Published as: *Nat Rev Cancer*. ; 12(1): 9–22.

HIF1α and HIF2α: sibling rivalry in hypoxic tumor growth and progression

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Preface

Hypoxia inducible factors (HIFs) are broadly expressed in human cancers, and HIF1α and HIF2α were previously suspected of promoting tumor progression through largely overlapping functions. However, this relatively simple model has now been challenged in light of recent data from genome-wide analyses of human tumors, genetically engineered mouse models of cancer, and systems biology approaches that reveal unique and sometimes opposing HIFa activities in both normal physiology and disease. These effects are mediated in part through regulation of unique target genes, as well as direct and indirect interactions with important oncoproteins and tumor suppressors, including MYC and p53. As HIF inhibitors are currently under clinical evaluation as cancer therapeutics, a more thorough understanding of unique roles performed by HIF1α and HIF2α in human neoplasia is warranted. This Review summarizes our rapidly changing understanding of shared and independent HIF1α and HIF2α activities in tumor growth and progression, and the implications for using selective HIF inhibitors as cancer therapeutics.

Introduction

Oxygen (O_2) levels are known to vary widely across sub-domains of solid tumors, due to rapid cell division and aberrant tumor angiogenesis and blood flow. Although extended exposure to complete O_2 deprivation (anoxia) can result in necrosis, viable hypoxic cancer cells often surround necrotic zones. Tumor hypoxia has long been associated with increased malignancy, poor prognosis and resistance to radiotherapy and chemotherapy (reviewed in[1,2]), prompting intensive research into cellular responses to O_2 deprivation. Particular interest has been focused on the mechanisms by wh ich hypoxic tumor cells alter their transcriptional profiles to modulate glycolysis, proliferation, survival and invasion to persist under conditions of hypoxic stress[3].

The Hypoxia Inducible Factor (HIF) transcription factors mediate the primary transcriptional responses to hypoxic stress in normal and transformed cells. HIFs are

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heterodimeric complexes composed of bHLH-PAS proteins including an O_2-Iabile alpha subunit (HIF1α, HIF2α, or HIF3α) and a stable beta subunit (HIF1β, also known as ARNT), which together bind hypoxia responsive elements (HREs) containing a conserved RCGTG core sequence (see Box 1). Hypoxic HIF activity is controlled primarily through post-translational modification and stabilization of HIF1α and HIF2α subunits, so that HIFa protein levels and overall HIF transcriptional activity increase as cells become more hypoxic. The central molecular mechanisms underlying the O_2-lability of HIFa subunits were first elaborated in 2001 by multiple groups, and are the subject of several recent reviews[4,5] (Box 1). Briefly, HIFa subunits are modified by HIF-specific prolyl-hydroxylases (PHDs) in the presence of O_2, leading to normoxic proteasomal degradation mediated in part by the Von Hippel Lindau tumor suppressor protein (pVHL) (Box 1). It is also important to note that elevated oncogenic signaling in cancer cells can induce HIFa expression through O_2-independent mechanisms including increased transcription and/or translation of HIFα mRNAB[6].

HIF1α was first described by Semenza and colleagues in 1995, and was shown to playa central role in mediating O_2-dependent transcriptional responses[7]. The identification of HIF2α by independent groups in 1997 (initially called endothelial PAS protein 1 (EPAS1)[8], HIF-related factor (HRF)[9], HIF1α-like factor (HLF)[10], and member of PAS family 2 (MOP2)[11]) indicated that HIF regulation was more complex. Whereas HIF1α appears to be expressed in nearly all cell types, RNA in situ hybridization on mouse embryos revealed that HIF2α expression is more restricted, and particularly abundant in blood vessels. This observation led to the hypothesis that the primary role of HIF2α is to modulate vascular endothelial cell (Ee) function, an idea supported in part by the close correlation of HIF2α and VEGF mRNA expression patterns[8]. A more complex view emerged as HIF2α protein expression was identified in multiple cell types in hypoxic rat kidney, lung, and colonic epithelia, as well as hepatocytes, macrophages, muscle cells and astrocytes[12], indicating that both HIF1α and HIF2α are co-expressed in a large number of cell types.

The majority of HIF transcriptional responses have been attributed to HIF1α and HIF2α; however, a third HIFα subunit (HIF3α) has also been described[13]. HIF3α mRNA is differentially spliced to produce multiple isoforms that either promote or inhibit the activity of other HIF complexes, although little is yet known about the impact of HIF3α on hypoxic tumor progression[14-17]. Similarly, a second ARNT protein (ARNT2) has been identified[18] and shown to regulate neuronal development[19] and exhibit overlapping activity with $ARNT_{20}$; however, its activity in human cancer cells has not been studied in depth[21]. Although it will be important to determine whether (and how) HIF3α and ARNT2 affect HIF-mediated responses in cancers, the available evidence suggests that HIF1α and HIF2α account for the vast majority of HIF-dependent effects on tumor growth and progression described to date.

Elevated expression of HIF1α and HIF2α protein has been observed in a broad array of human cancer cell types, and associated with poor prognosis in many cases (Table 1). Particular attention has been focused on renal clear cell carcinomas (RCCs), approximately 90% of which lose function of the Von Hippel-Landau tumor suppressor protein (pVHL), which binds prolyl-hydroxylated HIFα subunits and targets them for ubiquitin-mediated proteolysis[22] (Box 1). pVHL-deficient RCC cell lines consequently cannot degrade HIFα subunits in an 02-dependent manner, and have been used extensively to investigate the roles of HIF1α and HIF2α in tumor growth.

The observations summarized in Table 1 have led to the general view that elevated HIFα protein expression in tumor cells, whether induced by hypoxia or aberrant oncogenic signaling, actively drives tumor growth and progression by regulating the expression of

critical target genes. Disparate correlations have been observed in some tumor types; for example, HIF1α expression has been associated with both better and worse prognosis in separate analyses of renal and non-small cell lung cancers (see Table 1). The basis of these apparent discrepancies is not understood, but may reflect the consequences of HIF activity in different cancer subtypes, or at different stages of tumor progression. In some tumors, including gastric cancers and glioma, only one HIFα subunit is correlated with prognosis, suggesting it plays a particularly important role or predominant role in these tumor cell types. Interestingly, multiple recent studies have also revealed unexpected tumor suppressive activities of HIF1α and HIF2α in specific contexts[23-26]. Although initially viewed as having largely overlapping functions, there is now mounting evidence that HIF1α and HIF2α can promote highly divergent – even opposing – outcomes when expressed in the same cell type. It appears that HIF1α and HIF2α mediate these disparate responses partly through independent regulation of distinct target genes, but also through direct and indirect interactions with complexes containing important oncoproteins and tumor suppressors.

Direct regulation of gene expression by HIF1α and HIF2α

Numerous early studies revealed that either HIF1α or HIF2α could regulate the expression of many hypoxically induced genes, but that each HIFα isoform also had unique targets (Table 2)[27,28]. By swapping protein domains between HIF1α and HIF2α, several groups demonstrated that this transcriptional specificity resided in the N-terminal activation domain (N-TAO), suggesting that differential interactions with transcriptional co-factors likely determine differential gene activation[29,30]. Recently, multiple groups have used chromatin immunoprecipitation coupled to tiled microarrays (ChiP-chip) to assess HIFα binding across the genome[31-35]. These analyses confirmed the RCGTG core binding sequence, and revealed no additional sequences absolutely required for HIF binding[32,35].

