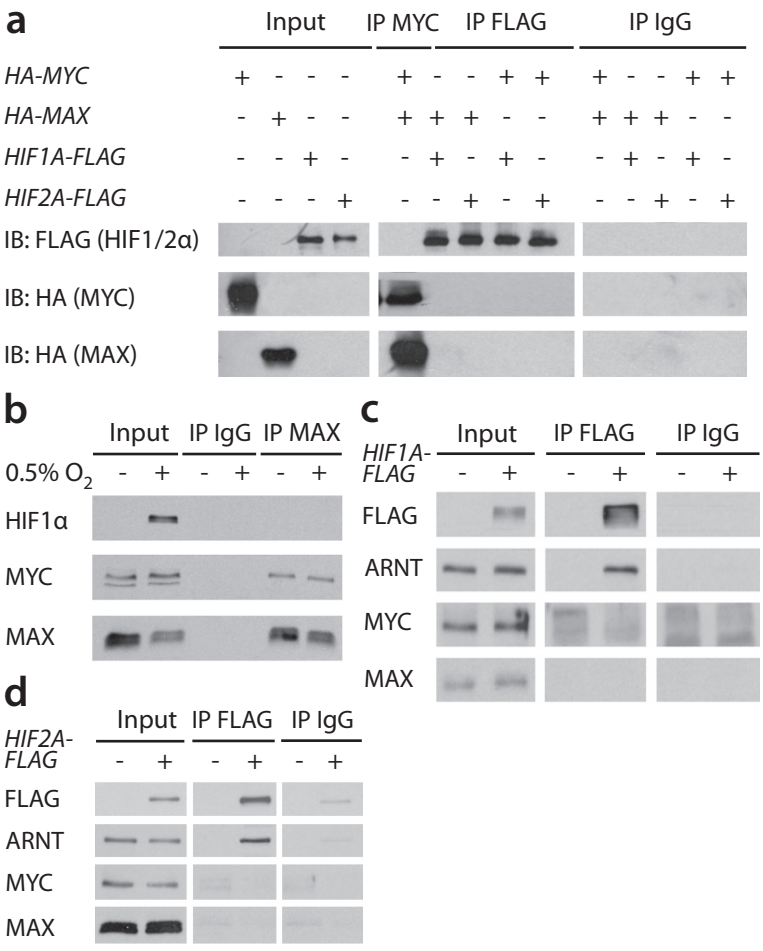

Figure 17. Hypoxic MYC degradation does not require HIF interaction with MYC or MAX. (**a**) *HA-MYC*, *HA-MAX*, *HIF1A-FLAG*, and *HIF2A-FLAG* were translated *in vitro* and co-immunoprecipitated as indicated. (**b**) HCT116 cells were grown at 21% or 0.5% O_2 for 6 h and the abundance of HIF1α and MYC in anti-MAX immunoprecipitates were determined. (**c**, **d**) HEK293T cells were transfected with FLAG-tagged *HIF1A* (**c**) or *HIF2A* (**d**), grown at 21% or 0.5% O_2 for 6 h, and the abundance of indicated proteins in anti-FLAG immunoprecipitates and total lysates determined.

MAX, HIF1α, and HIF2α using *in vitro* translated proteins (**Fig. 17a**). Co-immunoprecipitation studies in endogenous and overexpression settings confirmed MYC-MAX and HIF-ARNT binding, but we could not identify interactions between HIF1α or HIF2α and MYC or MAX (**Figs. 17b—d**). This result was somewhat surprising, because HIF1α has been suggested to bind to MYC and inhibit its transcriptional activity (Koshiji et al., 2004). Importantly, MYC-MAX dimerization in HCT116 cells was unaffected by 6 h of hypoxic exposure compared to normoxic control, but decreased after 24 h of hypoxia together with total MYC protein levels (**Fig. 17b** vs **8a—c**). These findings indicate that HIF proteins regulate MYC activity by multiple mechanisms, including direct competition and ubiquitin-mediated proteolysis, and the relative importance of these effects may vary in distinct cell types and different hypoxic conditions (see discussion).

MYC suppression under hypoxia is a protective mechanism against cell death. To study the biological consequences of MYC inhibition under hypoxia, we transduced HCT116 cells with retroviral vectors encoding HA-tagged versions of wild type MYC^{HAM} (i.e. HA-tagged MYC) or mutant MYC^{T58A} (i.e. Thr58 to Ala58 mutation). The absence of Thr58 phosphorylation renders MYC^{T58A} relatively resistant to degradation via the ubiquitin-proteasome pathway (Sears et al., 2000). Pulse chase analyses indicated that MYC^{T58A} turnover was almost identical to that of wild type MYC^{HAM} in normoxic settings (46 min vs 43 min), but in hypoxic conditions MYC^{T58A} was significantly more stable than MYC^{HAM} (43 min vs 35 min) (**Figs. 18a—b**). Western blotting verified that MYC^{HAM} and MYC^{T58A} were expressed at similar levels under normoxic conditions (**Fig. 18c**). Surprisingly, enhanced MYC expression in hypoxic HCT116-MYC^{T58A} cells did not result

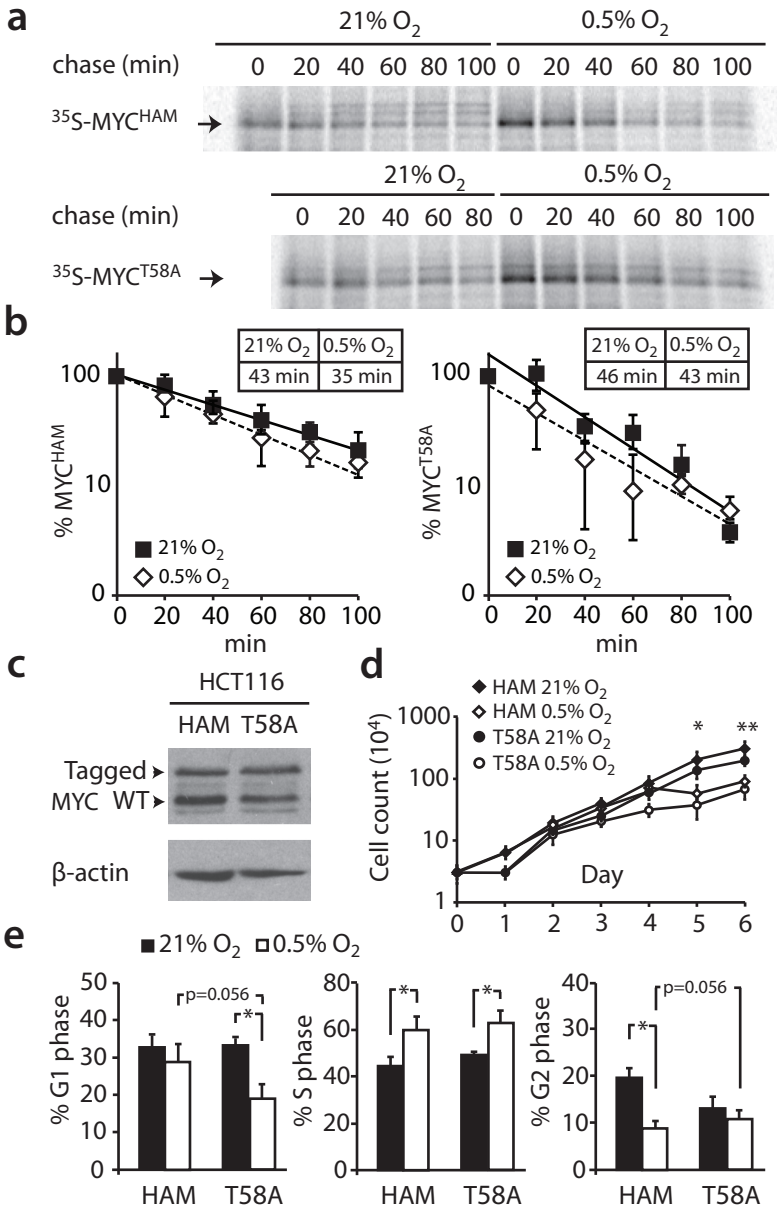

Figure 18. MYC^{T58A} mutation opposes hypoxia-induced MYC suppression. (**a**) HCT116 cells were transduced with retroviral vectors encoding MYC^{HAM} or MYC^{T58A}, grown at 21% or 0.5% O_2 for 24 h, pulsed with [^{35}S]Cys/Met and lysates obtained at the indicated times during the subsequent chase period. MYC was immunoprecipitated and visualized by autoradiography. (**b**) Average densitometric values and half-life measurements from 2 independent experiments are shown for HCT116-MYC^{HAM}(left) and HCT116-MYC^{T58A} (right). (**c**) MYC immunoblot of lysates from HCT116-MYC^{HAM} and HCT116-MYC^{T58A} cells. (**d**) HCT116-MYC^{HAM} and HCT116-MYC^{T58A} cells were seeded on Day 0 and counted at the indicated times. Mean results from two independent experiments are shown. (**e**) HCT116-MYC^{HAM} and HCT116-MYC^{T58A} cells were grown at 21% or 0.5% O_2 for 24 h and the percentage of cells in G1 (left), S (middle) and G2 phases (right) of the cell cycle were determined. Mean results from two independent experiments are shown. *, $p<0.05$ at 0.5% O_2. **, $p<0.05$ at 21% O_2.

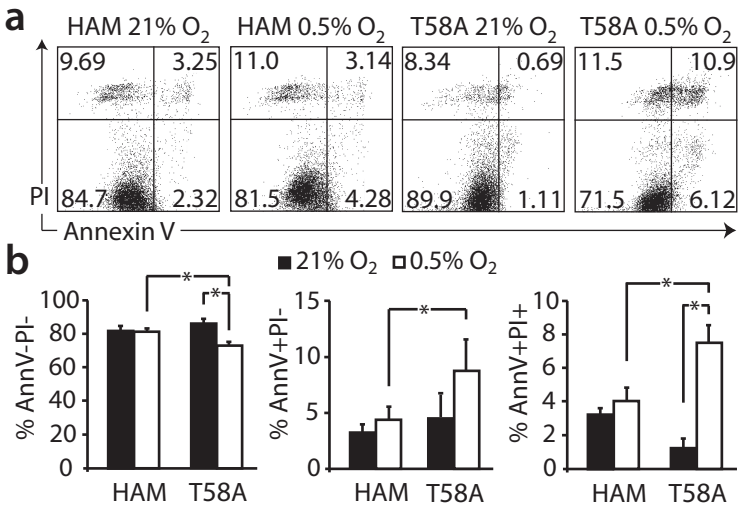

Figure 19. Overexpression of stabilized MYC promotes hypoxia-induced cell death. (**a**) The viability of HCT116-MYC^{HAM} and HCT116-MYC^{T58A} cells after 48 h growth at 21% or 0.5% O_2 was determined by annexin V and propidium iodide staining; data from one representative experiment are shown. (**b**) Mean results from three independent experiments are quantified.

in increased cell numbers as measured by serial cell counts. HCT116-MYC^{T58A} cell counts were lower than control cell counts at both 21% and 0.5% O_2 (**Fig. 18d**). BrdU incorporation experiments revealed that paradoxically, MYC^{T58A} cells displayed a trend towards increased G1/S cell cycle progression, whereas control cells underwent S/G2 arrest under hypoxia (**Fig. 18e**).

Because both hypoxia and MYC are known to induce apoptosis in certain conditions, we asked whether MYC overexpression in HCT116 cells contributed to apoptotic cell death. Cell viability was measured by annexin V and propidium iodide (PI) staining. The proportion of annexin V and PI negative (i.e. live) cells was reduced by the combination of O_2 limitation and MYC^{T58A} expression (**Figs. 19a—b**). Correspondingly, the proportion of dying (annexin V positive, PI negative) and dead (annexin V and PI positive) cells increased in hypoxic HCT116-MYC^{T58A} cells (**Fig. 19b**). To identify the mediators of cell death in response to hypoxia and MYC activity, we evaluated the expression of various BCL2 family proteins. Hypoxia induced *PUMA* expression in both control and MYC^{T58A} cells (**Fig. 20a**, upper panel). In contrast, expression of *NOXA*, a direct MYC target gene (Nikiforov et al., 2007), was induced only in hypoxic MYC^{T58A} cells (**Fig. 20a**, lower panel). These experiments suggest that high MYC activity under hypoxia results in an additional apoptotic stimulus, which correlates with increased *NOXA* induction.

