Hypoxia-inducible factors: central regulators of the tumor phenotype
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Low oxygen levels are a defining characteristic of solid tumors, and responses to hypoxia contribute substantially to the malignant phenotype. Hypoxia-induced gene transcription promotes characteristic tumor behaviors, including angiogenesis, invasion, metastasis, de-differentiation and enhanced glycolytic metabolism. These effects are mediated, at least in part, by targets of the hypoxia-inducible factors (HIFs). The HIFs function as heterodimers comprising an oxygen-labile α-subunit and a stable β-subunit also referred to as ARNT. HIF-1α and HIF-2α stimulate the expression of overlapping as well as unique transcriptional targets, and their induction can have distinct biological effects. New targets and novel mechanisms of dysregulation place the HIFs in an ever more central role in tumor biology and have led to development of pharmacological inhibitors of their activity.

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Introduction
Hypoxia occurs when available oxygen falls below 5%, triggering a complex cellular and systemic adaptation mediated primarily through transcription by hypoxia-inducible factors (HIFs). HIF-1α was first identified as a crucial regulator of erythropoietin expression in response to low oxygen [1]. HIF-2α and HIF-3α have also been described, with HIF-3α, also known as IPAS (inhibitory PAS domain protein), functioning as an inhibitor of transcription [2,3]. More than 100 HIF target genes have been identified in a variety of systems (Figure 1). These include genes that encode angiogenesis-promoting factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor, glycolytic enzymes such as aldolase A and phosphoglycerate kinase, and cell cycle regulators such as p21 and p27, in addition to other proteins involved in extracellular matrix remodeling, differentiation, invasion and apoptosis [4–7]. HIF-1α and HIF-2α, complexed with the β-subunits ARNT and (more rarely) ARNT2, bind DNA at hypoxia response elements (HREs) [8,9]. The biological significance and transcriptional effects of HIF-3α remain somewhat obscure, and only HIF-1α and HIF-2α are discussed further in this review.

HIF-α subunits are continuously transcribed and translated, and their stability is regulated by oxygen availability. Under normoxic conditions, two prolines (at positions 402 and 564 in human HIF-1α, and 405 and 531 in human HIF-2α) in the HIF-α oxygen-dependent degradation domain (ODD) are hydroxylated by a family of oxygen-dependent proline hydroxylases (PHD1, PHD2 and PHD3) [10–13], enabling binding and ubiquitination by the von Hippel-Lindau (VHL) tumor suppressor, a component of an E3 ubiquitin ligase complex [14] (Figure 2). Interaction of HIF-α subunits with the transcriptional co-activator p300 is also regulated by oxygen levels, and binding is inhibited by oxygen-dependent asparaginyl hydroxylation (asparagines 803 in human HIF-1α, and 851 in human HIF-2α) of the HIF transactivation domain by factor-inhibiting HIF (FIH) [15,16].

VHL disease is a hereditary cancer syndrome marked by clear-cell renal carcinoma (RCC), pheochromocytoma and hemangioblastoma. The VHL tumor suppressor protein (pVHL) is required for normoxic degradation of the HIF-α subunits and can also target atypical protein kinase Ca and some subunits of RNA polymerase for degradation [17]. The pathological stabilization of HIF-2α under normoxia is necessary for the growth of VHL-null RCC and hemangioblastoma. Re-expression of pVHL in an VHL−/− RCC cell line blocks xenograft formation in nude mice. However, xenograft growth is rescued by expression of a normoxically stable mutant of HIF-2α [18,19], but not stabilized HIF-1α [20]. Liver-specific deletion of Vhl is sufficient to generate hemangiomas in transgenic mice. This effect still occurs in mice with a combined Vhl and Hif-1α deletion, but is abrogated if Vhl is deleted in combination with the common β-subunit Arnt [21]. By contrast, pheochromocytoma results from an HIF-independent effect of pVHL on JunB [22]. The HIFs also play an important role in non-inherited malignancies. There is substantial clinical data associating HIF-α protein expression with poor outcomes in patients with a broad range of sporadic cancers. These include adenocarcinoma of the breast, lung and gastrointestinal tract, as well as central nervous system...
HIF-1α and HIF-2α activate overlapping but distinct genes: HIF-1α and HIF-2α share the regulation of target genes involved in angiogenesis, invasion and metastasis, whereas HIF-1α alone activated genes involved in glycolysis and apoptosis. HIF-2α uniquely expresses expression of the stem cell factor gene Oct4 and Cyclin D1, whereas it preferentially regulates the growth factor TGFα. Abbreviations: BNIP3, BCL2/adenovirus E1B 19 kDa interacting protein 3; CCD1, Cyclin D1; Fit1, fms-related tyrosine kinase 1; LDHA, lactate dehydrogenase A; PAI-1, plasminogen activator inhibitor 1; PDGF, platelet-derived growth factor; Tie2, Tunicam internal endothelial cell kinase 2.