Direct comparison of HIF1α and HIF2α binding in MCF7 breast cancer cells demonstrated that although some sites bind HIF1α exclusively, many others bind both HIFα subunits with equal affinity, despite the fact that HIF2α contributes to the hypoxic expression of relatively few genes in these cells[33]. Subsequent analysis using high-resolution ChiP-seq techniques revealed that HIFs bind to approximately 500 high-affinity sites across the genome, many of which are located at great distances (>100 kbp) from the genes they regulate[35]. Perhaps not surprisingly, HIF1α and HIF2α were shown to bind preferentially to specific genes each is known to preferentially regulate (Table 2): for example, a significantly higher level of HIF1α binding was associated with glycolytic pathway genes, whereas relatively greater HIF2α binding was observed at the *Oct4* locus. Strikingly, however, significant levels of both proteins were detected at essentially all HIF binding sites, further implicating differential interactions with specific co-factors, perhaps mediated by distinct posttranslational modifications, in controlling target gene specificity[35]. Interestingly, it appears that HIFs are recruited to genes already expressed in norm oxic cells (as revealed by DNAsel hypersensitivity), and are therefore unlikely to direct hypoxic changes in chromatin structure of target genes[32,35]. The spectrum of HIF target genes may therefore be determined largely by underlying cell type-specific patterns of chromatin structure, a speculation supported by the limited concordance (40-60%) of HIF binding sites detected in MCF7 and RCC cells[35]. Intriguingly, several reports indicate that HIF1α binds and regulates the expression of multiple Jumonji-domain containing histone demethylases (JMJHDs), which may contribute directly to changes in hypoxic target gene expression[31,34].

Assessing HIF1α and HIF2α function in tumor models

Multiple xenograft tumor models (Table 3) support the contention that HIF1α and HIF2α promote tumor progression by regulating both shared and unique target genes. As global deletion of the mouse *Hif1a* gene results in lethality at E9.5[36,37], and HIF2α deficiency causes embryonic and perinatal lethality[38-40], or severe developmental abnormalities[41] conditional alleles[37, 42] were required to investigate the specific effects of HIFα deficiency in autochthonous mouse tumor models. Only a small number of studies have been reported to date (Table 3), but these have demonstrated independent roles for HIF1α and HIF2α in different cancers, as well as stromal cell types, at various stages of tumor growth and progression. For example, HIF1α deletion in a mouse mammary tumor virus (MMTV) promoter-driven Polyoma middle T cancer model reduced overall tumor burden and pulmonary metastasis, even when equivalent tumor burdens were allowed to occur in control mice[43] (Table 3).

In a direct comparison of HIF1α and HIF2α function in a KRAS-driven lung tumor model, HIF1α deletion had surprisingly little effect on tumor burden and progression, whereas loss of HIF2α actually increased tumor growth and progression[26]. This latter effect correlates to HIF2α-driven expression of the *Scgb3a1* gene, which encodes the putative tumor suppressor secretoglobin 3a1 protein[44]. Surprisingly, overexpression of a stabilized HIF2α protein in the identical KRAS lung tumor model also promoted tumor angiogenesis and invasion by increasing expression of vascular endothelial growth factor (VEGF) and SNAIL[45], respectively. The observation that either HIF2α overexpression or deletion can promote tumor growth in the same tumor context, albeit by different mechanisms, suggests that effective targeting of HIFα subunits in cancer treatment may be complicated. Growth of pVHL-deficient mouse liver hemangiomas was similarly shown to be specifically dependent on HIF2α, but not HIF1α[46]

HIF1α and HIF2α deficiency in tumor-associated stromal cells also revealed isoform-specific effects on cancer progression (Figure 1). Initial gene expression studies revealed independent effects of HIF1α and HIF2α in primary human macrophages, as well as cultured murine macrophages[47]. HIF1α deletion in macrophages reduced overall tumor growth in a murine breast cancer model by reducing inducible nitric oxide synthase (iN OS) expression and consequent production of NO, which inhibits T cell responses *in vitro* and immune surveillance *in vivo*[48]. Intriguingly, HIF2α drives macrophage expression of arginase1[49], which catabolizes and thereby reduces pools of L-arginine, from which NO is produced. The two HIFα isoforms therefore appear to oppose one another to properly regulate overall macrophage NO levels. Interestingly, deletion of HIF2α (but not HIF1α) in mouse macrophages also significantly inhibits the expression of cytokine and chemokine receptors, including the macrophage colony-stimulating factor 1 receptor (M-CSFR, also known as CSF1 R) and CXCR4[50], thereby limiting macrophage migration into autochthonous liver and colorectal cancers and reducing overall tumor burden.

Loss of either HIF1α or HIF2α in mouse vascular endothelial cells (ECs) reduces tumor expansion in xenograft models, although through different mechanisms. EC-specific HIF1α deletion reduces VEGFR2 receptor expression, thereby inhibiting VEGF signaling and EC proliferation, survival and expansion in hypoxic tumor zones[51]. In contrast, loss of HIF2α function in ECs reduced expression of ephrin A1[52], delta-like ligand 4 (DLL4) and angiopoietin 2 (ANG2)[53], which correlated with unproductive sprouting and aberrant vessel remodeling and xenograft tumor growth. Collectively, these results reveal complex roles for HIF1α and HIF2α in distinct tumor and stromal cell types, although it will be important to test their function in additional tumor models.

Differential regulation of HIFα isoform expression

What molecular mechanisms contribute to the differential regulation of HIF1α and HIF2α? Control of HIF activity has been traditionally attributed to O_2-dependent posttranslational stabilization of HIFα subunits; however, recent data indicate that control of HIF1α and HIF2α expression can be selectively regulated at the level of transcription, translation, and protein stability (summarized in Figure 2A).

Differential transcription

Surprisingly, relatively little is known about the transcriptional regulation of the *Hif1a* and *Epas1* (encoding HIF2α) genes. Nuclear factor-KB (NF-KB) regulates the transcription of the *Hif1a* gene[54-57]. Moreover, Th1 cytokines stimulate this NF-κB-HIF1α pathway to activate a range of HIF1α target genes, whereas Th2 cytokines interleukin-4 (IL-4) and IL-1 0 differentially activate *Epas1* expression[49], although the precise mechanisms involved are not clear. Expression of the *Hif1a* locus, in contrast to *Epas1*, is also regulated by the SWIISNF chromatin remodeling protein BAF57[58] Additional investigation into differential *Hif1a* and *Epas1* transcription is certainly warranted.

Differential mRNA translation

It is well established that elevated HIFα mRNA translation rates increase HIFα protein levels and activity, particularly in cells with activated PI3K1AKT/mTOR signaling, a common feature of cancer cells (reviewed in[6]). Intriguingly, HIF1α expression in RCC cell lines appears to be regulated by both mTORC1 and mTORC2 kinase complexes, whereas HIF2α expression is mTORC2-dependent and mTORC1-independent[59] Other forms of differential translation control have been reported for HIFα proteins[60, 61]. For example, the iron response element binding protein 1 (IREBP1) was shown to bind a canonical iron response element (IRE) in the HIF2α 5′ UTR, thereby inhibiting translation[61]. This effect appears to be specific for HIF2α, as IREBP1 fails to bind the HIF1α transcript or regulate its translation, despite the presence of a near-consensus IRE in the HIF1α 5′ UTR[62] This regulation is also consistent with the identification of HIF2α as the primary regulator of erythropoiesis and cellular iron metabolism in vivo[42, 63-60].