Finally, we evaluated the contribution of *NOXA* and *PUMA* expression to hypoxic cell death in MYC^{T58A} cells. *NOXA* silencing alone was insufficient to improve HCT116-MYC^{T58A} cell viability. However, combined inhibition of *NOXA* and *PUMA* restored the viability of hypoxic HCT116-MYC^{T58A} cells to normoxic levels (**Fig. 20c**). In contrast,

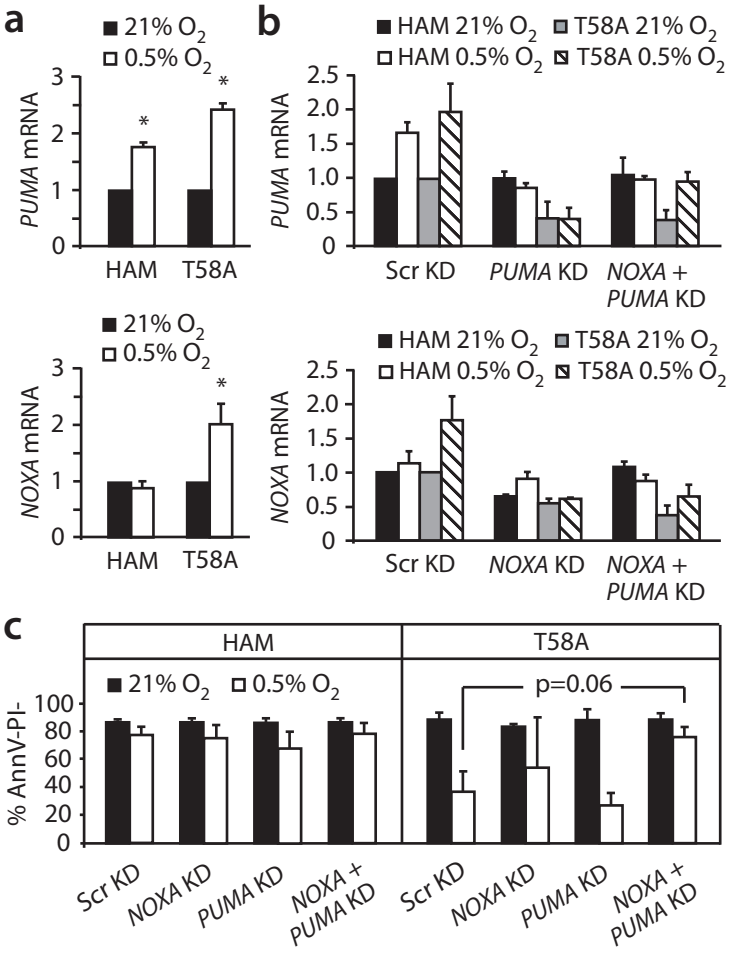

Figure 20. Hypoxia-induced cell death in HCT116-MYC^{T58A} cells is *NOXA*- and *PUMA*-dependent.
(**a**) HCT116-MYC^{HAM} and HCT116-MYC^{T58A} cells were grown at 21% or 0.5% O_2 and *PUMA* (top panel) and *NOXA* expression (bottom panel) determined by qRT-PCR. (**b**) HCT116-MYC^{HAM} and HCT116-MYC^{T58A} cells were transfected with scrambled control (Scr) or shRNA targeting *PUMA*, *NOXA*, or both, and knockdown efficiency determined using qRT-PCR. Mean results from two independent experiments are shown. (**c**) HCT116-MYC^{HAM} and HCT116-MYC^{T58A} cells were transfected with scrambled control (Scr) or shRNA targeting *PUMA*, *NOXA*, or both,and viability determined by annexin V and propidium iodide staining after 48 growth at 21% or 0.5% O_2. Mean results from three independent experiments are shown. *, $p<0.05$.

HCT116-MYC^{HAM} cell viability was unaffected by either hypoxic treatment or *NOXA* and *PUMA* knockdown (**Fig. 20c**). This finding suggests that the co-induction of *NOXA* and *PUMA* in hypoxic cells with high MYC activity contributes to hypoxia-induced cell death. Cells that are able to downregulate MYC under hypoxic conditions did not induce *NOXA* expression (**Fig. 20c**); in these cells, *PUMA* induction alone was insufficient to promote hypoxia-induced cell death. Collectively, the data presented here indicate that hypoxic MYC downregulation may be a physiologically important response in multiple cell types to circumvent *NOXA* induction and avoid NOXA- and PUMA-dependent cell death, thereby preserving cell viability under hypoxic stress.

DISCUSSION

MYC is an important regulator of cell division, metabolism, and apoptosis. These cellular processes are coordinated with nutrient and energy availability by regulatory mechanisms that modulate MYC in response to different types of stress. In this study, we demonstrated that MYC protein levels are suppressed under hypoxic conditions in both transformed and untransformed cells. We have extended our analysis of MYC activity during O_2 deprivation to show that Pol III-dependent transcription of MYC target genes is reduced in low O_2. MYC effects on Pol III-dependent transcription are known to be mediated via its direct interaction with the transcription factor TFIIIB, and do not require binding to MAX, MIZ1, SP1, or other related proteins (Gomez-Roman et al., 2003; Steiger et al., 2008). Therefore, the effect of O_2 on MYC-dependent transcription cannot be fully explained by previous models of hypoxic MYC inhibition, in which MYC-

containing complexes are disrupted either by direct HIF binding or induction of negative regulators *MXD1* and *MXI1* (Dang et al., 2008). We have identified the E3 ubiquitin ligase DDB1 and cathepsin proteases as novel regulators of hypoxic MYC degradation; we also independently confirmed that the E3 ligase FBXW7 contributes to increased MYC degradation under hypoxia (Li et al., 2009b). When MYC degradation under hypoxic conditions was prevented by forced expression of MYC^{T58A}, cell death occurred via *NOXA* and *PUMA* induction, indicating that hypoxic MYC suppression is important for maintaining cell survival under low O_2 tension.

MYC expression and activity are known to be controlled at virtually all levels of regulation (Meyer and Penn, 2008). However, MYC suppression in response to various stresses occurs via distinct pathways. For example, the absence of microRNA effects on hypoxic MYC abundance illustrates the complexity of MYC regulatory mechanisms in response to O_2 limitation and DNA damage (Kress et al., 2011). Interestingly, *MIR34B* or *MIR34C* inhibition enhanced normoxic MYC expression, suggesting that these microRNAs may be involved in homeostatic, but not hypoxic, MYC regulation. While our work indicated that hypoxic MYC inhibition occurred posttranslationally, it is possible that prolonged hypoxic exposure (i.e. greater than 48 h) and the accumulation of other stresses could create additional feedback responses on MYC expression. For example, hypoxic suppression of Wnt signaling via LEF1/TCF1 could eventually decrease *MYC* transcription (Kaidi et al., 2007). Hypoxia also inhibits mTORC1 activity, which could potentially suppress *MYC* expression due to global inhibition of cap-dependent translation (Arsham et al., 2003; Brugarolas et al., 2004; Wall et al., 2008). Although we did not observe these effects in our experimental conditions and timeframe, mechanisms

of MYC regulation under hypoxia may vary depending on the severity and duration of hypoxic stress.

We found that proteasomal MYC degradation under low O_2 tension was dependent on two E3 ubiquitin ligases, FBXW7 and DDB1. The molecular mechanism behind their hypoxic responsiveness remains unclear, because neither *DDB1* nor *FBXW7* were transcriptionally upregulated in hypoxia. Other, non-proteasomal, modes of MYC regulation included degradation mediated by cathepsins. Cathepsins are endosomal and lysosomal proteases that play important roles in autophagy and cell death. Although cathepsin activity has long been associated with nonspecific intracellular protein degradation, emerging evidence suggest that they can participate in the proteolytic processing of specific substrates in a controlled fashion (Reiser et al., 2010). We found that hypoxia induced the transcription of multiple cathepsin genes. Importantly, chemical inhibition of cathepsins restored MYC stability to normoxic levels. Therefore, MYC protein stability is controlled by ubiquitylation-dependent and -independent proteolysis under hypoxic conditions.

Our finding that hypoxic MYC inhibition requires the concerted activity of HIF1α and HIF2α, while consistent with some previous reports (Li et al., 2009b; Zhang et al., 2007), lies in contrast to the unique and antagonistic contributions of HIF1α and HIF2α to MYC activity in renal clear cell carcinoma (ccRCC) (Dang et al., 2008). Although HIF2α expression in HCT116 cells contributed to MYC inhibition, the exclusive expression of HIF2α in ccRCC cells clearly enhances MYC transcriptional activity (Gordan et al., 2007). One possible explanation is that the relative expression levels of HIF1α and HIF2α may determine whether MYC is inhibited or activated during O_2

deprivation. Further work is required to understand whether HIF1α stabilization modulates the activity of HIF2α with respect to MYC, and whether HIF2α activity exerts distinct effects on MYC in different tissue and cellular contexts. The importance of cellular context in hypoxic MYC regulation is further highlighted by the existence of physical interactions between HIF and MAX in ccRCC cells, and the absence thereof in HCT116 cells.

Elevated MYC expression in HCT116-MYC^{T58A} cells exacerbated cell death under low O_2 levels. Hypoxia promotes apoptosis by modulating p53 activity and expression of downstream effectors, including the BH3-only proteins BNIP3, PUMA, and NOXA (Bruick, 2000; Kim et al., 2004; Yu et al., 2003). We have confirmed that hypoxia activates expression of the pro-apoptotic gene *PUMA*. Moreover, high MYC activity during O_2 limitation resulted in additional apoptotic stimuli via increased *NOXA* induction. This observation is consistent with a previous report that *NOXA* is a direct MYC target gene (Nikiforov et al., 2007). Knockdown experiments indicate that the combination of high MYC activity and hypoxic stress promote cell death via *NOXA*- and *PUMA*-dependent mechanisms.

The role of MYC in promoting cell death during hypoxia suggests a survival benefit in MYC suppression under low O_2 tension. In support of this argument, inhibition of MYC-induced apoptosis in pancreatic β cells promotes tumor development *in vivo* (Pelengaris et al., 2002). Alternatively, tumors with high MYC activity frequently lose expression of p53, ARF, or downstream apoptotic mediators such as BAX and PUMA in order to escape cell death (Eischen et al., 1999; Eischen et al., 2001; Garrison et al., 2008; Schmitt et al., 1999). Intriguingly, induction of the pro-apoptotic protein *Bim* is

attenuated in MYC^{T58A}-overexpressing mouse embryonic fibroblasts (MEFs) compared to MEFs overexpressing wild type MYC (Hemann et al., 2005), suggesting that at least in some contexts, stabilizing MYC mutations can inhibit MYC-dependent cell death. We did not observe consistent effects on *BIM* induction in HCT116-MYC^{T58A} cells. Instead, we found that *NOXA* and *PUMA* induction in MYC^{T58A}-overexpressing cells enhanced apoptosis in low O_2 conditions. We propose, therefore, that hypoxia-induced MYC inhibition is an adaptive mechanism to ensure cell survival at the expense of unrestricted growth.

Chapter Three

HIFs regulate keratinocyte terminal differentiation and epidermal barrier function

SUMMARY

Proper formation of the cornified envelope is essential to epidermal barrier development and the protective function of the skin. The cornified envelope is formed by terminally differentiating keratinocytes during late gestation and early life. In this chapter, I describe experiments which indicate that the embryonic epidermis is normally O_2-deprived. Targeted deletion of *Hif1a* and *Hif2a* in murine epidermis revealed defects in keratinocyte differentiation and epidermal barrier formation. Mice lacking *Hif1a* and *Hif2a* in the epidermis exhibited dry flaky skin, impaired permeability barrier, and decreased corneocyte integrity. These defects were associated with stratum granulosum attenuation and reduced filaggrin expression. Hypoxic treatment of primary keratinocytes induced filaggrin (*Flg*) gene expression in a HIF1α- and HIF2α-dependent manner, suggesting that one mechanism by which *Hif1a* and *Hif2a* loss causes epidermal barrier defects in mice lies in *Flg* dysregulation. These data reveal an unexpected role for HIFs in the regulation of filaggrin expression and epidermal development. Therefore, low O_2 tension is an important component of the epidermal microenvironment that contributes to skin development and function.

INTRODUCTION

The epidermis is a stratified squamous epithelium. Together with hair follicles, sebaceous glands, and dermal connective tissue, it forms the largest organ in the body. Skin performs many important functions, including thermoregulation, sensory perception, immunity, and protection from physical trauma. The protective barrier of the epidermis derives mainly from the most superficial epithelial layer, the cornified envelope. This barrier is constantly regenerated from differentiating keratinocytes; abnormalities in this process have been associated with skin diseases including ichthyosis, psoriasis, and atopic dermatitis (Ghadially et al., 1996; Palmer et al., 2006; Smith et al., 2006).

Keratinocyte growth, differentiation, and desquamation are precisely coordinated (Fuchs, 2008). Dividing basal keratinocytes detach from the basement membrane, commit to terminal differentiation, and eventually slough off the body surface. The course of keratinocyte differentiation can be delineated spatially and morphologically, as well as by the expression of specific keratin intermediate filaments at distinct stages of differentiation. For example, proliferating basal keratinocytes express keratin 5 (KRT5) and keratin 14 (KRT14), while newly differentiating keratinocytes in the spinous and lower granular layers express keratin 1 (KRT1) and keratin 10 (KRT10) (Fuchs and Green, 1980). Terminally differentiated keratinocytes in the upper granular layer and the cornified envelope express cornification proteins such as involucrin (IVL), loricrin (LOR), and filaggrin (FLG). Notably, FLG binds intermediate filaments in the upper granular layer, thereby condensing the keratinocyte cytoskeleton into a strong, flattened matrix (Steinert et al., 1981). Other cornified envelope proteins bind this matrix and

become crosslinked to epidermal sphingolipids, resulting in structural integrity and barrier properties of the epidermis.

Numerous regulatory and signaling pathways govern the sequence of epidermal specification, differentiation, and cornification. For example, Wnt and BMP signaling specify epidermal cell fate in early development (Nguyen et al., 2009; Wilson and Hemmati-Brivanlou, 1995). Notch and p63 transcriptional programs control the switch from basal to suprabasal keratinocyte cell fate (Mills et al., 1999; Rangarajan et al., 2001; Yang et al., 1999), while formation of the cornified envelope is regulated by transcription factors such as KLF4 and IKKα (Gareus et al., 2007; Segre et al., 1999). The epidermal microenvironment is also an important determinant, especially at the first and last steps of epidermis formation. Integrin and TGF-β signaling at the basement membrane influence the commitment of dividing keratinocytes to terminal differentiation. At the end of the developmental program, cornified envelope formation is regulated by extracellular calcium gradient as well as steroid hormone concentration (Elias et al., 1998; Kömüves et al., 2000).