(CNS) malignancies and squamous cell tumors of the cervix, head and neck [5]. Data from mouse allograft studies have been less consistent. In some cases, disruption of Hif-1α inhibited allograft growth [23,24], but in others it promoted it [25,26]. Consistent inhibition of tumor growth has been observed following the stabilization of HIF-1α in normoxia due to Vhl loss [20,27–29]. Similarly, overexpression of either HIF-1α or HIF-2α in
glioma models correlates with decreased tumor growth [25,30].

Regulation of HIF stability and expression
The normoxic degradation of the HIF-α subunits is well characterized, but its inhibition under hypoxia is an area of active investigation, and remains controversial. Given that oxygen is required for hydroxylation, it is a limiting substrate under anoxic (0% O2) conditions. However, HIF-α proteins are stabilized in a reactive oxygen species (ROS)-dependent fashion well above this threshold. Early evidence showed that inhibitors of mitochondrial ROS generation were able to block hypoxic HIF-α stabilization [31]. However, such drugs might have toxic effects independent of those on HIF-α regulation. It has also been suggested that these drugs might cause redistribution of oxygen away from the mitochondrion, leaving more oxygen available for PHD activity and thus maintaining it under moderate hypoxia [32,33]. However, genetic studies have shown that disruption of electron transport chain (ETC) complex III, cytochrome c and Rieske iron-sulfur protein also blocks hypoxic HIF stabilization [34*,35*], whereas disruption of ETC complex IV did not [36*]. These data suggest that respiration is not required for HIF-α stabilization but that the delivery of electrons to cytochrome c is, supporting a requirement for ROS, but not oxygen consumption, in hypoxic HIF-α stabilization. Further evidence comes from the analysis of jumD−/− mice, which show enhanced ROS production, leading to abnormal HIF-α expression under normoxia. In this case, enhanced intracellular H2O2 levels were shown to inhibit PHD activity by decreasing the availability of Fe2+, which is required for hydroxylation to occur [37*]. This is also a plausible mechanism for PHD regulation in hypoxic cells.

HIF-α stabilization under normoxic conditions is both necessary and sufficient for RCC development following VHL inactivation, as described above. pVHL can also be inhibited by the E2−EPF ubiquitin carrier protein, which targets pVHL for proteasome-mediated degradation [38**]. Overexpression of this protein occurs in breast, lung, ovarian and CNS cancers, and correlates strongly with tumor grade and poor patient outcomes [39]. The alteration of metabolic pathways impinging on PHD activity can also promote normoxic HIF-α stabilization and tumor formation. In addition to requiring oxygen and Fe2+, the PHDs require 2-oxoglutarate as a substrate and ascorbic acid as a co-factor to catalyze HIF-α hydroxylation, and produce succinate and carbon dioxide in addition to hydroxylated proline residues. Inactivation of fumarate hydratase, a rare cause of inherited RCC, promotes HIF-α stabilization due to inhibition of the PHDs by fumarate, which competes with 2-oxoglutarate for active-site binding [40]. Similarly, inactivation of succinate dehydrogenase, which occurs in some renal, thyroid and colon cancers, leads to succinate accumulation and product inhibition of the PHDs [41].