Differential stability

As HIF1α and HIF2α protein levels are both modulated in a similar way by PHD-pVHL-dependent mechanisms (Box 1), the observation that HIF1α and HIF2α proteins accumulate at different O_2 levels in specific cell types came as a surprise. Pahlman and colleagues first demonstrated that HIF2α protein is stabilized at moderate (2-5% O_2) levels, whereas HIF1α accumulates only at lower (0-2% O_2) levels in HeLa and neuroblastoma cells[67, 68] (similar results were later reported for glioma cells[69]). Hypoxic neuroblastoma[68] and lung adenocarcinoma cells[60] maintain elevated HIF2α protein levels during long-term hypoxic culture (48 hours); in contrast, HIF1α levels increase acutely upon hypoxic exposure, but then decline after several hours. The HIF-mediated expression of antisense transcripts from the *Hif1* a (but not *Epas1*) locus, results in *Hif1a* mRNA destabilization and may explain the gradual and specific reduction of HIF1α protein[60]

Two HIF1α specific E3 ubiquitin ligases have been described recently that may also contribute to the differential stability of HIF1α and HIF2α. HIF-associated factor (HAF) binds and destabilizes HIF1α under normoxic and hypoxic conditions in a pVHL-independent, proteasome-dependent manner, but has no effect on HIF2α levels[70]. Instead, HAF binds HIF2α at a distinct C-terminal region and promotes HIF2α transcriptional activity, effectively switching cells from a HIF1α to a HIF2α transcriptional program[71]. In addition, heat shock protein 70 (HSP70) and carboxyl terminus of Hsc70-interaction protein

(CHIP), a recently identified E3-ubiquitin ligase, were shown to bind and degrade HIF1α (but not HIF2α) under conditions of prolonged hypoxia in cultured cells, whereas rapid reoxygenation destabilized both HIF1α and HIF2α proteins in a PHD-pVHL-dependent manner[72] Precisely how these novel ubiquitylation events are regulated, either by hypoxia or other stimuli, and how they affect HIF activity in cancer progression is not yet known.

Posttranslational modifications and differential HIFα activity

The regulation of HIFα subunits by posttranslational proline and asparagine hydroxylation, catalyzed by PHD and factor inhibiting HIF (FIH, also known as HIF1AN) enzymes, respectively (Box 1), has been extensively reviewed elsewhere[4, 63, 73-70]. Interestingly, specific PHD enzymes exhibit biased activity toward HIF1α and HIF2α; for example, PHD3 preferentially hydroxylates HIF2α in multiple celilines[76]. Peet and colleagues have also shown that FIH preferentially hydroxylates HIF1α in certain cell lines, owing to the identity of the amino acid immediately C-terminal to the hydroxylated asparagine (valine in HIF1α, alanine in HIF2α)[77]. These results suggest that differential N-hydroxylation might regulate HIF1α and HIF2α activity, although the largely HIF-independent neurological phenotypes of FIH-deficient mice[78] indicate that other factors are likely involved.

In addition to hydroxylation, both HIF1α and HIF2α are subject to an array of distinct, O_2-independent posttranslational modifications, and growing evidence indicates that at least some of these are specific for either HIF1α or HIF2α, and may promote their differential activity (Figure 2B), These include:

Phosphorylation

Early work showed that both HIF1α and HIF2α are phosphorylated[79, 80], and recent work suggests that isoform-specific phosphorylation may impact tumor progression, Specifically, Huang and colleagues demonstrated that HIF1α represses Myc-dependent expression of the DNA damage repair protein nibrin (NBS1) by displacing the SP1 transcription factor from the MYC transcriptional complex[81], HIF2α, in contrast, is inhibited from interacting with SP1 through phosphorylation on T324 by protein kinase D1 (PKD1), a modification dependent on a neighboring proline residue unique to HIF2α (Figure 2C), When a proline residue was introduced into the corresponding position in HIF1α, PKD1 also phosphorylated the modified HIF1α protein, which consequently lost the ability to displace SP1 from MYC[81], Other specific phosphorylation events catalyzed by MAPK[82], casein kinase 1 (CK1)[83] and ataxia telangectasia mutated (ATM)[84] have been demonstrated to modulate HIF1α activity, although it is not yet known whether these also occur in HIF2α, It will be important to determine the degree to which these various phosphorylation events distinguish HIF1α or HIF2α activation, and whether they represent another mechanism of parallel regulation in cancer cells.

Acetylation

HIFα activity is also modulated by multiple sirtuins, a family of redox-sensitive, NAD+-dependent deacetylases and/or ADP-ribosyltransferases. Mammalian cells express a family of sirtuins (SIRT1-7) that regulate complex changes in gene expression, metabolism, and cellular redox status, and have also been implicated in controlling longevity, although this idea remains highly controversial[85]. SIRT1 forms a complex with HIF2α and deacetylates conserved lysine residues in the N-TAD, which enhances HIF2α transcriptional activity *in vitro* and *in vivo*[86] SIRT1 was also reported to deacetylate lysine residues in HIF1α, which resulted in HIF1α transcriptional repression[87] although this effect was not universally observed[86] (Figure 3).

The apparently opposing effects of SIRT1 on HIF1α and HIF2α could skew cells toward either HIF1α or HIF2α transcriptional programs in response to changing metabolic activity in hypoxic tumors. Park and colleagues[87] proposed a positive feedback mechanism in which HIF1α promotes glycolysis, reducing NAD+/NADH ratios under hypoxia and inhibiting SIRT1, thereby further augmenting HIF1α activity. Presumably, inhibiting SIRT1 under these conditions would also decrease HIF2α activity, although the relative sensitivity of endogenous HIF1α and HIF2α proteins to SIRT1-mediated effects over a range of O_2 levels is not yet clear, and the kinetics of these responses may differ. It would be interesting to determine whether deacetylation by SIRT1 contributes to the high relative abundance of HIF2α at intermediate O_2 levels. There appear to be yet more wrinkles in this story, as both HIF1α and HIF2α were shown to bind the *SIRT1* gene promoter and induce its expression under hypoxia[88], and AKT activity can induce both HIF1α and SIRT1 expression by downregulating miR199a-5p expression[89].

Other sirtuins have also been shown to regulate HIFα activity. Mostoslavsky and colleagues identified SIRT6 as a HIF1α repressor, and showed that SIRT6 deficiency increased HIF1α-dependent glucose uptake and glycolytic activity at the expense of mitochondrial respiration[90]. Although the precise mechanisms regulating interactions between SIRT6 and HIF1α are not yet clear, SIRT6 deficiency increases both HIF1α synthesis and stability, suggesting that the effects of SIRT6 may be at least partly indirect. In addition, the mitochondrial SIRT3 deacetylase indirectly regulates HIFα stabilization by suppressing the formation of mitochondrial reactive oxygen species (ROS)[91] which, in turn, promote HIF1α stabilization[92-94]. For this reason, SIRT3-deficient cells display HIF1α-dependent increases in glucose transport, glycolysis and proliferation[95,96]. The implications of these findings for tumor progression have not been explored in depth, but are likely to be both complex and important.