The epidermal microenvironment is further characterized by low oxygen (O_2) availability. Studies in humans and rodents have indicated that O_2 saturation in adult epidermis ranges from 0.5% to 5% (Evans et al., 2006). The molecular response to low O_2 is mediated primarily by hypoxia inducible factors (HIFs) (Majmundar et al., 2010). These are heterodimeric transcription factors comprised of an O_2-labile subunit (HIF1α or HIF2α) and a constitutively-expressed HIF-β subunit, also known as aryl hydrocarbon receptor nuclear translocator (ARNT). In low-O_2 conditions, HIF1/2α stabilization results in the activation of many genes involved in metabolism, cell growth and proliferation,

angiogenesis, differentiation, and apoptosis. These changes simultaneously suppress cellular O_2 demand and increase O_2 delivery. HIF1α activity in the epidermis is important in cutaneous O_2 sensing, skin innate immunity, wound healing, and melanoma transformation (Bedogni et al., 2005; Boutin et al., 2008; Elson et al., 2000; Peyssonnaux et al., 2008). In comparison, little is known about the function of HIF2α in the skin. However, both HIF1α and HIF2α have well-characterized roles in the determination and differentiation of other O_2-deprived tissues such as the placenta, skeletal muscle, and bone (Amarilio et al., 2007; Dahl et al., 2005; Majmundar et al., 2012; Provot et al., 2007).

Does O_2 availability regulate skin development? In this study, we investigated the effect of hypoxic culture on keratinocyte differentiation. O_2-deprived keratinocytes specifically upregulated *Flg* expression in a HIF1α and HIF2α dependent manner. Low O_2 conditions occurred naturally during epidermal development and correlated with HIF1α and HIF2α expression in the epidermis. In this context, genetic deletion of *Hif1a* and *Hif2a* led to decreased *Flg* expression, reduced corneocyte integrity, and impaired epidermal barrier function. Consequently, HIF is a novel regulator of keratinocyte differentiation and epidermal barrier formation.

MATERIALS AND METHODS

Mice. *Krt14-Cre*$^{+}$ transgenic mice were obtained from JAX. *Arnt*$^{fl/fl}$, *Hif1a*$^{fl/fl}$, and *Hif2a*$^{fl/fl}$ mice have been described previously (Gruber et al., 2007; Ryan et al., 2000; Tomita et al., 2000). For genotyping, genomic DNA was isolated from epidermal and dermal tissue using phenol-chloroform extraction. PCR was performed using allele-specific primers

(sequences available on request). All animal experiments were performed in compliance with Institutional Animal Care and Use Committee regulations and approved by the University of Pennsylvania institutional review board.

Cell culture. Primary human keratinocytes were maintained in a 1:1 mixture of K-SFM (Invitrogen) supplemented with human recombinant Epidermal Growth Factor and Bovine Pituitary Extract and Medium 154 (Invitrogen) supplemented with Human Keratinocyte Growth Supplement Kit (Invitrogen) in a humidified incubator at 37°C, 5% CO_2. Primary murine keratinocytes were maintained in MCDB 153 media (Sigma) supplemented with 5% chelexed fetal bovine serum (Hyclone), 100 μM ethanolamine (Sigma), 100 μM phosphorylethanolamine (Sigma), 2 mM L-glutamine (Invitrogen), 10 ng/mL human recombinant Epidermal Growth Factor (Invitrogen), 1 μM hydrocortisone (Invitrogen), 5 μg/mL insulin (Invitrogen), 0.2 nM cholera toxin (Sigma), 2 nM 3,3'5-triiodo-L-thyronine (Sigma), and 45 μM calcium chloride (Sigma). Murine keratinocytes were maintained in a humidified incubator at 34°C, 8% CO_2; experiments were performed at 37°C, 5% CO_2. For hypoxia treatment, cells were cultured in an InVivo2 400 hypoxia workstation (Ruskinn) at 37°C, 0.5% O_2, 5% CO_2.

Isolation of primary newborn keratinocytes. Primary keratinocytes were isolated from neonatal mice as described (Lichti et al., 2008) with modifications. Briefly, whole skins of P0 to P2 mice were floated on Dispase II (BD Biosciences) overnight at 4°C. Epidermis was separated from dermis, rinsed in PBS, and incubated in trypsin-EDTA solution (Invitrogen) for 5—10 min at 37°C. Keratinocytes were physically dissociated from the basement membrane using a scalpel and passed through a 70 μm cell strainer (BD Falcon) to obtain single cells.

Visualization of tissue pO_2. 1-((2-hydroxy-3-piperdinyl)propyl)-2-nitroimidazole hydrochloride (Hypoxyprobe Inc) was injected intraperitoneally into pregnant mice at E14, E16, E18, and intraperitoneally into P0 pups. After 4 h, embryos/pups were collected and frozen in OCT (Sakura). Skin cryosections were stained using anti-hypoxyprobe-FITC antibody (Hypoxyprobe Inc).

Histological and immunohistochemical analysis. Tissues were fixed in 4% paraformaldehyde for 1 h and embedded in paraffin. 5 um sections were stained with hematoxylin-eosin or antibodies directed against ARNT (SC-8076; Santa Cruz), FLG, HIF1α (Ab2185; Abcam), HIF2α (NB100-132; Novus), IVL, Ki67 (Novocastra), KRT1, KRT6, KRT10, and LOR (all from Covance unless otherwise noted). Antibody binding was detected with HRP peroxidase using DAB as substrate (Vector Labs) or with AlexaFluor 488 anti-rabbit immunoglobulin (Invitrogen).

Quantitative RT-PCR. Total RNA from primary keratinocytes or homogenized epidermal tissue was extracted with Trizol reagent (Invitrogen) and cDNA produced using High capacity RNA-to-cDNA kit (Applied Biosystems). Analysis of gene expression was performed in a 7900HT Sequencer (Applied Biosystems) using specific primers (sequences available on request). Expression levels of *18S rRNA* and *HPRT1* were used for normalization.

Western blotting. 10-50 μg of total protein extract were electrophoresed on 10-14% sodium dodecyl sulfate-polyacrylamide gels. Primary antibodies used were directed against ARNT (NB100-110; Novus), β-actin (Sigma), FLG, IVL, HIF1α C-Term (Cayman), HIF2α (NB100-122; Novus), KRT5, KRT10, and LOR (all from Covance unless otherwise noted). Optical densities were quantified using ImageJ software (NIH).

Chromatin immunoprecipitation (ChIP). ChIP was performed as described (Forsberg et al., 2000). Cells were crosslinked in 1% formaldehyde for 10 min. Precleared chromatin was immunoprecipitated with anti-HIF1α (NB100-105; Novus) or ARNT (NB100-110; Novus) antibody and protein A agarose beads (Roche) for 16 h at 4°C. Immunoprecipitated and total DNA were quantified in a 7900HT Sequencer using specific primers (sequences available on request).

Outside-to-in permeability barrier assay. Epidermal permeability was assessed by toluidine blue staining as described (Takagi et al., 2003). E17 mouse embryos were sequentially dehydrated in 25%, 50%, 75%, and 100% methanol, rehydrated in PBS, immersed in 0.1% toluidine blue/PBS, then rinsed extensively in PBS. Epidermal permeability was categorized as high (dorsal staining), moderate (above 50% ventral staining), or low (below 50% ventral staining).

Corneocyte sonication. Cornified envelope was purified and sonicated as described (Koch et al., 2000). Briefly, skin from the tail, ear, or hind limb of P3 mice were floated on Dispase II (BD Biosciences) for 1 h at 37°C. Epidermis was separated from dermis, rinsed in PBS, and boiled in CE extraction buffer (100 mM Tris–HCl pH 8.5, 5 mM EDTA, 20 mM DTT, 2% SDS). Insoluble corneocytes were isolated by centrifugation, resuspended in extraction buffer, and sonicated in a bath sonicator (Diagenode) at 4°C on setting High for 20 s.

Ovalbumin treatment and serum IgE ELISA. Ovalbumin (OVA) challenge was performed as described (Palmer et al., 2006). 20 to 50 ug of OVA (fraction V; Sigma) in PBS was applied daily to ventral skin of control or DKO littermates for five consecutive

days. OVA challenge was repeated twice with intervening intervals of 7 d each. Total serum IgE was measured using PharMingen antibodies (BD Biosciences).

Statistical analysis. Data are presented as mean+SEM. Differences between groups were analyzed for significance using Student's t test, with $p < 0.05$ considered to be significant.

RESULTS

Developing murine epidermis is naturally low in O_2. On day 14 of gestation (E14), the murine epidermis exists as a single layer of epidermal keratinocytes underneath a protective periderm layer. By E16, stratification of the true epidermis has begun such that all epidermal layers are present by E18 (Mack et al., 2005). The cornified envelope is present by E18 but undergoes further maturation until after birth (P0) (Candi et al., 2005). To characterize the O_2 microenvironment during this developmental process, we made use of the O_2-sensitive dye pimonidazole hydrochloride, which detects areas of O_2 saturation below 2% O_2. Positive pimonidzaole staining indicated that the epidermis experienced low O_2 tension between E16 and P0 (**Fig. 21a**). Consistent with this finding, HIF1α and HIF2α protein were detectable in newborn epidermis (**Fig. 21b**). Furthermore, primary keratinocytes exposed to hypoxic conditions stabilized HIF1α and HIF2α (**Fig. 21c**). These results suggest that murine epidermal development occurs in an O_2-deprived setting.

Hypoxic keratinocytes upregulate filaggrin expression. To determine the effect of low O_2 on epidermal development, we examined the expression of early and late differentiation genes in hypoxic keratinocytes. Keratin 1 (*Krt1*) and keratin 10 (*Krt10*) are

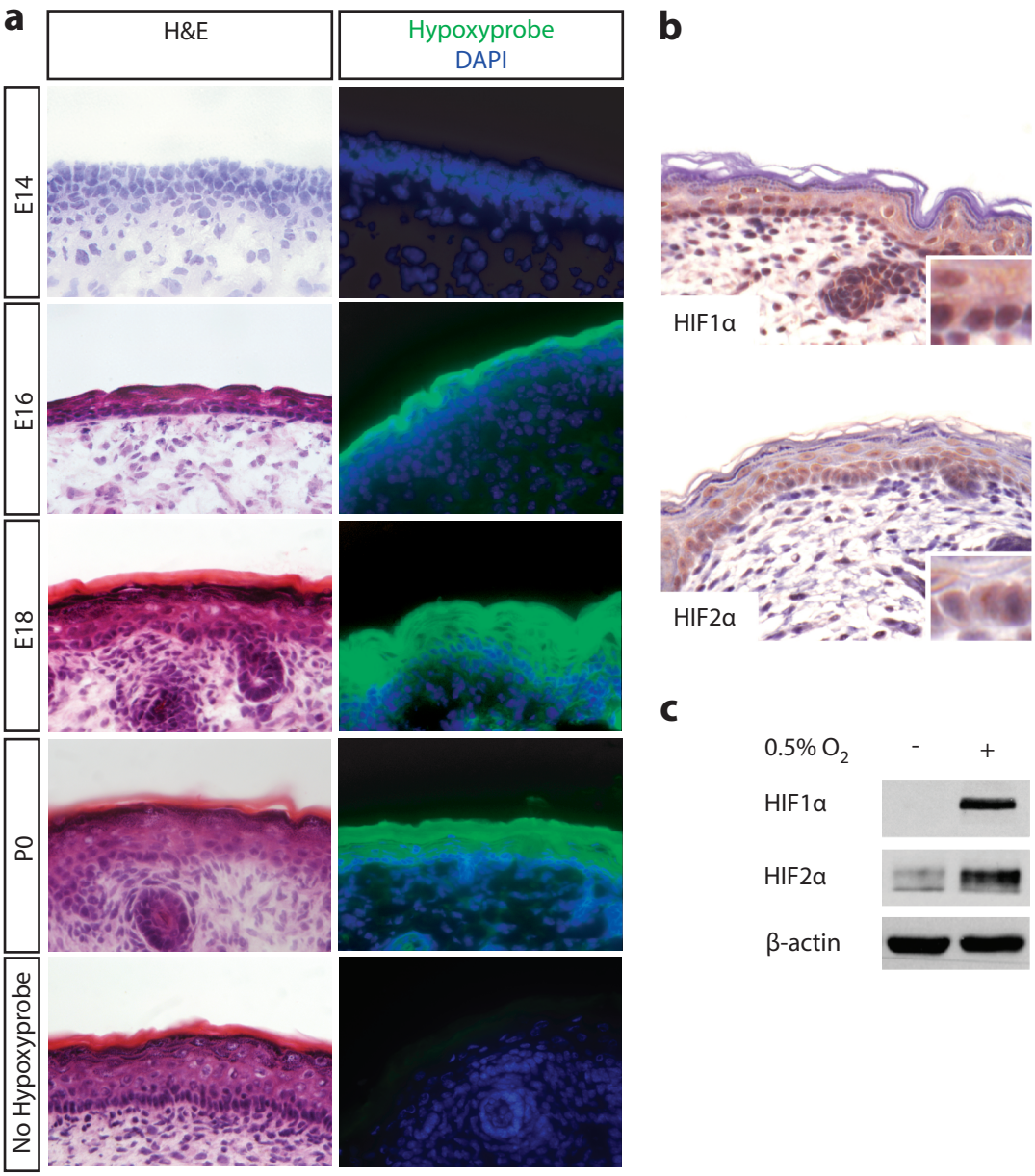

Figure 21. Murine epidermis develops under naturally low O_2 tension. (**a**) Oxygen sensitive 1-((2-hydroxy-3-piperdinyl)propyl)-2-nitroimidazole hydrochloride (Hypoxyprobe) was injected intraperitoneally into pregnant mice at E14, E16, E18, and intraperitoneally into P0 pups. After 4 h, embryos or pups were collected and frozen skin sections stained using hematoxyin and eosin, anti-hypoxyprobe-FITC antibody (green), and DAPI (blue). Animals of the same age that had not been injected with hypoxyprobe were used as negative controls. (**b**) Paraffin-embedded dorsal skin sections from wild type P2 pups were immunostained using antibodies against HIF1α and HIF2α. (**c**) Primary murine keratinocytes were grown at 21% or 0.5% O_2 for 48 h and cell lysates immunuoblotted using antibodies against HIF1α and HIF2α.