Control of HIF-α translation
The mammalian target of rapamycin (mTOR) kinase responds to nutrient and growth factor availability to regulate translation. Normoxic HIF-α expression is promoted by disruption of mTOR regulation, resulting from increased HIF-α translation rates despite unaltered levels of degradation. This is likely to occur in many tumors that show hyperactivation of receptor tyrosine kinases, and thus translation [42], but is also seen in several inherited tumor syndromes. Loss of the tuberous sclerosis 2 (TSC2) tumor suppressor protein, an inhibitor of mTOR activity, causes normoxic stabilization of the HIF-α subunits by enhancing their translation rate, leading to the formation of highly vascular tumors [43]. Enhancement of HIF-α translation under hypoxia by disruption of the promyelocytic leukemia (PML) tumor suppressor can also promote tumor growth. Originally identified as part of a leukemogenic fusion protein, PML has since been appreciated to have a tumor suppressive effect and is lost in multiple sporadic tumors [44]. Genetic disruption of Pml correlates with increased VEGF and HIF-α expression through attenuation of the hypoxic inhibition of mTOR, normally effected by the sequestration of mTOR in PML-containing nuclear subdomains [45**]. Thus, the regulation of HIF-α translation is likely to have a contributing role in a broad range of tumor types.

HIF-1α versus HIF-2α
Discovered first and expressed ubiquitously, HIF-1α is by far the best characterized α-subunit. HIF-2α expression is limited to endothelium, kidney, heart, lung and gastrointestinal epithelium, and some cells of the CNS [3,46,47]. Differences exist in their targets, with HIF-1α uniquely activating glycolytic enzyme genes and HIF-2α preferentially activating VEGF, transforming growth factor-α (TGFα), lysyl oxidase, Oct4 and Cyclin D1 [7,48,49***,50,51,52*]. Similarly, the effects of Hif-1α and Hif-2α gene disruption are substantially different: Hif-1α knockout consistently leads to impaired cardiac and vascular development and E10.5 lethality [23,26,53]. By contrast, Hif-2α loss leads to group of strain-specific phenotypes, including embryonic lethality due to bradycardia and vascular defects, perinatal lethality due to impaired lung maturation, and embryonic and postnatal lethality caused by multi-organ failure and mitochondrial dysfunction [54–57].

Differences in gene targets and knockout phenotypes suggest that HIF-2α promotes a distinct phenotype in tumors expressing it. This has been observed in CNS, colorectal, non-small cell lung and head and neck tumors, where expression of HIF-2α is more strongly associated with poor patient outcomes than is expression of HIF-1α [5,58]. Embryonic stem cell-derived teratomas with the murine Hif-2α cDNA ‘knocked in’ to the Hif-1α locus exhibit a fourfold increase in mass compared with Hif-1α-expressing controls, largely due to increased proliferation [59]. These data suggest that HIF-2α preferentially
promotes tumorigenesis. Enhanced proliferation probably results from increased expression of TGFα and Cyclin D1. Additional effects on the tumor phenotype might result from HIF-2α-mediated induction of the stem cell factor Oct4 and promotion of c-Myc transcriptional activity, as described below [49**] (JD Gordan et al., unpublished).

**HIF transcriptional targets**

A series of microarray studies have defined HIF target gene families [6,7,50,60–63]. Erythropoiesis, angiogenesis, and glycolytic metabolism are controlled through multiple gene targets, with differential activation being based on cell type and which HIF-α subunit is expressed. Continued analysis is expanding our understanding of how some of these responses are mediated. HIF-1α-mediated induction of glycolytic metabolism has been well appreciated, but the inhibition of aerobic metabolism through the induction of pyruvate dehydrogenase kinase (PDK1) was only recently described. PDK1 phosphorylates pyruvate dehydrogenase, inhibiting the conversion of pyruvate to acetyl-CoA. The inhibition of aerobic metabolism at moderate levels of hypoxia might free limited oxygen supplies for other cellular processes and avoid the accumulation of toxic metabolites [64*].