It is possible that other acetylation and deacetylation events regulate HIF activity: for example, the mouse arrest defective-1 (mARD1) protein was reported to destabilize HIF1α by acetylating K532[97], an event apparently reversed by recruitment of HDAC1 to HIF1α by metastasis-associated protein 1 (MTA1)[98] Other researchers, in contrast, observed neither interaction between mARD1 and HIF1α, nor any effects of hypoxia on mARD1 activity, and the importance of this regulatory event remains in dispute[99]. Finally, a growing number of reports indicate that HIFα proteins are subject to numerous other posttranslational modifications, including sumoylation, S-nitrosylation, and neddylation[100-106] although whether any of these differentially regulate HIF1α and HIF2α is as yet unknown.

HIFs, oncogenes, and tumor suppressors - balancing HIF1α and HIF2α

Although HIF1α and HIF2α clearly influence tumor progression by directly regulating unique and shared target genes (Table 2), recent evidence indicates that these HIFα proteins also affect tumor progression by exerting distinct, often opposing effects on critical oncoproteins and tumor suppressors including MYC, p53, and mTOR.

HIFα and c-Myc

In many cell types, hypoxia suppresses proliferation. Koshiji et al. were the first to demonstrate that acute HIF1α stabilization at 1% O_2 produces cell cycle arrest by inhibiting the protooncoprotein MYC[107], a bHLH/leucine zipper (bHLH/LZ) transcription factor that is overexpressed in >40% of human cancers. MYC controls the *G1/S* cell cycle transition by forming heterodimers with the related protein MAX, binding conserved E-box sequences (CTCGAG), and promoting expression of genes encoding cyclin D2 (*CCND2*), E2F1, and ornithine decarboxylase 1 (*ODC1*), for example. MYC simultaneously inhibits the expression of *CDKN1A* and *CDKN1B* genes encoding cyclin-dependent kinase inhibitors

(CKIs) p21 and p27, respectively[108], in part by displacing the SP1 protein from the transcription factor MIZ1. MYC also promotes proliferation by inducing the expression of essentially all glycolytic enzymes and enhancing protein synthesis, thereby increasing cell growth.

Under hypoxic conditions, HIF1α binds to SP1, displacing MYC from multiple target genes including *CDKN1A, MSH2, MSH6*, and *NBS1*[81,109]. (Figure 3B). Gordan et al. subsequently showed that HIF1α rapidly disrupted the association of MYC with MAX and MIZ1, thus reducing MYC promoter occupancy at the *CDKN1A, CDKN1B, CDKN2B* (which encodes p15), *ODC1, CCND2*, and *E2F1* genes[110] A more chronic adaptation results from HIF1α mediated induction of MXI1, which interacts with MAX at E-boxes to inhibit the expression of *ODC1* and peroxisome proliferator-activated receptor-y coactovator 1β(*PGC-1β*)[111, 112], suppressing mitochondrial biogenesis and function. Moreover, HIF1α promotes MYC degradation under chronic hypoxia[111,112]. Through these multiple mechanisms, HIF1α effectively limits MYC-dependent anabolic metabolism, protein synthesis and cell division, an important hypoxic adaptation. Intriguingly, HIF1α also drives expression of the glycolytic pathway genes, permitting hypoxic cells to inhibit MYC driven macromolecular synthesis whilst producing ATP from glycolysis.

Surprisingly, transformed cells expressing HIF2α exclusively exhibit enhanced MYC activity, with more rapid entry into S phase of the cell cycle, increased *CCND2, E2F1*, and *ODC1* gene expression, and elevated MYC promoter occupancy[110] Moreover, HIF2α promotes cell cycle progression in hypoxic cells via transcriptional effects on both MYC activated *(CCND2, E2F1)* and repressed *(p21, p27)* target genes, and interactions with MAX, SP1, and MIZ1. This impact on MYC likely contributes to HIF2α-mediated neoplastic progression of renal clear cell carcinoma (RCC) tumorigenesis following loss of the *VHL* tumor suppressor[113]. Of note, RCC cells exclusively expressing HIF2α also displayed reduced genomic instability, correlating with increased MYC-dependent expression of genes encoding DNA repair proteins (including BRCA 1, BARD1, XRCC2, BUB1, and CENPE)[113]. These resu lts reveal a critical collaborative role for HIF2α and MYC in promoting genomic integrity and resistance to replication stress.

How do HIF1α and HIF2α exert these opposing roles on MYC? Multiple mechanisms appear to be involved: for example, HIF1α binds to SP1 via the PAS-B domain, whereas HIF2α fails to do so because it is phosphorylated by PKD1, blocking its ability to interact with SP1[81]. In contrast, HIF2α forms a complex with MAX, causing a dose-dependent stabilization of MYC-MAX and MYC-MAX-SP1 complexes, resulting in increased MYC-MAX binding at *CCND2, E2F1, p21*, and *p27*[110]. These effects occur rapidly and can be detected after only 1-2 hours at 0.5% O_2, suggesting they are independent of HIF2α transcriptional activity, which peaks at approximately 16 hours at 0.5% O_2. A specific role for MXI1 in this differential regulation is currently unclear, as both HIF1α and HIF2α appear to contribute to MXI1 expression in *VHL*-deficient RCC cells[112]. How the "competition" between HIF1α and HIF2α is moderated in a given cell type, in terms of their respective influence on MYC activity, is equally mysterious at present.

The relative expression levels of MYC and HIFα proteins also play an important role in regulating tumor cell proliferation and metabolism. Many cancer cells exhibit subtle alterations in MYC levels as a consequence of elevated oncogenic signaling, whereas other cells express MYC at very high levels due to chromosomal amplifications, translocations, and mutations within *MYC* coding exons[108]. It appears that high levels of MYC sequester and tightly bind MAX, thereby relieving potential inhibition by HIF1α[114]. For example, most genes induced by ectopic *MYC* expression were not transcriptionally repressed by hypoxia in a B-cell tumor model. The picture is more complex, however, as HIF1α can

actually cooperate with MYC to induce the expression of specific target genes, including those encoding the glycolytic enzyme hexokinase 2 *(HK2)*, pyruvate dehydrogenase kinase 1 *(PDK1)*, and VEGFA[114]. Similarly, high levels of NMYC override HIF1α inhibition of cell cycle progression while cooperating with HIF1α to promote phosphoglycerate kinase 1 *(PGK1), (HK2)*, and lactate dehydrogenase A *(LDHA)* expression in neuroblastomas with *MYCN* gene amplification[115]. In summary, when *MYC* family members are highly overexpressed, they not only overcome the inhibitory effects of HIF1α, but MYC and HIF1α collaborate to favor glycolysis and continued proliferation under decreased O_2 availability. In contrast, tumors with lower MYC levels are susceptible to HIF1α inhibition, explaining the anti-tumorigenic effects of HIF1α in certain cancers such as RCC[116].