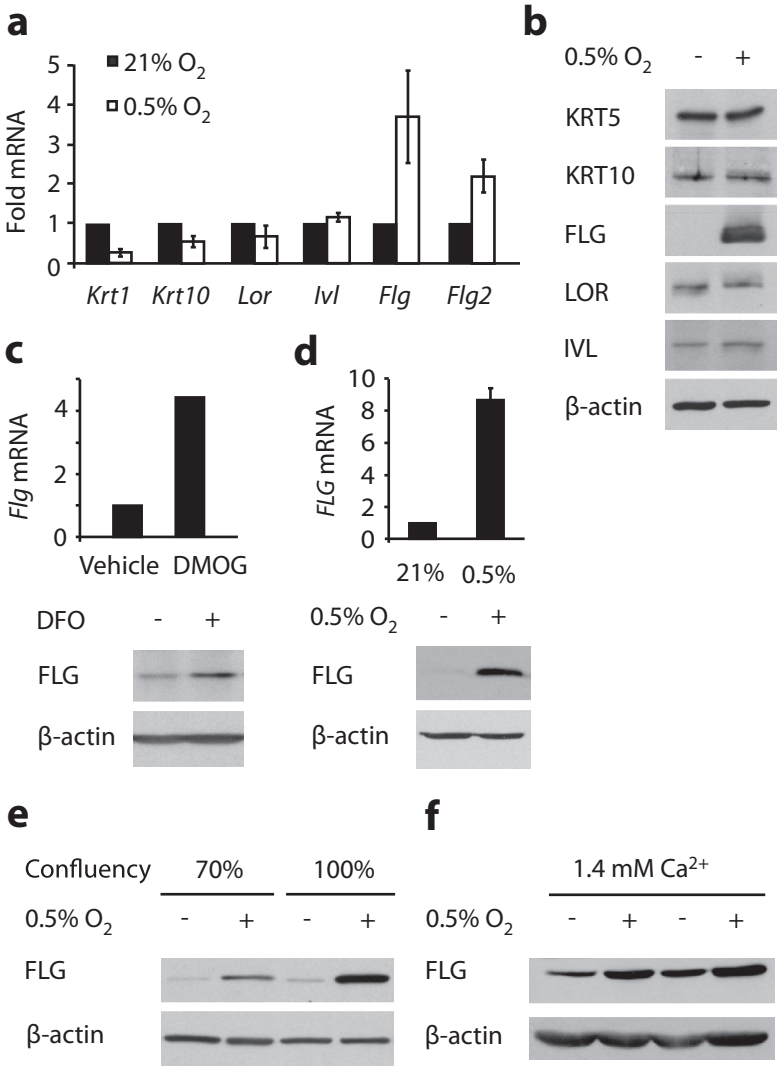

Figure 22. Hypoxia induces filaggrin expression via HIF. (**a**) Primary murine keratinocytes from wild type P2 pups were grown at 21% or 0.5% O_2 for 48 h and epidermal gene expression assessed by qRT-PCR. Mean results from two independent experiments are shown. (**b**) Primary murine keratinocytes were grown at 21% or 0.5% O_2 for 48 h and lysates immunoblotted for epidermal proteins as indicated. (**c**) Primary human keratinocytes were grown at 21% or 0.5% O_2 for 48 h and filaggrin expression evaluated by qRT-PCR and western blotting. (**d**) Primary murine keratinocytes were grown in 1 mM DMOG or 100 μM DFO for 48 h and filaggrin expression determined using qRT-PCR and western blotting respectively. (**e**) Primary murine keratinocytes from wild type P2 pups were grown at 21% or 0.5% O_2 for 48 h to 70% or 100% confluency and lysates immunoblotted for FLG. (**f**) Primary murine keratinocytes were cultured in media containing 1.4 mM Ca^{2+} at 21% or 0.5% O_2 for 48 h and lysates immunoblotted for FLG.

expressed in early differentiating cells, whereas loricrin (*Lor*), involucrin (*Ivl*), and filaggrin (*Flg*) expression are induced at the terminal stages of epidermal differentiation. qRT-PCR analysis using primary keratinocytes cultured under normoxic and hypoxic conditions revealed that low-O_2 conditions specifically induced expression of *Flg* and the related filaggrin-2 (*Flg2*) gene (**Fig. 22a**). FLG upregulation was also observed at the protein level, while the expression of several other epidermal proteins remained unchanged (**Fig. 22b**). Treatment with 2-oxoglutarate-dependent dioxygenase inhibitors, including dimethyloxalylglycine (DMOG) and desferrioxamine (DFO), also induced filaggrin expression (**Fig. 22c**). Similar upregulation of filaggrin mRNA and protein were observed in hypoxic human keratinocytes (**Fig. 22d**).

Although FLG expression in cultured keratinocytes is known to be induced by cell confluency and extracellular calcium (Resing et al., 1993), we found that hypoxic treatment of primary keratinocytes resulted in FLG upregulation in both subconfluent and confluent cultures (**Fig. 22e**). Similarly, hypoxic treatment further increased FLG expression even at high calcium concentration (**Fig. 22f**). We concluded that hypoxic conditions induce *Flg* expression; furthermore, this effect is independent of other known microenvironmental regulators.

Filaggrin expression is HIF-dependent. To characterize the requirement for HIF in hypoxic *Flg* induction, we generated mice lacking HIF1α and HIF2α in the epidermis using the *Cre* transgene driven by the keratin 14 (*Krt14*) promoter. *Krt14* is expressed in the basal layer of the epidermis. Mice lacking either HIF1α or HIF2α in the epidermis have been described previously and do not develop noticeable epidermal abnormalities (Boutin et al., 2008; Rezvani et al., 2011). *Krt14-Cre*$^{+}$;*Hif1a*$^{fl/wt}$;*Hif2a*$^{fl/fl}$ mice were crossed

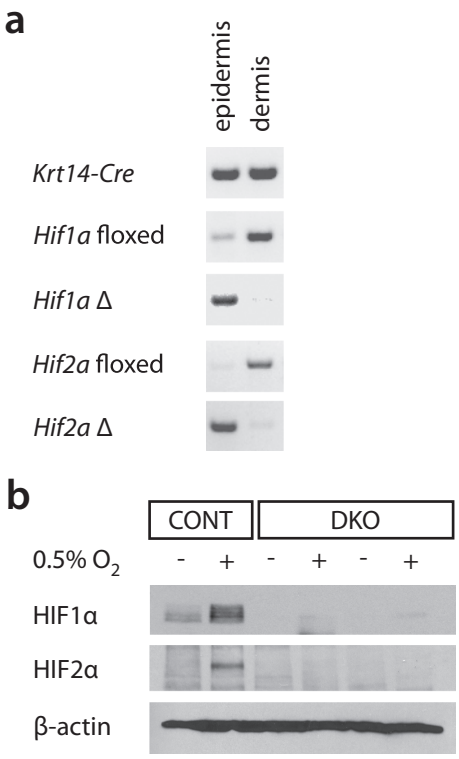

Figure 23. Epidermal *Hif1a* and *Hif2a* double knockout (DKO) mice. (**a**) PCR analysis of epidermal and dermal DNA with allele-specific primers indicated site-specific and efficient recombination. (**b**) Primary murine keratinocytes from control (CONT) or DKO P2 pups were grown at 21% or 0.5% O_2 for 48 h and lysates immunoblotted for HIF1α and HIF2α.

to produce control (*Krt14-Cre*$^{+}$;*Hif1a*$^{fl/wt}$;*Hif2a*$^{fl/fl}$) or epidermis-specific double knockout mice (DKO; *Krt14-Cre*$^{+}$;*Hif1a*$^{fl/fl}$;*Hif2a*$^{fl/fl}$). Importantly, control mice, which lack epidermal HIF1α, are phenotypically indistinguishable from wild type mice. *Cre*-mediated recombination deleted *Hif1a* and *Hif2a* efficiently and specifically in the epidermis (**Fig. 23a**). As expected, keratinocytes obtained from these mice did not stabilize HIF1α or HIF2α under hypoxic conditions (**Fig. 23b**).

FLG expression in the epidermis was significantly reduced in the absence of *Hif1a* and *Hif2a* (**Fig. 24a**). Consistent with a role for HIF in transcriptional regulation of *Flg*, DKO epidermis showed reduced levels of *Flg* transcript, profilaggrin (pro-FLG), and FLG monomer (**Figs. 24b—c**). *Flg* induction was similarly abrogated in hypoxic keratinocytes lacking either *Hif1a* and *Hif2a* or *Arnt* (**Fig. 24d**). These results indicate that HIF is required for *Flg* expression both *in vivo* and *in vitro*. FLG accumulation is also regulated by posttranslational processing of pro-FLG to monomeric FLG; this process is catalyzed by the epidermal serine proteases caspase 14 (*Casp14*) and serine protease inhibitor matriptase (*Prss8*) (Denecker et al., 2007; List et al., 2003). Both *Casp14* and *Prss8* gene expression were unchanged in DKO epidermis (**Fig. 24e**). However, *Spink5*, an inhibitor of epidermal serine proteases, was more highly expressed compared to control mice (**Fig. 24e**). *Spink5* deficiency is correlated with increased monomeric FLG in the epidermis (Chavanas et al., 2000). Therefore, the upregulation of *Spink5* in DKO mice may reduce the conversion of pro-FLG to FLG. Epidermal HIF activity thus exerts transcriptional and posttranslational control over FLG expression.

HIF-deficient mice develop skin abnormalities. DKO mice were indistinguishable from control littermates at birth. After 4—5 days (P4—P5), the hind

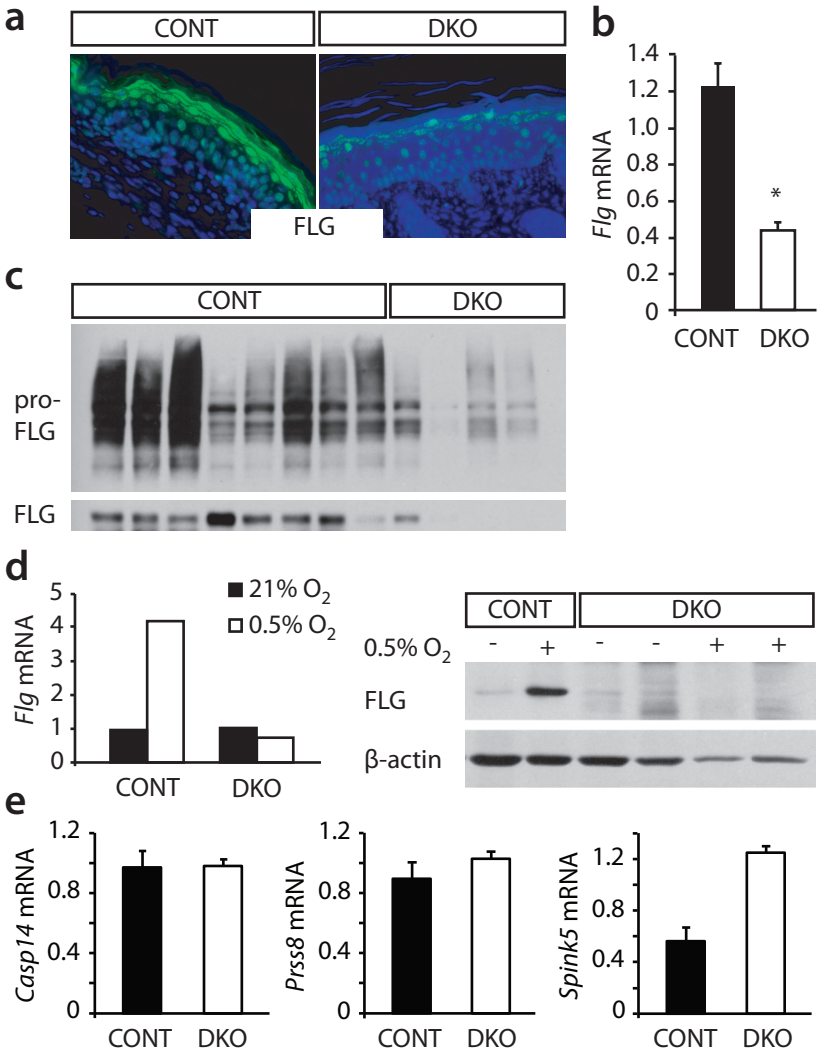

Figure 24. HIF is required for filaggrin expression *in vivo* and *in vitro*. (**a**) Paraffin-embedded skin sections from the ears of control and DKO P5 pups were immunostained using antibodies against FLG. (**b**) Epidermal RNA extracted from control and DKO P3 pups were subjected to qRT-PCR using specific primers for *Flg*. (**c**) Epidermal protein extract from control and DKO P3 pups were immunoblotted for FLG. (**d**) Primary murine keratinocytes from control (CONT) or DKO P2 pups were grown at 21% or 0.5% O_2 for 48 h and filaggrin expression determined by qRT-PCR (left) or western blotting (right). (**e**) Expression of epidermal proteases involved in FLG processing in epidermal tissue from P3 control or DKO pups were determined using qRT-PCR.

limbs and tails of DKO mice exhibited dry, flaky skin (**Fig. 25a**). By P8, DKO mice were marked by diminutive but thickened external ears, constricted digits of the hind limb, and tail constrictions that eventually led to auto-amputation (**Fig. 25b**). This set of phenotypes is associated with epidermal barrier defects in other mouse models, and most closely matched descriptions of flaky tail (*ft*) mice (Lane, 1972). Interestingly, *ft* mice possess a spontaneous mutation in the *Flg* gene (Presland et al., 2000). Although desquamation and epidermal constriction regressed concomitant with hair growth, adult DKO mice retained underdeveloped external ears and dry tail skin.