Metastasis is a defining characteristic of cancer and is also promoted by tumor hypoxia. Metastasis is a coordinated process wherein chemokines direct cell migration, adhesion molecules mediate attachment in distant organs, and proteases and other secreted enzymes degrade or alter the extracellular matrix. Studies in breast cancer and RCC demonstrated that the chemokine receptor CXCR4, a major metastatic mediator, is upregulated by HIF [66], whereas analysis of lung epithelium further showed that matrix metalloproteinases (MMPs) 2 and 9 are regulated by hypoxia [67]. Another key mediator of metastasis is lysyl oxidase, an HIF target strongly associated with hypoxia and poor patient outcome in several tumor types. Lysyl oxidase alters extracellular matrix components such as elastin and collagen, and its inhibition blocks in vitro migration and in vivo metastasis from subcutaneous xenografts or after tail vein injection [52*].

HIF targets known to be important in development also have a substantial role in tumor biology. Oct4, an HIF-2α target gene, encodes a POU-domain transcription factor that is a key regulator of stem cell behavior. Well known for a role in embryonic stem cells, Oct4 has more recently been observed in some adult stem cell populations [68]. In studies of a knock-in model in which murine Hif-2α was expressed from the Hif-1α promoter, a dramatic disruption of embryonic development was observed, correlating with an enhancement of TGFα, VEGF and Oct4 expression. In vitro models of these developmental phenotypes were mostly reversed by short hairpin RNA knockdown of Oct4 [49**]. Interestingly, Oct4 knockdown also substantially reversed the growth advantage seen in subcutaneous teratomas derived from the knock-in embryonic stem cells compared with teratomas from Hif-1α wild type embryonic stem cells [49**]. The mechanism by which Oct4 modulates tumor behavior is not yet clear, but one intriguing possibility is that it promotes the growth of a ‘cancer stem cell’ population, and thus self-renewal and chemotherapy resistance.

In addition to its direct gene targets, HIF can regulate the transcription factors Notch and c-Myc. HIF-1α was found to require Notch and its target genes in models of hypoxia-induced muscle and neural cell de-differentiation. In fact, HIF-1α interacts directly with the intracellular domain of Notch1, increasing its half-life and transcriptional activity [69*]. The in vivo implications of this interaction remain to be understood, but given Notch’s role in development and tumor biology they are likely to be significant [70]. The implications of HIF-1α inhibition of c-Myc are somewhat clearer. Though HIF-1α has long been connected with cell cycle arrest, the mechanism by which this occurs has not been well understood. HIF-1α directly inhibits c-Myc, causing de-repression of its targets p21 and p27 [71**]. c-Myc targets involved in mismatch repair are also modulated by HIF-1α, suggesting a role for HIF in hypoxia-induced genetic instability [72*]. In assessing the effects of HIF-2α on c-Myc, we have observed that HIF-2α promotes c-Myc transcriptional activity, which might also contribute to HIF-2α-mediated tumor progression (JD Gordan et al., unpublished).

**HIF and cancer therapy**

Pharmacologic inhibition of the HIF target VEGF has proven efficacy as a cancer therapeutic [73] and has generated interest in targeting global HIF activity. Direct approaches such as inhibition of p300-mediated co-activation [74] and DNA binding [75] are being explored, as is HIF inhibition through repression of its translation. HIF-α subunits appear to be particularly sensitive to translational regulation, because the use of pharmacological mTOR inhibitors can block HIF-α expression even following VHL loss [76]. In fact, the mTOR inhibitor CCI-779 resulted in a statistically significant survival advantage in patients with metastatic renal cancer [77].