HIF1α, HIF2α, and p53

Low O_2 and other stresses associated with tumor growth (such as growth factor withdrawal, nutrient deprivation, and acidosis) activate p53, a critical tumor suppressor that is mutated or silenced in a majority of human cancers[117]. While it is maintained at low levels in normal cells by MDM2-mediated degradation, p53 is posttranslationally modified and stabilized in response to numerous stimuli, including abnormal proliferation signals, osmotic stress, DNA damage, and hypoxia[118]. p53 forms homotetramers that bind and regulate numerous genes involved in metabolism, DNA repair, cell cycle arrest, and cell death, thereby coordinating cellular responses to microenvironmental stress[117]

HIF1α and HIF2α display opposing effects on the p53 pathway. Numerous studies have shown that p53 accumu lation occurs within hypoxic regions of solid tumors, and correlates with cells undergoing apoptosis, although this may only occur when also accompan ied by acidosis and nutrient deprivation[119]. An et al. orig inally suggested that transcriptionally active wild type p53 is stabilized through a physical association with HIF1α[120] Sanchez-Puig further reported that the HIF1α ODD and N-terminal TAD domains bind to p53 tetramers under physiological conditions[121]; however, subsequent reports suggested that MDM2 mediates the interaction between p53 and HIF1α by acting as a bridge between the two transcription factors[122]. Whereas HIF1α fails to bind p53 *in vitro*, it directly binds MDM2, suppressing MDM2-dependent ubiquitylation of p53 *in vivo* and p53 nuclear export. Surprisingly, MDM2 overexpression actually promotes p53 accumulation and target gene stimu lation when HIF1α is activated in hypoxic cells[122]. Furthermore, HIF1α appears to enhance p53 activation by ionizing radiation (IR), resulting in increased p53 phosphorylation and p53-mediated apoptosis[123] IR significantly increases HIF1α activity in tumors due to increased reactive oxygen and nitrogen species, and rad iation combined with hypoxia lead to increased p53 phosphorylation in a HIF1α dependent manner, by a mechanism that rema ins unclear.

It should also be noted that the relationship between HIF1α and p53 provides a potential negative feedback loop for HIF1α activity. Ravi et al. have suggested that p53 can induce HIF1α turnover, by promoting its MDM2-mediated ubiquitylation and proteasomal degradation[124]. Therefore, p53 loss in colon cancer cells enhanced HIF1α levels and augmented VEGFA expression and tumor angiogenesis, suggesting that inactivating p53 mutations can contribute to the “angiogenic switch” during colorectal tumorigenesis.

In contrast to HIF1α, HIF2α does not bind MDM2[125], and appears to inhibit p53 indirectly by multiple mechanisms. Bertout et al. demonstrated that elevated HIF2α expression inhibits p53 phosphorylation and stabilization in ReG cell lines, whereas knocking down HIF2α expression increases p53 transcriptional activity and target gene expression[125]. Furthermore, HIF2α deficient cells exhibit elevated ATM activity and DNA double strand break formation, as well as increased levels of ROS after IR. HIF2α has been reported to regulate antioxidants such as superoxide dismutase 1 (SOD1), SOD2, glutathione peroxidase

1, and catalase in developing embryos and neonates[41]. However, in RGG cells, HIF2α instead decreases ROS accumulation by regulating the expression of distinct antioxidant enzymes (heme oxygenase 1, ceruloplasmin, glutathione peroxidase 8, and peroxiredoxin 3). Importantly, HIF2α expression in RCC tumor samples correlates with decreased p53 phosphorylation and target gene expression, and may contribute to radioresistance in HIF2α expressing RCCs[125].

In parallel studies, Roberts et al. showed that HIF2α also suppresses p53 expression and function via indirect effects on MDM2[126]. AKT-mediated phosphorylation of MDM2 promotes its nuclear localization and enhanced p53 degradation, and represents an important pro-survival effect of AKT. AKT activation occurs downstream of growth factor receptors like EGFR and platelet-derived growth factor receptor (PDGFR), which are stimulated by transforming growth factor-α (TGF-α) and PDGFβ, specific transcriptional targets of HIF2α in RCC cells. Thus, HIF2α overexpression in *VHL*-deficient RCC can inhibit p53 through a growth factor receptor-AKT-MDM2 pathway, in addition to maintaining redox homeostasis. In aggregate, these findings suggest that HIF2α likely contributes to RCC tumor cell survival during both radiation and chemotherapy by multiple mechanisms.

HIFs regulate mTOR

Cell division requires high levels of protein synthesis and anabolic metabolism, which is regulated by the serine/threonine kinase mTOR in response to nutrient and growth factor availability. mTORC1 promotes ribosome biogenesis, mRNA translation, and nutrient import, while inhibiting autophagy[127]. Elevated mTORC1 activity is observed in the majority of human tumors, due to activation of upstream oncogenes (PI3K, AKT) and/or loss of tumor suppressors (PTEN, LKB1)[128]. In particular, the tuberous sclerosis proteins TSC1 and TSC2 together inhibit mTORC1 activity to limit cell growth under conditions of environmental stress, including reduced growth factor, glucose, amino acid, and O_2 levels[127].

Hypoxia suppresses mTORC1 through multiple mechanisms. For example, decreased ATP levels in severely hypoxic cells activate AMP-activated kinase (AMPK)[129], which phosphorylates TSC2 (as well as the mTORC1-associated factor RAPTOR) to inhibit mTORC1 activity. In addition, HIF1α (but not HIF2α) induces expression of the *DDIT4* gene[130], which encodes REDD1, a protein that represses mTORC1 by promoting the release of sequestered TSC2 from 14-3-3 proteins[131]. Finally, the hypoxia-inducible proautophagic protein BNIP3 binds and inhibits RAS homolog enriched in brain (RHEB), resulting in decreased mTORC1 activity[132]. HIF1α dependent inhibition of mTORC1 may benefit cells by reducing ATP-intensive protein synthesis, while increasing autophagy, under conditions of hypoxic stress.

Growing evidence suggests that HIF2α may, in contrast, stimulate mTORC1 to promote cellular proliferation in O_2-deprived cells. The focal adhesion kinase (FAK) family interacting protein of *200 kd* (FIP200) gene has been identified as a HIF2α target through microarray studies$_{27}$ and FIP200 has been proposed to interact with TSC1, thereby disrupting TSC1ITSC2 complexes and promoting mTORC1 activation[133]. In addition, FIP200 may promote TSC1 degradation by the ubiquitin-proteosome pathway[134]. HIF2α could also selectively enhance mTORC1 activity by positive effects on growth factor signaling, as HIF2α induces the expression of TGF-α, PDGF-β, and IGF-1, leading to AKT and mTORC1 activation in renal cancer cells[126]. Although additional work is clearly needed to further elucidate the molecular mechanisms by which HIF2α promotes mTORC1 functions, these results reveal another example in which HIF1α and HIF2α antagonize one another to balance hypoxic responses in key growth regulatory pathways.