Histologically, DKO mice showed specific attenuation of the stratum granulosum (**Fig. 25c**). Reduced cornified envelope thickness in DKO tail epidermis was consistent with observed desquamation (**Figs. 25a—c**). The density of developing hair follicles was unchanged between control and DKO mice (data not shown). Importantly, stratum granulosum and cornified envelope changes were not correlated with changes in keratinocyte proliferation or cell fate. No significant difference in the number of Ki67-positive basal keratinocytes was observed between control and DKO mice (**Fig. 26**). The early differentiation marker KRT10 was appropriately expressed in suprabasal keratinocytes (**Fig. 26**). KRT6, a marker associated with dysregulated epidermal differentiation (Heyden et al., 1994), was absent in DKO epidermis (data not shown). Taken together, these results suggested that HIF deficiency in the epidermis specifically affected terminal keratinocyte differentiation in the granular layer and cornified envelope.

DKO mice exhibit defective permeability barrier and cornified envelope integrity. To investigate whether HIF loss affects epidermal barrier function, we

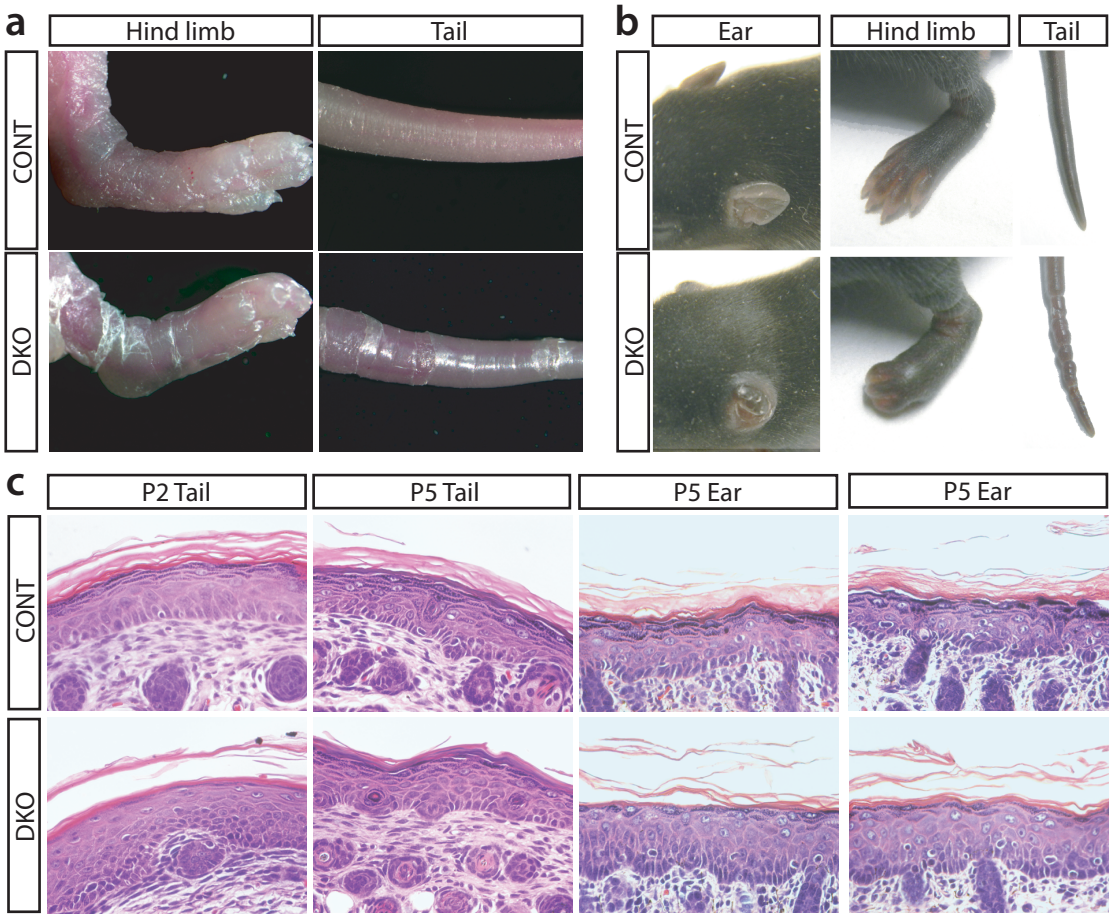

Figure 25. Gross and histological characterization of DKO mice. (**a**) Flaky skin on hind limbs and tails of DKO P5 pups compared to control littermates. (**b**) Thickened, shortened pinna of the ear, taut skin of the feet, and constricted, flaking tail skin in DKO P8 pups compared to control littermates. (**c**) Paraffin-embedded tail or ear skin sections from control or DKO were stained using hematoxyin and eosin. Stratum granulosum attenuation was observed in DKO epidermis compared to control littermates.

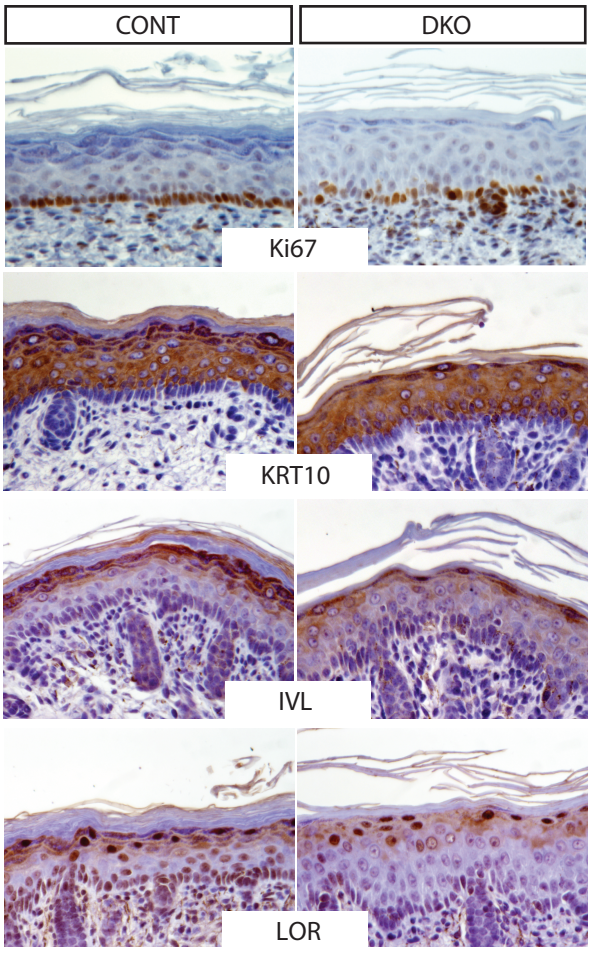

Figure 26. Immunohistochemical characterization of DKO epidermis. Paraffin-embedded skin sections from the ears of control (CONT) and DKO P5 pups were immunostained using the indicated antibodies. No observable differences were detected in Ki67, IVL, and KRT10 staining between CONT and DKO epidermis.

performed dye penetration assay on E17 embryos. In this assay, development of the outside-to-in skin barrier is reflected by impermeability to toluidine blue dye. Most control embryos efficiently excluded toluidine blue, consistent with normal development (Hardman et al., 1998). In contrast, many DKO embryos were completely or partially stained, although both control and DKO mice displayed phenotypic variability (**Fig. 27a**). By classifying embryos into those with high, moderate, or low dye permeability, we found that DKO mice were at increased risk of defective epidermal barrier, as indicated by high permeability to toluidine blue (**Fig. 27b**).

Inside-to-out barrier function was evaluated by measuring the rate of trans-epidermal fluid evaporation. We found that newborn control pups maintained constant body weight over 3.5 hours, while DKO pups suffered steady weight (i.e. water) loss during the measurement period (**Fig. 27c**). Therefore, the loss of epidermal HIF activity resulted in impaired epidermal barrier development.

To study the mechanical properties of the skin barrier, we isolated terminally differentiated keratinocytes from control and DKO cornified envelope. The cornified envelope consists of corneocytes (i.e. terminally differentiated keratinocytes) crosslinked to a lipid matrix. We hypothesized that reduced FLG expression would render *Hif*-deficient corneocytes more susceptible to mechanical stress. After mild ultrasonic disruption in a water bath, control corneocytes were largely intact while DKO corneocytes were mostly fragmented or destroyed (**Fig. 27d**). Taken together, these experiments indicate that epidermal barrier function is compromised in DKO mice, probably due to the loss of cornified envelope integrity.

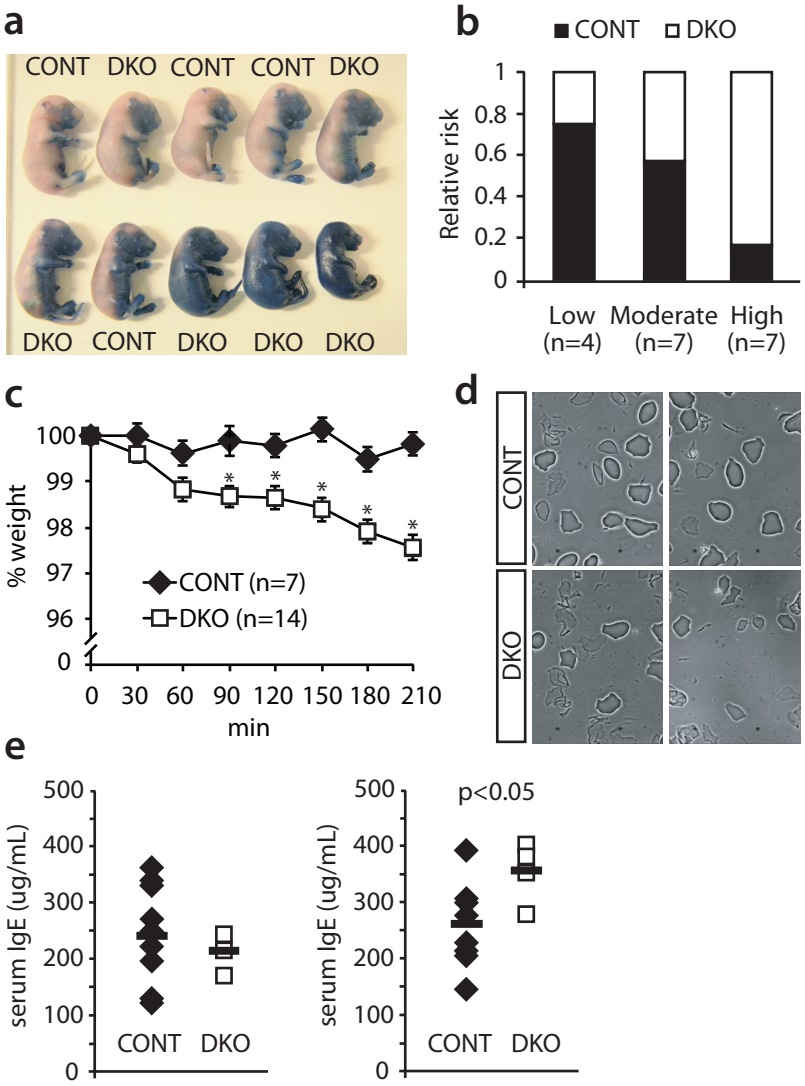

Figure 27. Impaired epidermal permeability barrier and stratum corneum integrity in DKO mice. (**a**) Control (CONT) and DKO littermates were collected at E17.5 and stained with toluidine blue dye. (**b**) Embryos were categorized into low, moderate, or high permeability groups and results from multiple experiments quantified. (**c**) Newborn CONT and DKO mice were weighed at regular intervals as indicated. (**d**) Cornified cells were isolated from CONT or DKO P3 epidermis, sonicated, and visualized under a microscope. (**e**) CONT and DKO littermates were treated topically with ovalbumin for 5 d for 3 cycles and serum IgE levels determined after treatment using ELISA. Untreated animals were included as controls.