Mouse models have shown that HIF can have a substantial impact on the response to cytotoxic cancer therapies. Ionizing radiation treatment in subcutaneous tumor models causes HIF-1α stabilization through ROS induction. HIF-1α induction leads to release of cytokines, including VEGF, that promote endothelial cell survival and thus blunt the therapeutic effect of ionizing radiation [78*]. The stabilization of HIF-1α in endothelial cells is also likely to occur following ionizing radiation and can substantially promote tumor growth [79*]. By contrast, HIF-1α enhances the effect of ionizing radiation on tumor cells themselves. In a similar model, induction
of HIF-1α promotes p53 phosphorylation and stabilization, as well as cell death following ionizing radiation. These effects, combined with HIF effects on the endothelium, suggest a particular advantage to combination treatments using ionizing radiation followed at a later point by HIF inhibition [80]. Thus, the inhibition of the HIFs, either at the level of protein expression or transcriptional activity, should be considered on a case-by-case basis, depending on tumor type and other therapies used concurrently.

**Conclusion**

In addition to important roles in development, hematopoiesis, and ischemic disease, the HIFs also have a broad range of effects on tumor biology. They are directly responsible for tumor angiogenesis and metastasis, and contribute substantially to metabolic changes, the evasion of apoptosis, and genomic instability. Despite the appreciation of their relevance to tumor biology, novel targets and mechanisms are reported frequently. Their pharmacological inhibition represents an opportunity and a challenge, and an important area for future study.

**Update**

In a recently published report, Holmquist-Mengelbier and colleagues [81] describe a novel difference between HIF-1α and HIF-2α, with important effects in neuroblastoma. They describe the stabilization of HIF-2α at relatively mild levels of hypoxia (5% oxygen) and over prolonged time courses, compared with HIF-1α. Using a tissue microarray, they show a strong correlation between HIF-2α protein expression, vascularization, and poor clinical outcomes. It will be of great interest to assess the importance of this effect in other tumor types.

**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


Genetic and cellular mechanisms of oncogenesis


34. Mansfield KD, Guzy RD, Pan Y, Young RM, Cash TP, Schumacker PT, Simon MC: Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic HIF-α activation. Cell Metab 2005, 1:393-399. See annotation [36].


36. Brunelle JK, Bell EL, Quesada NM, Vercauteren K, Tirantí V, Zeviani M, Scarulla RC, Chandel NS: Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. Cell Metab 2005, 1:409-414. In a comprehensive series of murine- and patient-derived genetic models, these authors, together with Mansfield et al. [34] and Guzy et al. [35], show a requirement for ROS in hypoxic HIF-α stabilization.


45. Bernardi R, Guernah I, Jin D, Grisendi S, Alimonti A, Teruya-Feldstein J, Cardon-Cardo C, Simon MC, Rafi S, Pandolfi PP: PML inhibits HIF-1α translation and neangiogenesis through repression of mTOR. Nature 2006, 442:779-782. The authors show that PML regulates HIF-α expression under hypoxic conditions through modulation of mTOR, providing mechanistic insight into translational control and an important tumor suppressor.


In identifying pyruvate dehydrogenase kinase as an HIF-1α target, the authors, together with Papandreu et al. [69*], describe the mechanism whereby HIF inhibits aerobic metabolism under hypoxia, limiting oxygen consumption and ROS production.


See annotation [64*].


Here, Gustafsson and colleagues describe an important new mechanism for HIF-α effects on development. By modulating Notch activity, the HIF-α subunits can promote or block differentiation in a context-dependent fashion.


This article, and one the following year by the same group [72*], shows an important role for HIF-1α in modulating the activity of the c-Myc proto-oncogene.


See annotation [69*].


This study shows a new role for HIF-1α in tumor biology, showing that it substantially promotes p53 activity and cell death following ionizing radiation.


With cre-loxP-mediated deletion of HIF-1α from endothelial cells, Tang and colleagues show that HIF-1α expression in endothelial cells is key in tumor vascularization.


This study shows the importance of HIF-2α in an additional tumor type. Furthermore, it suggests that some of the differences between HIF-1α and HIF-2α are due to differential stability under moderate hypoxia rather than distinct target genes.