HIFα and growth control

Why would the two HIFα subunits result in opposite effects on the c-Myc, p53, and mTORC1 pathways? The inhibitory activity of HIF1α towards these growth regulatory systems represents important energy conservation mechanisms in light of decreased ATP production during periods of O_2 limitation, which are likely to be compounded by decreased availability of nutrients (glucose, amino acids, lipids) and growth factors in hypoxic subdomains of solid tumors. In contrast, the "pro-growth" effects of HIF2α may contribute to the ability of endothelial cells to proliferate during neoangiogenesis in ischemic tissues. It is interesting that HIF2α accumulates at higher O_2 levels than HIF1α, which may allow its selective activation in blood vessels. In addition, the ability of HIF2α to promote cell growth in RCCs may explain why HIF1α expression is often silenced in these tumors. Selective[135], as well as genome-wide[136], sequence and copy number analyses have identified truncating *Hif1α* mutations in a small percentage of RCCs, as well as *Hif1α* heterozygosity in others[137], supporting the hypothesis that inhibition of HIF1α function is a selective advantage for some RCCs. It is also tempting to speculate that the recent identification of *Epas1* single nucleotide polymorphisms (SNPs) as a predisposing factor for RCC development[136] could reveal genetic alterations that increase or expand HIF2α function.

Therapy

As HIF complexes are instrumental in cancer cell adaptation to hypoxic tumor microenvironments, the ability to *selectively* inhibit HIF activity would appear to be of clinical benefit[1, 139]. Historically, DNA-binding proteins *have* been difficult to target, but a large collection of compounds *have* been reported to inhibit HIF transcriptional activity, either directly or indirectly. For example, compounds including topoisomerase inhibitors (camptothecan, topotecan)[140] and DNA intercalators (ech inomycin, daunorubicin, doxorubicin)[141] block HIF heterodimerization and transcriptional activation, and interfere with xenograft tumor growth in a HIF-dependent manner. These observations are particularly interesting, given their frequent use as sequence non-specific DNA damaging agents in chemotherapy. Oncogenic signal transduction pathways also promote mTORC1-dependent HIF1α mRNA translation; consequently, receptor tyrosine kinase inhibitors (Herceptin, *Gleevec*, erlotinib, gefitinib) and mTOR inhibitors (rapamycin, temsirolimus, everolimus)[142,143] are thought to reduce tumor angiogenesis, and possibly other hypoxic responses, by indirectly reducing HIFα protein synthesis. Other drugs have been shown to increase HIFα degradation, including HDAC inhibitors[144,145], and compounds that disrupt HIFα binding to HSP90 (geldanamycin)[146, 147]. These studies indicate that HIF activity is susceptible to inhibition using a variety of drugs already approved for cancer treatment; however, the extent to which these drugs limit the growth and progression by specifically inhibiting HIF activity in autochthonous tumors is as yet unknown, and needs to be investigated.

Given the disparate effects of HIF1α and HIF2α on tumor growth and progression described in this review, it will also be critical to determine whether potential HIF inhibitors affect both HIFα subunits equally. There may be situations where *se lective* inhibition of either HIFα protein wou ld be especially beneficial; for example, inhibiting HIF1α may be particularly advantageous for highly glycolytic hypoxic tumors, whereas inhibiting only HIF2α is likely to be useful in treating RCCs. Intriguingly, IIiopouios and colleagues identified a series of small compounds that interfere with HIF2α mRNA translation by enhancing IREBP1 binding to the iron response element found in the 5′ UTR of HIF2α, but not HIF1α[62] Selective agents of this kind could be particularly useful in cancers that express both HIF1α and HIF2α, where they *have* distinct roles. Furthermore, Dewhirst et al *have* shown that rad iation induces HIF1α and VEGFA, protecting endothelial cells from

radiation-mediated apoptosis[148]. Treatment of mice harboring tumors with the HIF inhibitor YC-1 enhanced vessel destruction and slowed tumor growth; in another study[148], the HIF inhibitor PX-478 reduced VEGFA expression, rendering xenografts more sensitive to ionizing radiation. As stated previously, endothelial VEGFA expression appears to be regulated primarily by HIF1α, suggesting that its selective inhibition would be beneficial.

Finally, HIF inhibition may be advantageous only up to a certain point. As discussed earlier, HIF2α overexpression, as well as HIF2α deletion, increases the growth of KRAS driven murine lung tumors, although by different mechanisms. Too much HIF2α increases VEGF and SNAIL expression, promoting angiogenesis and tumor invasion, whereas complete loss of HIF2α reduces the expression of the tumor suppressor *Scgb3a1*, a direct HIF2α target gene. These data suggest that successful HIF inhibition in cancer treatment may involve a narrower therapeutic window than initially envisioned. Although currently in early stages, the prospect of pharmacological HIF inhibition for cancer treatment, whether targeting HIF1α and HIF2α together, or either subunit individually, is an exciting one.

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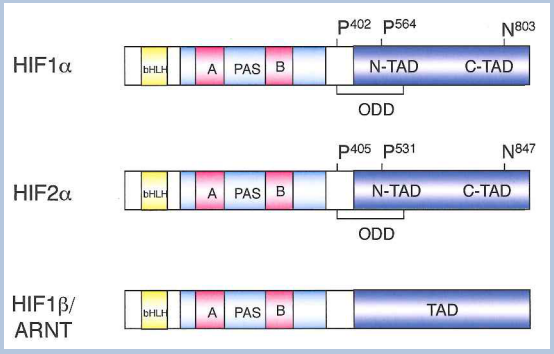
Box 1. O_2-dependent HIF regulation

Using molecular O_2 and 2-oxoglutarate as substrates, **HIF** prolyl hydroxlase (PHD) enzymes[4] hydroxylate two specific proline residues that reside in the 02-dependent degradation domain (ODD) of HIF-a proteins. These hydroxylation events occur on P402 and P564 in HIF1α, and P405 and P531 in HIF2α, respectively, and are required for the Von Hippel-Lindau (pVHL) tumor suppressor protein, the recognition component of an E3-ubiquitin ligase, to bind and degrade HIFα subunits under normoxic conditions. Hypoxia inhibits PHD activity through a number of mechanisms, including substrate limitation (reviewed in[4]), resulting in HIFα stabilization, heterodimerization with HIF1β/ARNT, and increased **HIF** transcriptional activity. Hypoxic conditions also inhibit a second hydroxylation of a conserved HIFα C-terminal asparagine residue by the FIH hydroxylase, an event that blocks the interaction between HIFα and the transcriptional co-activators p300/CBp[149-151]. Thus, whereas PHD-mediated hydroxylation destabilizes HIFα subunits, FIH-mediated hydroxylation inhibits their transcriptional activity.



Box 2. HIFs in normal and cancer stem cells

Stem cells reside in complex microenvironments or niches, and multiple studies revealed that O_2 levels influence the ability of stem and/or progenitor cells to remain quiescent or undergo differentiation, depending on cell type[152]. Again, HIF1α and HIF2α exhibit distinct roles in stem cell regulation. HIF1α appears to playa dominant role in modulating[152]. WNT-β-catenin signaling in hypoxic ES cells and isolated neural stem cells (NSCs) of the embryonic mesencephalon and adult hippocampus[153]. WNT-β-catenin activity is closely associated with low O_2 regions in the subgranular zone of the hippocampus, an important NSC niche, and *Hif1α* deletion impairs WNT-dependent processes, such as NSC proliferation, differentiation, and neuronal maturation. It should be noted that the opposite resu lt has been reported for colon cancer cells, where HIF1α inhibits WNT-β-catenin activity[154], indicating that the interaction between HIF1α and WNT in stem cells is functionally distinct from more differentiated cells, including neoplastic cells. The basis for this difference is currently unknown. HIF1α has also been proposed to increase the intracellular stability of activated NOTCH1 and to promote NOTCH target gene activation of myogenic and neural precursor cells[155]. This has been extended to thymic lymphomas in p53 mutant mice where HIF1α promotes NOTCH1 activation and target gene expression[156]. However, the data on neuroblastoma stem cells suggest that both HIF1α and HIF2α can augment NOTCH pathway signaling.