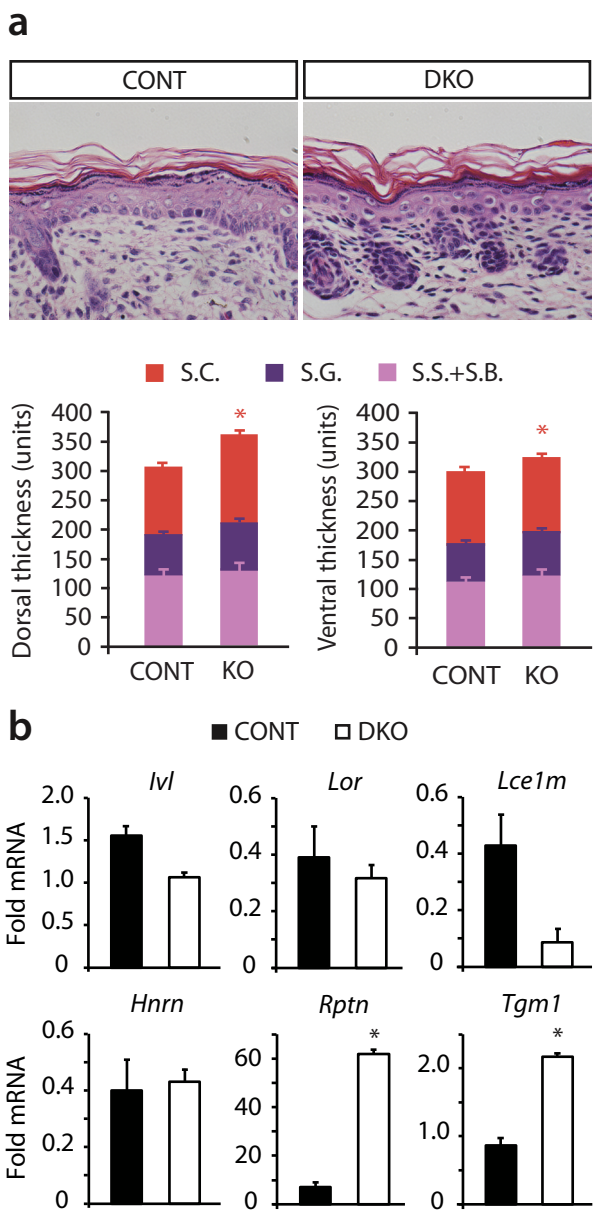

Figure 28. Expression of late cornified envelope genes in DKO epidermis. (**a**) DKO P7 pups exhibited hyperkeratotic dorsal epidermis compared to control littermates. Three thickness measurements were taken per high power field over 6 randomly selected fields. Data are presented as mean+SEM. SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basalis. (**b**) Expression of late cornified envelope genes (*Ivl*, *Lor*, *Lce1m*, *Hnrn*, *Rptn*, *Tgm1*) in epidermal tissue from control or DKO P3 pups were determined using qRT-PCR.

Epidermal barrier disruption is a common feature of inflammatory skin disorders. For example, mutations in FLG confer significantly increased risk of atopic dermatitis (Palmer et al., 2006). We applied the common allergen ovalbumin (OVA) to the skin of control and DKO mice and measured serum IgE levels as a marker of atopic response. There was no significant difference in baseline serum IgE levels between untreated control and DKO mice (**Fig. 27e**). OVA application did not alter serum IgE levels in control mice; however, OVA treatment significantly elevated IgE levels in DKO mice (**Fig. 27e**). This result suggests that epidermal barrier defects in DKO mice persist beyond the neonatal period. Furthermore, the loss of HIF activity in the epidermis may create a favorable setting for the development of atopic skin diseases.

Compensatory changes in gene expression and keratinization in DKO epidermis. Surprisingly, we observed orthokeratotic hyperkeratosis in DKO dorsal and ventral skin (**Fig. 28a**). We also conducted qRT-PCR analysis of genes important in cornified envelope formation using control and DKO epidermis. Of the five genes examined, two (*Ivl*, *Lor*) were unchanged, one (*Lce1m*) was downregulated, while two (*Rptn*, *Tgm1*) were expressed at significantly higher levels (**Fig. 28b**). *Rptn* encodes the cornified evelope protein repetin, which belongs to the same family of intermediate filament-aggregating proteins as FLG. *Tgm1* encodes transglutaminase 1, an enzyme that crosslinks lipids and proteins in the cornified envelope. Loss of *Tgm1* results in defective epidermal barrier and loss of corneocyte integrity (Kuramoto et al., 2002). We hypothesize that when HIF is absent, the detrimental effects of *Flg* downregulation are partly compensated for by the upregulation of other cornified envelope proteins, such that the severity of the skin defect is limited. This hypothesis is consistent with previous

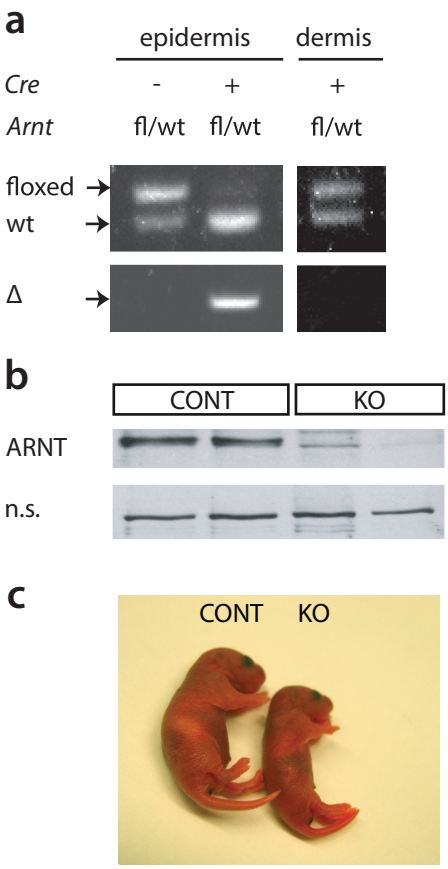

Figure 29. Epidermal *Arnt* knockout (KO) mice. (**a**) PCR analysis of epidermal and dermal DNA with allele-specific primers indicated site-specific and efficient recombination. (**b**) Cell lysates from primary murine keratinocytes obtained from control (CONT) or KO P0 pups were immunoblotted for ARNT. (**c**) Newborn KO pups appeared dimunitive and lacked milk spot compared to control littermates.

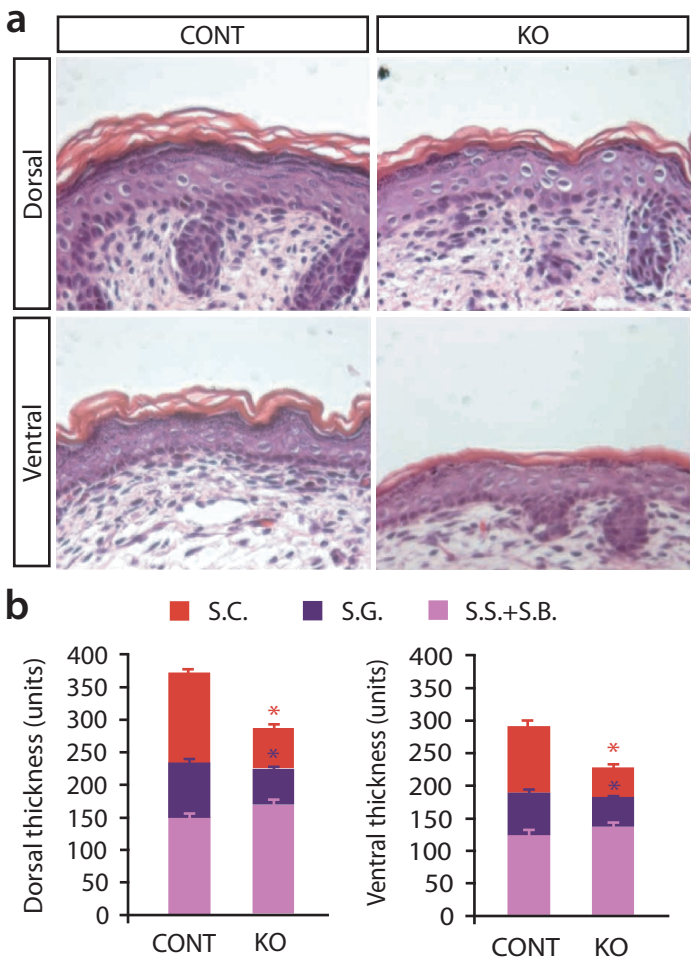

Figure 30. Attentuated strata corneum et granulosum in KO mice. (**a**) Paraffin-embedded dorsal and ventral skin sections from control and epidermal *Arnt* KO P0 pups were stained using hematoxyin and eosin. (**b**) 3 thickness measurements were taken per high power field over 6 randomly selected fields. Data are presented as mean+SEM (n=8 for each genotype). *, $p<0.05$.

reports that the absence of key epidermal proteins can result in the compensatory upregulation of other cornified envelope components (Koch et al., 2000; Kuramoto et al., 2002).

Comparison with *Arnt*-deficient mice. *Arnt* is required for ceramide synthesis in the epidermis and its absence results in epidermal barrier defects in mice (Geng et al., 2006; Takagi et al., 2003). However, ARNT mediates cellular responses to environmental toxins as well as O_2 deprivation. Therefore, the contribution of low O_2 to *Arnt* epidermal defects remained unclear. To characterize the role of ARNT in epidermal development in the context of hypoxic gene regulation, *Krt14-Cre*$^{+}$ mice were crossed to *Arnt*$^{fl/fl}$ mice to produce control (*Krt14-Cre*$^{+}$;*Arnt*$^{fl/wt}$) and KO (*Krt14-Cre*$^{+}$;*Arnt*$^{fl/fl}$) mice. *Cre*-mediated recombination efficiently and specifically deleted *Arnt* in the epidermis (**Figs. 29a—b**). Consistent with previous reports, *Arnt* KO mice were perinatal lethal (**Fig. 29c**). Histological examination of newborn pups revealed that similar to DKO mice, *Arnt* KO epidermis showed attenuation of stratum granulosum and cornified envelope thickness (**Fig. 30**). Similar to DKO mice, no difference in staining was observed for the proliferation marker Ki67, or for early differentiation proteins KRT1 and KRT10 (**Figs. 31a—b**). The skin areas that showed positive staining for the cornified envelope proteins IVL and FLG were reduced in KO mice (**Figs. 31a—b**). Importantly, *Arnt* deficient keratinocytes were unable to activate *Flg* expression under hypoxic conditions (**Figs. 32a—b**). While previous studies have proposed that epidermal barrier defects in *Arnt* KO mice are a result of deficiencies in lipid processing, these findings suggest that reduced FLG expression in O_2-deprived keratinocytes also contributes to this phenotype.

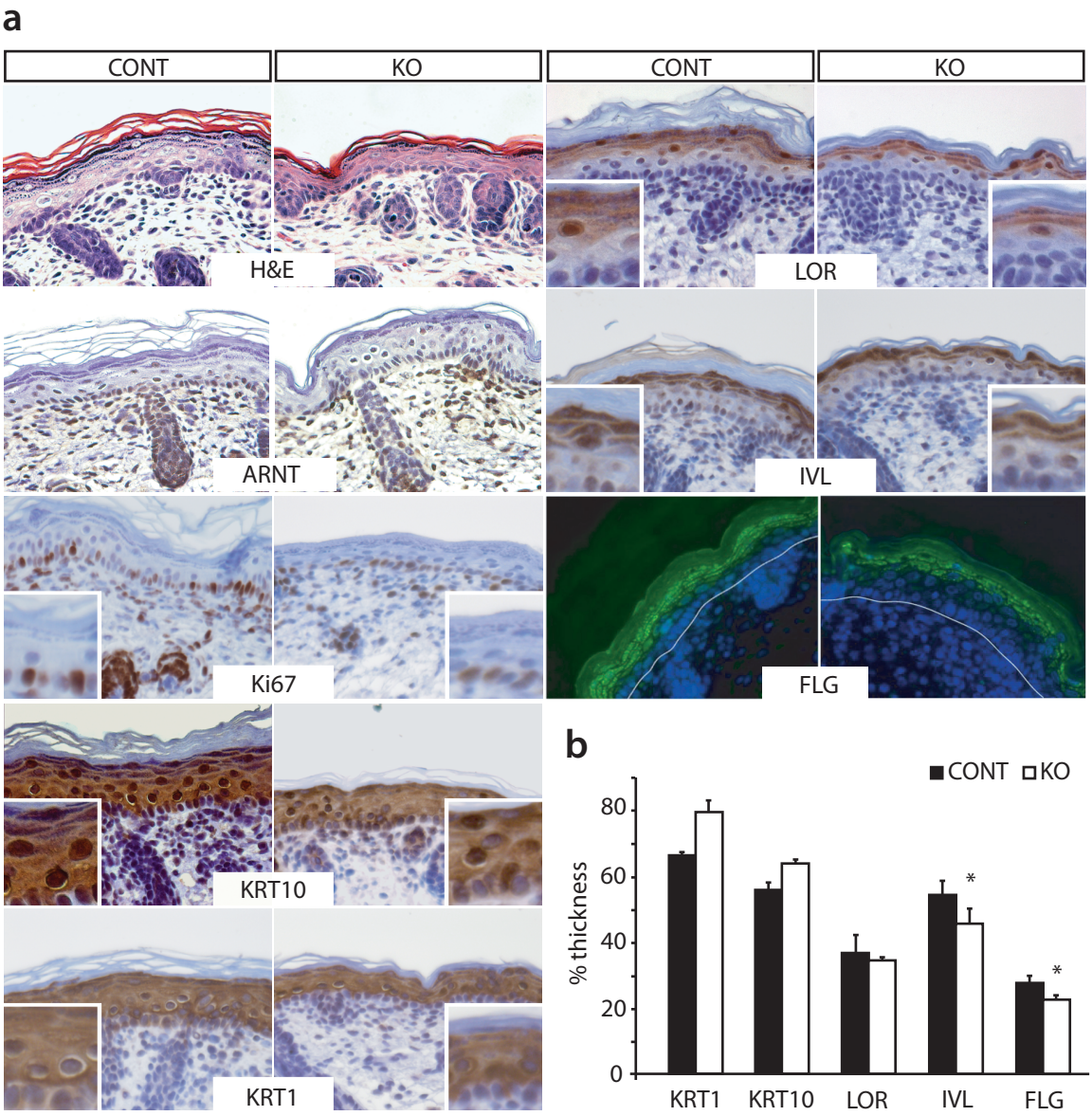

Figure 31. Immunohistochemical characterization of *Arnt*-deficient epidermis. (**a**) Paraffin-embedded dorsal skin sections from control and epidermal *Arnt* KO P0 pups were stained using hematoxyin and eosin and antibodies for various epidermal proteins as indicated. (**b**) Histological results were quantified by measuring the thickness of the whole epidermis and that of positively staining regions. 3 thickness measurements were taken per high power field over 6 randomly selected fields. Data are presented as mean+SEM (n=8 for each genotype). *, $p<0.05$

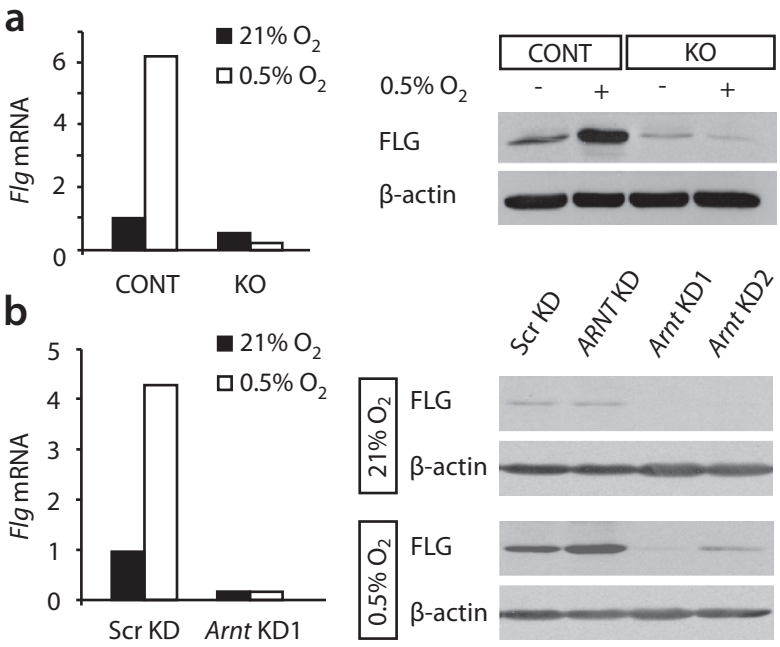

Figure 32. *Arnt* is required for hypoxic filaggrin induction. (**a**) Primary murine keratinocytes from control (CONT) or *Krt14-Cre*$^{+}$;*Arnt*$^{fl/fl}$ (KO) P0 pups were grown at 21% or 0.5% O_2 for 48 h and filaggrin expression determined by qRT-PCR (left) or western blotting (right). (**b**) Primary murine keratinocytes were transduced with lentivirus encoding scrambled sequence (Scr), sh*ARNT* (human), or sh*Arnt* (mouse) and grown under 21% or 0.5% O_2 for 48 h. Filaggrin expression was determined by qRT-PCR (left) or western blotting (right).

DISCUSSION

In this study, we demonstrated the importance of hypoxic signaling in skin development. We showed that a low-O_2 microenvironment exists in the murine epidermis during embryonic development. Low-O_2 conditions stabilize HIF1α and HIF2α, the absence of which impaired terminal keratinocyte differentiation and formation of the epidermal barrier. These results demonstrate that in addition to its influences on innate immunity (Peyssonnaux et al., 2007), erythropoiesis (Boutin et al., 2008), and cancer (Bedogni et al., 2005), HIF activity in keratinocytes also regulates epidermal development.

Although epidermal O_2 saturation has been studied for many decades, the reasons for low O_2 tension in the epidermis remain poorly understood (Evans and Naylor, 1967; Evans et al., 2006; Stewart et al., 1982). Possible causes of low O_2 tension include the lack of epidermal vascularization, high O_2 consumption in dividing basal keratinocytes, and low pO_2 in amniotic fluid (Bejar et al., 1971). Unlike other microenvironmental regulators of keratinocyte differentiation such as extracellular calcium and basement membrane adhesion, which coordinate the expression of a set of genes, HIF activity under low-O_2 conditions induces *Flg* expression, but not other major cornified envelope proteins. While HIF1α and HIF2α can sometimes act in distinct and opposing fashion (Keith et al., 2012), they promote *Flg* expression and epidermal barrier development in a redundant manner. Epidermal deletion of *Hif1a* or *Hif2a* individually does not cause developmental defects in mice (Boutin et al., 2008; Rezvani et al., 2011). Interestingly, loss of *Hif1a* alone results in increased epidermal atrophy and delayed wound healing in aged mice (Rezvani et al., 2011). While this finding could suggest that

distinct requirements exist for HIF1α and HIF2α at different developmental stages, it is equally possible that these changes are secondary to the non-redundant roles of epidermal *Hif1a* in ischemic wound healing (Biswas et al., 2010), immune function (Peyssonnaux et al., 2007), and cell migration (Fitsialos et al., 2008).

FLG is a critical contributor to keratinocyte differentiation and epidermal barrier function. Its regulation is complex and involves multiple transcription factors: examples include Jun, c-Fos, OCT1, OCT6, and specific isoforms of p63 (Sandilands et al., 2009). We identified HIF1α and HIF2α as novel participants in this transcriptional network. Hypoxic *Flg* induction was similarly abrogated in *Arnt* deficient keratinocytes, which strongly suggests that the HIF1/2α—ARNT heterodimer governs *Flg* gene expression transcriptionally. However, the exact mechanism by which HIF promotes *Flg* transcription remains unclear. HIF1α and ARNT were not enriched at putative hypoxia response elements in the *Flg* promoter region (data not shown). Current experiments to address this question include systematic mutagenesis of *Flg* promoter and RNAi-mediated silencing of known *Flg* regulators under hypoxic conditions.

FLG mutations and deletions are common causes of atopic dermatitis and ichthyosis vulgaris (Palmer et al., 2006; Smith et al., 2006). Ichthyosis vulgaris is characterized by fine scaling on the extremities, absence of keratohyalin granules, and attenuation of the stratum granulosum (Compton et al., 2002), features reminiscent of DKO phenotypes. DKO mice also phenocopied flaky tail mice, which harbor a spontaneous *Flg* mutation, on both gross and histological levels (Lane, 1972; Presland et al., 2000). Although similar phenotypes and epidermal barrier defects can also result from genetic manipulation of *Ela2* (Bonnart et al., 2010), *Ets1* (Nagarajan et al., 2010),

Tgm1 (Matsuki et al., 1998), *Casp14* (Denecker et al., 2007), *Lor* (Suga et al., 2000), and *Prss8* (List et al., 2003), expression of these genes were either unchanged in DKO mice or altered in the contrary direction. Taken together, these results suggest that defective keratinocyte differentiation and epidermal barrier malformation in DKO mice could be attributed to reduced *Flg* expression. To formally prove this hypothesis, we plan to perform a genetic rescue via intra-amniotic injection of lentivirus encoding *Flg* (Endo et al., 2008). Driven by the human IVL promoter, this transgene should restore FLG expression in the granular layer and cornified envelope during embryogenesis. We predict that this intervention should restore epidermal barrier function and normal skin structure in DKO mice.

Surprisingly, *Arnt* KO mice were invariably perinatal lethal while most DKO mice survived into adulthood. This finding suggests that ARNT and HIF activity in the epidermis only partially overlap: while both are required for *Flg* induction and epidermal barrier formation, ARNT may fulfil additional vital functions. For instance, ARNT regulates ceramide synthesis in the epidermis as well as AHR-dependent effects on keratinocyte differentiation in culture (Geng et al., 2006; Sutter et al., 2009; Takagi et al., 2003). Another possibility is that the depletion of HIF activity may be more complete in *Arnt* KO than DKO mice. DKO mice were weaned at sub-Mendelian ratios, suggesting that some degree of perinatal lethality may exist in correlation with higher deletion efficiency (**Table 2**).

Flg belongs to a family of "fused" genes that encode intermediate filament-aggregating proteins of the epidermis. Other members of this family include *Rptn* and *Hnrn*; *Tchh*, another family member, is mainly expressed in the hair follicle. The

Table 2. Predicted and observed numbers of *Krt14-Cre*$^{+}$;*Hif1a*$^{fl/wt}$;*Hif2a*$^{fl/fl}$ progeny.

Genotype	**Expected** (n=66)	**Observed** (n=66)
Krt14-Cre^{+};*Hif1a*$^{fl/fl}$;*Hif2a*$^{fl/fl}$	11.5 (25%)	5 (11%)
Krt14-Cre^{+};*Hif1a*$^{fl/wt}$;*Hif2a*$^{fl/fl}$	23 (50%)	28 (61%)
Krt14-Cre^{+};*Hif1a*$^{wt/wt}$;*Hif2a*$^{fl/fl}$	11.5 (25%)	13 (28%)

expression of *Rptn*, but not *Hnrn*, was dramatically upregulated in DKO epidermis. *Tgm1*, a crosslinker of cornified envelope constituents, was also significantly upregulated. We interpreted these unexpected changes in *Rptn* and *Tgm1* expression to be part of a compensatory response to *Flg* downregulation in DKO mice. Compensatory induction of cornified envelope proteins in mice with defective epidermal barriers is known to result in epidermal hyperkeratosis (Kuramoto et al., 2002; Suga et al., 2000). Consistent with this hypothesis, trunk epidermis of DKO mice indeed exhibited hyperkeratosis. Interestingly, this phenotype reverted with the onset of hair growth and epidermal dedifferentiation between 15 and 18 days after birth (data not shown). However, serum IgE measurements taken after cutaneous ovalbumin application indicated that DKO mice remained permissive to epicutaneous sensitization up to two months of age. This finding suggested that despite phenotypic resolution, defects in the epidermal barrier persisted beyond neonatality.

The link between epidermal barrier defects and atopic skin diseases became firmly established with the identification of *FLG* as a susceptibility gene (Palmer et al., 2006). However, only 30% of European patients with atopic dermatitis bear *FLG* mutations (Bieber, 2008). Recent genome-wide association studies have uncovered additional loci in *OVOL1*, *C11orf30*, *HNRN*, *TNFRSF6B*, *ZGPAT*, *TMEM232*, *SLC25A46*, *ACTL9*, and *KIF3A* that confer risk for atopic dermatitis (Esparza-Gordillo et al., 2009; Paternoster et al., 2012; Sun et al., 2011). Some of these gene products are closely involved in epidermal differentiation. For example, OVOL1 suppresses loricrin expression (Nair et al., 2006), while HNRN is a component of the cornified envelope (Henry et al., 2011). FLG regulation has also been implicated at multiple levels: besides mutations in FLG itself,

polymorphisms and mutations in its negative regulator *SPINK5* are associated with ichthyosis and atopic dermatitis (Chavanas et al., 2000, 5; Walley et al., 2001). Based on the data presented here, we hypothesize that HIF pathway mutations or polymorphisms may be similarly associated with skin diseases arising from FLG dysregulation and epidermal barrier defects. *EPAS1* (encoding HIF2α) and *VHL* variants have been identified in renal clear cell carcinoma, Chuvash polycythemia, familial erythrocytosis, and high-altitude adaptations (Ang et al., 2002; Beall et al., 2010; Percy et al., 2008; Purdue et al., 2011; Simonson et al., 2010; Yi et al., 2010). Genetic investigation of this possibility will enhance our understanding of the mechanisms by which the low-O_2 microenvironment of the epidermis regulates skin development and homeostasis.

Chapter Four

Concluding remarks

The experiments described in the previous chapters concern how cells respond to low O_2 tension in two different biological contexts. First, do cancer cells modulate oncogenic pathways in response to O_2 deprivation? Second, do normal cells sense O_2 deprivation as a developmental cue? In the first set of experiments, I used colon carcinoma cell lines to demonstrate that the MYC oncoprotein is suppressed during low O_2 tension. This change is correlated with reduced MYC transcriptional activity, cell cycle arrest, and cell viability under hypoxic stress. I demonstrated that hypoxic MYC downregulation is a consequence of increased proteasomal activity via the E3 ubiquitin ligases FBXW7 and DDB1, as well as cathepsin gene upregulation under hypoxic conditions. In the second set of experiments, I used murine genetic models to show that HIFs are required for normal skin development. Mice lacking HIF1α and HIF2α specifically in their epidermis displayed defective keratinocyte differentiation, reduced expression of the major cornified envelope protein filaggrin (FLG), and impaired epidermal barrier function. Collectively, this work has furthered our understanding of the cellular mechanisms by which hypoxia modulates MYC activity in cancer cells, and also provided a basis for studying hypoxic responses in the novel context of epidermal development.

In this chapter, I shall attempt to address the implications of these findings for future research in terms of the immediate context as well as the broader scientific view.

HIF, O_2 and MYC

I have demonstrated that MYC is degraded under hypoxic conditions by cathepsin activity, as well as the E3 ligases FBXW7 and DDB1. An immediate task will be to establish the molecular mechanisms by which cathepsin expression and MYC ubiquitylation are regulated by low O_2 tension. Experiments described in chapter two have provided the first conclusive evidence for hypoxic induction of cathepsins D and S at the mRNA and protein levels. However, it remains uncertain whether cathepsins are direct HIF target genes. Microarray data suggest that hypoxic induction of cathepsin expression is HIF-dependent: cathepsin gene expression was reduced in hypoxic A498 cells in which HIF activity had been ablated (Bertout et al., 2009; GEO accession GSE16622). Another potential regulatory mechanism may lie in hypoxic repression of endogenous cathepsin inhibitors. One report suggested that hypoxic treatment reduces cystatin C levels in tumor cells, without defining the functional significance of this effect (Wickramasinghe et al., 2005). Therefore, further experiments in this area will enhance our understanding of hypoxic regulation of cellular proteolytic activity.