In contrast, HIF2α (but not HIF1α) regulates the POU transcription factor OCT4 (also known as POU5F1)[157]. OCT4 is essential for maintaining an undifferentiated cell fate in embryonic stem (ES) cells, the embryonic epiblast, and primordial germ cells (PGCs). Finally, HIF2α is selectively expressed in $CD133^+$ glioblastoma “stem” cells, whereas HIF1α is detected in both tumorigenic (i.e. stem) and non-tumorigenic populations, suggesting HIF2α has a unique role in the $CD133^+$ fraction[69]. Similarly, human neuroblastomas exhibit small numbers of tumor initiating/stem cells expressing neural crest markers (ID2, NOTCH1, HES1, and Vimentin) and HIF2α[158]. Upon HIF2α inhibition, these cells undergo early sympathetic neuronal differentiation, and express markers such as HASH1 (also known asASCL1), ISL1, and SCG10 (also known as STMN2). **It** is noteworthy that the $CD133^+$ glioblastoma and putative neuroblastoma tumor initiating/stem cells express high levels of HIF2α, although they reside in peri-endothelial niches[159]. While the extent of O_2 saturation with in these capillaries is unknown, the data are consistent with the idea that HIF2α accumulates at higher levels of O_2 than HIF1α. Alternatively, HIFα expression in distinct cancer cell subpopulations may be controlled by non-hypoxic stimuli, such as aberrant metabolism[160].

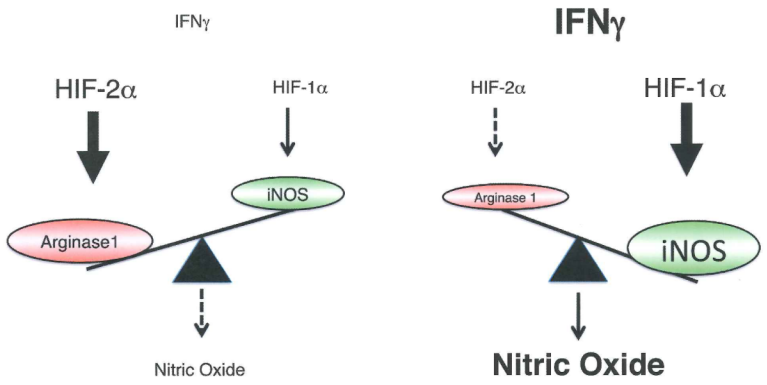


Figure 1. HIF1α and HIF2α exhibit antagonistic functions in nitric oxide (NO) production

Under low IFNγ conditions, HIF2α is more abundant and induces arginase1 expression, resulting in NO production. Under high IFNγ conditions, HIF2α is diminished and HIF1α dominates so that iNOS can utilize arginine for NO generation. These physiologically antagonistic functions allow the HIFα subunits to coordinately regulate NO production in a cytokine-induced and transcription-dependent fashion.

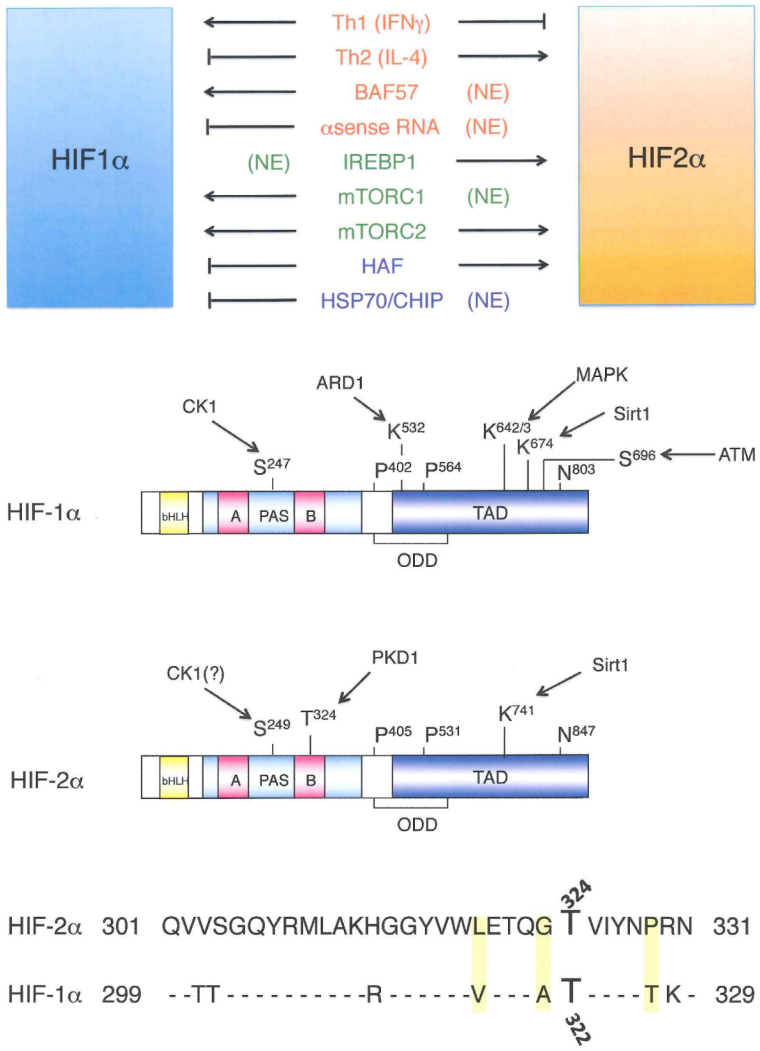


Figure 2. HIF1α and HIF2α are post-translationally modified, and differentially regulated by multiple mechanisms

(A) Multiple mechanisms differentially regulate HIF1α and HIF2α at the levels of transcription or mRNA stability (red), mRNA translation (green), and protein stability (blue). In most cases, these regulatory events have opposite effects on HIF1α and HIF2α expression, or appear to be specific for only one HIFα isoform. See text for details. (NE), no effect. (8) Summary of phosphorylations, acetylations, and hydroxylations of the two HIFα subunits by CK1, ARD1, PHDs, FIH, MAPK, SIRT1, PKD1, and ATM. It should be noted that ARD1 acetylates HIF1α, while SIRT1 deacetylates both HIF1α and HIF2α. (C) Sequence alignment of HIF2α residues 301-331 with a similar region of HIF1α; shaded residues are unique to HIF2α and allow the selective phosphorylation of HIF2α T324 by PKD1.