Similarly, little is known about the mechanisms by which hypoxia enhances MYC degradation via the E3 ligases FBXW7 and DDB1. MYC ubiquitylation by FBXW7 requires upstream phosphorylation events at Thr58 and Ser62 (Sears et al., 2000). These modifications promote MYC recognition and binding by the ubiquitinase complex (Sears et al., 2000). Surprisingly, although hypoxic treatment increases MYC ubiquitylation, T58/S62 phosphorylation is unchanged. On the other hand, knockdown experiments indicated that FBXW7, as well as DDB1, is required for increased MYC turnover under hypoxia. These results suggest that hypoxic stimulation of MYC ubiquitylation is

independent of upstream phosphorylation signals. One possible scenario may be that hypoxia directly enhances MYC association with its E3 ligases. To test this hypothesis, immunoprecipitation experiments can be performed to assess MYC binding to FBXW7 and DDB1. Alternatively, hypoxia could modulate the intracellular localization of MYC and its E3 ligases. Although I hypothesized that hypoxia promotes MYC cytoplasmic accumulation, so that it becomes more accessible to ubiquitylase complexes and cytosolic proteases, I found that nuclear and cytoplasmic distribution of MYC was unaltered by hypoxic treatment (data not shown). MYC and proteasomes can co-localize within the nucleoli of MYC -overexpressing cells (Arabi et al., 2003). It remains unclear whether hypoxia affects this process, and whether E3 ligases including FBXW7 and DDB1 can be identified in these structures also.

The finding that MYC^{T58A} overexpression promotes cell death in hypoxia indicates that hypoxic MYC suppression in certain tumor settings may lead to survival advantages. Furthermore, a recent report suggested that MYC suppression may have additional pro-tumorigenic effects (Liu et al., 2012). The authors found that MYC expression inhibits cancer cell migration and metastasis via transcriptional repression of $\alpha_v\beta_3$ integrin (Liu et al., 2012). However, this finding is controversial, because MYC overexpression is known to promote tumor metastasis by downregulating E-cadherin expression (Ma et al., 2010). Interestingly, hypoxia also suppresses E-cadherin, presumably via MYC-independent mechanisms (Esteban et al., 2006). HIF also directly induces the expression of lysyl oxidase (LOX) in hypoxic cancer cells, thereby promoting metastasis (Erler et al., 2006). To generalize, HIF activity and MYC inhibition may cooperate in some O_2-deprived tumors to activate multiple pathways that enhance cancer

cell survival and migration. One conceivable model could be that HIF activity upregulates *LOX* expression, suppresses MYC-dependent integrin expression, and inhibits E-cadherin expression via *SNAIL1*, thus stimulating cancer cell motility in a coordinated manner.

It will be important to demonstrate the survival and/or migratory benefits of hypoxic MYC suppression *in vivo*. The generation of HCT116 cells overexpressing MYC under the control of a hypoxia responsive promoter (e.g. *PGK1*) would provide a useful reagent for defining the relationship between hypoxia, MYC activity, and tumorigenesis in a xenograft model. HCT116 cells retain an intact p53 pathway, which may explain why MYC^{T58A} overexpression in these cells promotes cell death under hypoxic stress. High MYC expression in normal cells triggers apoptosis; in contrast, many MYC-driven cancers have gained the ability to suppress p53 activity (Eischen et al., 1999; Zindy et al., 1998). This fact illustrates the important notion that the consequences of MYC activity under hypoxic stress depend on the sum of genetic and epigenetic changes in the cell. Under hypoxic stress, tumor cells that have inactivated pro-apoptotic pathways will be able to utilize MYC to control proliferation, metabolism, and mutagenesis. However, those cells that have not evolved resistance to apoptosis must suppress MYC expression in order to survive.

This distinction between cancer cells which have evolved a reliance on MYC pathways, and those which have not, is likely to be clinically important. In the context of antitumor therapy, many studies have demonstrated that MYC-driven cancers are also MYC-addicted (Felsher, 2010). In other words, inhibition of MYC activity in these cells causes death (Gao et al., 2009; Soucek et al., 2008; Wise et al., 2008). The development of

MYC therapeutics has been energized by the recent discovery that inhibition of BET bromodomain regulatory proteins exerts potent activity against MYC. Emerging data indicate that BET inhibitors induce dramatic regression of MYC-driven multiple myeloma and lymphomas (Delmore et al., 2011; Mertz et al., 2011; Zuber et al., 2011). However, this and other studies suggest that in cancer cells which do not strictly depend on MYC activity, MYC suppression may lead to pro-tumorigenic effects. Therefore, anti-MYC therapy should be approached with care.

Another direction for future research will be to assess the impact of O_2 deprivation on MYC pathway activity from a global perspective. MYC target genes involved in ribosomal biogenesis, DNA replication, and cell cycle progression were suppressed by hypoxic treatment. Intriguingly, two new studies have proposed that MYC does not act on a defined group of 'target genes'; rather, MYC amplifies the existing gene expression profile in any cell (Lin et al., 2012; Nie et al., 2012). MYC overexpression results in increased MYC binding to active promoters, P-TEFb recruitment, and transcriptional elongation (Lin et al., 2012; Nie et al., 2012). As a result, MYC promotes global upregulation of actively transcribed genes. These studies explain why MYC gene expression signatures in various cell types differ greatly (Dang et al., 2012), but also radically challenge our understanding of MYC transcriptional biology. Importantly, although MYC acts universally at all active promoters, MYC binding affinity is stronger in the presence of conserved E-box sequences (Lin et al., 2012). Therefore, promoter sequence may potentiate gene sensitivity towards MYC-dependent upregulation. An unbiased survey of MYC occupancy and gene expression changes under low-O_2 conditions will provide invaluable information on the extent of O_2-dependent effects on

MYC. For example, does hypoxia preferentially regulate genes with strong/weak MYC binding, or does it reduce gene expression globally via MYC suppression? Furthermore, how does the transcriptional amplification model explain the observation that HIF1α and MYC cooperate to activate glycolytic gene expression under hypoxic conditions (Kim et al., 2007)?

In contrast to cancer cells, MYC activity in normal tissues lies under the strict control of upstream pathways such as Wnt, BMP, and Notch signaling (Hayward et al., 2008; Wang et al., 2004). Proper regulation of MYC expression by these signaling pathways is critical for embryonic development and adult tissue homeostasis. Interestingly, regions of low O_2 tension exist in the developing embryo and adult tissue niches (Simon and Keith, 2008), suggesting that 'physiological hypoxia' may also participate in the control of MYC expression. Therefore, I hypothesized that the epidermis, which is mildly hypoxic, might be such an environment in which MYC and HIF intersect. Although keratinocytes downregulated MYC under hypoxic conditions as expected, epidermal deletion of HIF1α and HIF2α did not produce changes in the skin that were indicative of MYC perturbation. DKO mice did not display keratinocyte hyperproliferation and hyperplasia, hair loss, or spontaneous skin lesions (Arnold and Watt, 2001; Bull et al., 2005; Waikel et al., 2001; Zanet et al., 2005). This result suggested that either hypoxic effects on MYC activity in keratinocytes are independent of HIF1α and HIF2α, or the hypoxic environment of the epidermis does not influence MYC activity. Instead, DKO mice developed epidermal defects that bore significant resemblance to filaggrin (*Flg*) mutant animals.

The observation that hypoxia regulates *Flg* expression via HIF1α and HIF2α raises a number of interesting questions to address in future studies. The critical next step would be to identify the molecular mechanisms by which HIF induces *Flg* transcription. As described in chapter three, chromatin immunoprecipitation experiments failed to identify HIF1α or ARNT at putative hypoxia response elements (HREs) in the *Flg* promoter. This finding suggests that HIF1α and HIF2α do not transactivate *Flg* via canonical HRE binding. One possibility is that HIF1α and HIF2α modulate the activity of other transcription factors that are important for *Flg* expression. Computer-based predictions of transcription factor binding at the *Flg* promoter have identified a list of candidate proteins. Some of these proteins, such as AP1 and ETS1, are hypoxia-responsive in other biological settings (Elvert et al., 2003; Yao et al., 1994). Based on these predictions, a limited RNAi screen can be performed to identify transcription factors that are required for hypoxic *Flg* induction. Additionally, promoter mutagenesis studies will help to define hypoxia-responsive elements in the *Flg* promoter for more detailed investigation.

Another research priority will be to establish an *in vivo* link between HIF, *Flg*, and the epidermal barrier. Although hypoxic induction of *Flg* in keratinocytes requires HIF1α and HIF2α, DKO defects in epidermal and barrier development—similar as they are to *Flg* mutant phenotypes (Lane, 1972; Smith et al., 2006)—cannot be attributed conclusively to reduced FLG expression in these mice. As described in chapter three, lentivirus encoding *Flg* can be injected intra-amniotically into DKO embryos. Functional rescue of the epidermal barrier in DKO mice with *Flg* overexpression will demonstrate

the dependence of the DKO phenotype on FLG levels. This work is in the planning stage in collaboration with Flake and colleagues (Endo et al., 2008).

The realization that *Flg* expression is HIF-dependent may allow its function to be studied in novel contexts. Intriguingly, microarray data indicate that *FLG* is upregulated in *VHL* mutant clear cell renal cell carcinoma (ccRCC) (Hu et al., 2003). In these tumors, *VHL* loss results in the normoxic stabilization of HIF1α and HIF2α. Therefore, ccRCC may represent another biological setting in which HIF activity controls FLG expression. Little is known about the role of FLG in non-epidermal tissues. For example, does it bind and organize intermediate filaments, or does it possess another function in non-keratinocytes? Given the importance of intermediate filaments in mediating tumor invasion and metastasis (Kleeberger et al., 2007; Schoumacher et al., 2010), it is tempting to speculate that interactions between FLG and intermediate filaments may influence cancer cell migration. If *FLG* expression in ccRCC can be confirmed, then further investigation could involve biochemical characterization of potential interactions between FLG and intermediate filaments in ccRCC, investigation of FLG effects on cytoskeletal changes, and importantly, studying the role of FLG expression in ccRCC development and metastasis.

Past research on epidermal development has relied on the transit amplification model of keratinocyte differentiation. In this model, keratinocyte stem cells in the basal layer of the epidermis give rise to transit amplifying cells, which proliferate for several cycles before committing to terminal differentiation (Jones et al., 2007). However, recent lineage tracing experiments failed to identify transit amplifying cells in the epidermis (Clayton et al., 2007). Instead, the authors found that basal keratinocytes undergo

asymmetric division at a constant rate, and this process is sufficient to maintain skin homeostasis. Surprisingly, deletion of *Hif1a* and *Hif2a* in basal keratinocytes did not affect keratinocyte proliferation or commitment to differentiate; only the terminal developmental step of epidermal cornification was impaired. The requirement for HIF1α and HIF2α clearly differs between various progenitor cell pools, even when a hypoxic niche is present (Majmundar et al., 2012; Mazumdar et al., 2010b). Intriguingly, an RNAi screen for regulators of hair follicle stem cell self-renewal *in vitro* identified *Egln3* (encoding PHD3) as a positive regulator of 'long-term self-renewal', whereas *Hif1a* had an opposite role (Chen et al., 2012). While many caveats apply, this result suggests that HIF activity in hair follicle stem cells may promote differentiation. DKO mice (which lack *Hif1a* and *Hif2a* in the epidermis as well as the hair follicle) exhibit an equal number of developing hair follicles as control littermates at birth. However, I have not examined whether hair follicle stem cell quiescence is enhanced in DKO animals.

Whereas targeted deletion of *Hif1a* and *Hif2a* during embryogenesis by non-inducible *Krt14-Cre* resulted in epidermal developmental defects, the inducible *Krt14-Cre*^{ERT} transgene would provide a powerful tool for investigating the role of HIF1α and HIF2α in adult epidermis. The adult epidermis, similar to its embryonic counterpart, is naturally O_2-deprived. However, the hypoxic response in adult and embryonic keratinocytes may differ. For example, neonatal DKO mice exhibit desquamation, epidermal constrictions, and granular layer attenuation, but adult DKO mice do not. Therefore, it will be important to determine whether distinct roles for HIF1α and HIF2α exist during skin development and adult homeostasis. Using a similar strategy, Trumpp

and colleagues have revealed that MYC is required for intestinal crypt development, but not for crypt homeostasis in the adult (Bettess et al., 2005).

Inducible deletion of epidermal HIF1α and HIF2α can also be used to study disease processes in the adult epidermis. Given the high prevalence of skin malignancies in the northern hemisphere (Rogers et al., 2010), it will be important to examine the role of the hypoxic microenvironment in skin carcinogenesis. For example, the requirement for epidermal HIF1α and HIF2α in skin tumorigenesis can be assessed in the context of $KRAS^{G12D}$ mutation and p53 loss (Caulin et al., 2007). A similar genetic approach has identified a tumor suppressive role for HIF2α in lung adenocarcinoma (Mazumdar et al., 2010a). Alternatively, skin carcinogenesis can be induced by DMBA/TPA administration. A recent study demonstrated that DMBA/TPA treatment in mice bearing endothelial cell-specific deletion of *Epas1* (encoding HIF2α) leads to smaller skin papillomas (Skuli et al., 2012). By comparing these results with the effects of epidermal *Hif1a* and *Hif2a* deletion, we shall better understand the contributions of different cellular compartments to skin carcinogenesis.

Finally, the discovery that HIFs regulate epidermal cornification and barrier formation raises an important clinical question. With regard to tissue engineering, skin can be grown *in vitro* using three-dimensional keratinocyte culture. In this system, cornified envelope formation is induced by raising the epidermis above the air-liquid interface (Harriger and Hull, 1992). Given that low O_2 tension upregulates *Flg* expression in keratinocytes, cornification of artificial skin may be enhanced with hypoxic treatment. This manipulation may improve barrier function in skin grafts and lead to better

protection against fluid loss and pathogen entry in surgical and burns patients (Metcalfe and Ferguson, 2007; Sabolinski et al., 1996).

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