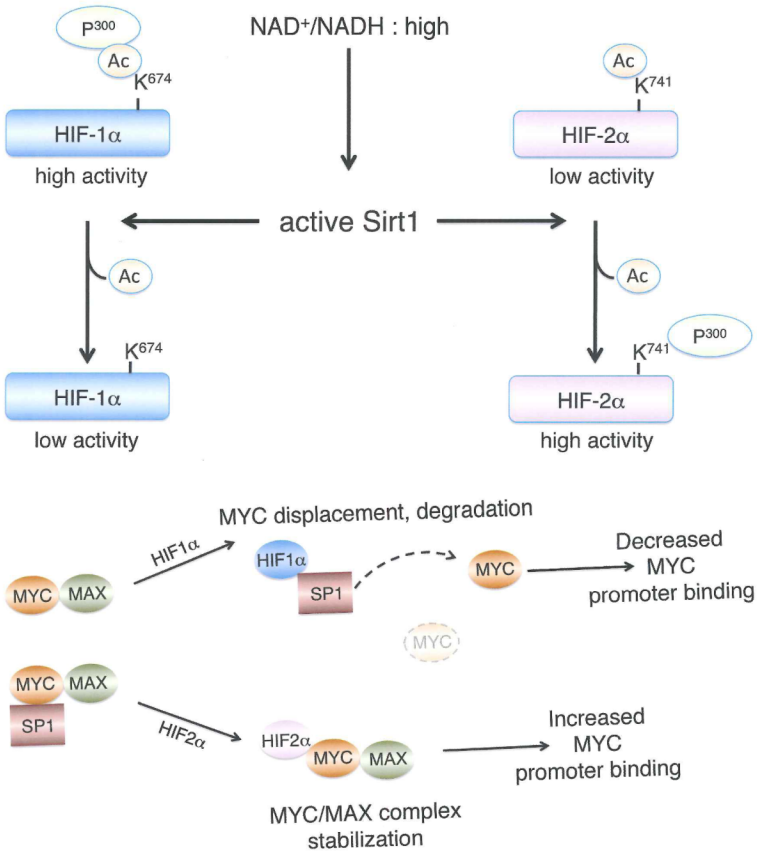


Figure 3. Differential regulation of HIF1α and HIF2α by SIRT1

(A) High levels of NAD^+ inactivate SIRT1, resulting in decreased HIF1α transcriptional activity and enhanced HIF2α stimulation of target genes like erythropoietin. (8) Distinct effects of HIF1α and HIF2α on MYC complex formation and promoter occupancy. Hypoxic cells exclusively expressing HIF1α exhibit decreased MYC activity due to diminished association with MAX and SP1, as well as reduced MYC stability. HIF1α also induces MXI1 expression, which inhibits MYC target gene expression (see text for details). Cells expressing HIF2α exhibit increased MYC complex formation and target gene activation, although the mechanisms involved are not fully understood.

Table 1

Correlation between HIFa protein expression and poor prognosis in human cancers*

Cancer type	HIF1α	HIF2α	References
Astrocytoma	+	+	161,162
Bladder	+	ND	163
Breast	+	+	164,165
Cervical	+	+[a]	166,167
Colorectal	+	+	168
Gastric	+	NC	169, 170
Gastric	+	ND	171
GIST	+	ND	172
Glioblastoma	ND	+	162
Glioma	NC[b]	+[b]	69
Head/neck	+	+	173,174
Hepatocellular	ND	+	175
Lung (NSCLC)	+	+	176
Lung (NSCLC)	+	ND	177
Lung (NSCLC)	NC	+	178
Melanoma	+	+	179
Neuroblastoma	FP	+	180
Ovarian	+	ND	181
Ovarian	+	+[d]	182
Pancreatic	+	ND	183,184
Prostate	+	+[d]	185
Renal	FP	ND	186
Renal	+	ND	187

NC, no correlation; ND, not determined; FP, correlation between HIFα expression and favorable prognosis

*Also see references (3) and (138).

[a]HIF2α expression in macrophages

[b]Correlation using mRNA levels

[c]Correlation of HIF1α expression with favorable prognosis

[d]Correlation with cytoplasmic HIF2α expression

Table 2

Representative shared and unique target genes regulated by HIF1α and HIF2α

Gene	Function	HIF1α	HIF2α	Cell type
GLUT1	Glucose transport	+	+	RCC[27], mouse ES[36, 37]
ADRP	Lipid metabolism	+	+	RCC[27]
CAXII	pH homeostasis	+	+	RCC[27]
FILAG	Cytoskeletal structure	+	+	RCC[27]
IL-6	Immune cytokine	+	+	RCC[27]
ADM1	Angiogenesis	+	+	RCC[27]
VEGF	Angiogenesis	+	+	RCC, Hep3B[27-29]
VEGF	Angiogenesis	+	−	Mouse EC[51], mouseES[36,37]
BNIP3	Autophagy, apoptosis	+	−	RCC[28]
HK1	Glycolysis	+	−	mouse ES[36, 37]
HK2	Glycolysis	+	−	RCC[27], mouse ES[36, 37]
PFK	Glycolysis	+	−	RCC[27], mouse ES[36,37]
ALDA	Glycolysis	+	−	RCC[27], mouse ES[36, 37]
PGK1	Glycolysis	+	−	RCC[27], mouse ES[36, 37]
LDHA	Glycolysis	+	−	RCC[27], mouse ES[36, 37]
INOS	NO production	+	−	Macrophages[49]
ARG	Inhibitor of NO production	−	+	Macrophages[49]
EPO	Erythropoiesis	−	+	Kidney[41, 42, 65], liver[188]
OCT4	Stem cell identity	−	+	Mouse ES[157]
SCGB3A1	Secretoglobin 3A1	−	+	NSCLC[26]
TGFα	Growth Factor	−	+	RCC[28, 189]
CCND1	Cell cycle progression	−	+	RCC[28]
DLL4	NOTCH signaling, EC branching	−	+	Mouse ECS[53]
ANG2	Blood vessel remodeling	−	+	Mouse ECS[53]

Table 3

Mouse models testing altered expression of HIFα proteins in tumour growth and progression

Tumour or cell type	HIFlα status	HIF2α status	Phenotypes	Refs
Xenograft tumours				
Teratoma	Loss-of-function knockout	Wild-type	Reduced growth and angiogenesis	37,190
Teratoma	Wild-type	Loss-of-function knockout	Increased growth	25
Fibrosarcoma	Loss-of-funct ion knockout	Wild-type	Reduced growth	191
RCC	Gain offunction	Wild-type	Reduced growth	23,28
RCC	Wild-type	Gain offunction	Increased growth	28,192
Autochthonous tumours				
MMlV-PyMT mammary tumours	Conditional knockout	Wild-type	Reduced metastasis	43
KRAS-driven NSCLC	Conditional knockout	Wild-type	No effect	26
KRAS-driven NSCLC	Wild-type	Conditional knockout	Increased tumour burden and progression	26
p53-driven thymic lymphoma	Heterozygous germline knockout	Wild-type	Decreased tumour incidence	156
Tumour-associated stromal cells				
Tumour-associated macrophages	Conditional knockout	Wild-type	Reduced NO production. increased T cell-mediated tumour immunosurveillance and reduced autochthonous mammary tumour growth	48,49
Tumour-associated macrophages	Wild-type	Conditional knockout	Decreased macrophage infiltration into autochthonous liver and colon tumours and decreased tumour growth	50
Vascular ECs	Conditional knockout	Wild-type	Decreased xenograft tumour angiogenesis and growth	51
Vascular ECs	Wild-type	Conditional knockout	Non-productive angiogenic sprouting and impaired vessel remodelling	53

EC, endothelial cell: MMTV, mouse mammary tumour virus; NO, nitric oxide; NSCLC, non-small-cell lung cancer; PyMT, polyoma middle T antigen; RCC, renal cell carcinoma.