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Genetic modification of hypoxia signaling in animal models and its effect on cancer

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Abstract Conditions that cause hypoxemia or generalized tissue hypoxia, which can last for days, months, or even years, are very common in the human population and are among the leading causes of morbidity, disability, and mortality. Therefore, the molecular pathophysiology of hypoxia and its potential deleterious effects on human health are important issues at the forefront of biomedical research. Generalized hypoxia is a consequence of highly prevalent medical disorders that can severely reduce the capacity for O_2 exchange between the air and pulmonary capillaries. In recent years, some of the key O_2-dependent signaling pathways have been characterized at the molecular level. In particular, the prolyl hydroxylase (PHD)-hypoxia-inducible factor (HIF) cascade has emerged as the master regulator of a general gene expression program involved in cell/tissue/organ adaptation to hypoxia. Hypoxia has emerged as a critical factor in cancer because it can promote tumor initiation, progression, and resistance to therapy. Beyond its role in neovascularization as a mechanism of tumor adaptation to nutrient and O_2 deprivation, hypoxia has been linked to prolonged cellular lifespan and immortalization, the generation of "oncometabolites", deregulation of stem cell proliferation, and inflammation, among other tumor hallmarks. Hypoxia may contribute to cancer through several independent pathways, the inter-connections of which have yet to be elucidated. Furthermore, the relevance of chronic hypoxemia in the initiation and progression of cancer has not been studied in depth in the whole organism. Therefore, we explore here the contributions of hypoxia to the whole organism by reviewing studies on genetically modified mice with alterations in the key molecular factors regulating hypoxia.

Keywords Hipoxia · Cáncer · Genetically modified mouse models

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Hypoxia

Oxygen is essential for the normal function of eukaryotic cells because it has a central role as the final electron acceptor in mitochondrial respiration [1]. For this reason, eukaryotic cells have evolved elaborated oxygen-sensing mechanisms that are designed to rapidly respond to decreases in oxygen levels, i.e., hypoxia [2, 3]. Hypoxia-Inducible Factors (Hifs) are the main effectors of oxygen homeostasis, allowing cellular adaptation to hypoxia by regulating the expression of more than one hundred genes involved in various biological processes such as the enhancement of oxygen delivery (angiogenesis) and cellular energy metabolism (for example, the promotion of anaerobic metabolism) [3–5].

Hypoxia-Inducing Factor-1 (Hif-1) is a heterodimeric complex consisting of two basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) subunits called Hif-1α and Hif-1β/Arnt [6, 7]. Hif-1α has two other isoforms called Hif-2α and Hif-3α that are all associated with oxygen sensing [8–10]. Both proteins, Hif-α (Hif-α refers to either Hif-1α or Hif-2α) and Hif-1β, are constitutively expressed during both normoxia and hypoxia [11–13] (Fig. 1). Under

normoxic conditions, Hif-α is post-translationally hydroxylated by two different proteins: Prolyl Hydroxylase Domain (Phd) and Factor-Inhibiting Hif (Fih) [11–13]. There are three different Phds (called 1, 2, and 3) that are implicated in the hydroxylation of Hif-α at two conserved prolines at positions 402 and 564 [13–15]. This modification tags the protein and is then recognized by the von Hippel–Lindau tumor suppressor protein (pVhl), which recruits an E3 ubiquitin ligase complex that causes Hif-α proteasomal degradation [13, 16] (Fig. 1). The other hydroxylase, Fih, modifies a buried residue, asparagine 803, in a hydrophobic region formed by the interaction of the Hif-α C-terminal sequence with the p300 coactivator, which enhances gene transcription [13, 15, 17]. Both Phds and Fih are Fe^{2+}- and 2-oxoglutarate-dependent dioxygenases that promote the oxygen-dependent degradation and oxygen-dependent inactivation of Hif, respectively. However, under hypoxic conditions, Hif hydroxylases are inactive, and thus Hif-α becomes stabilized, forming a complex with Hif-1β/Arnt. This complex translocates to the nucleus where it regulates the expression of downstream target genes.

During solid tumor growth, hypoxic conditions can occur locally because of insufficient oxygen delivery to rapidly proliferating tumor cells [18]. Tumor cells respond to these hypoxic conditions activating the hypoxia HIF signaling pathway. Hif activation results in the induction of angiogenesis with the formation of new blood vessels allowing an increase in oxygen supply. In addition, Hif activation affects cellular metabolism, inducing a metabolic switch from mitochondrial respiration to anaerobic glycolysis that allows tumor cell survival [5, 19]. Therefore, the HIF pathway controls several biological functions that are critical for tumor cell growth and survival, thus making it an attractive therapeutic target [20].

Animal models of cancer are critical for the study of cancer biology and genetic testing of novel therapeutic strategies. There are three major types of animal cancer models: direct injection of cancer cells into 'healthy'-athymic animals [18, 21], implantation of three-dimensional tumors into the animal (xenograft models) [18, 22], and genetically engineered mouse models (GEMM) [22–24]. There are two types of GEMMs: transgenic and targeted. Each type of cancer model has advantages and

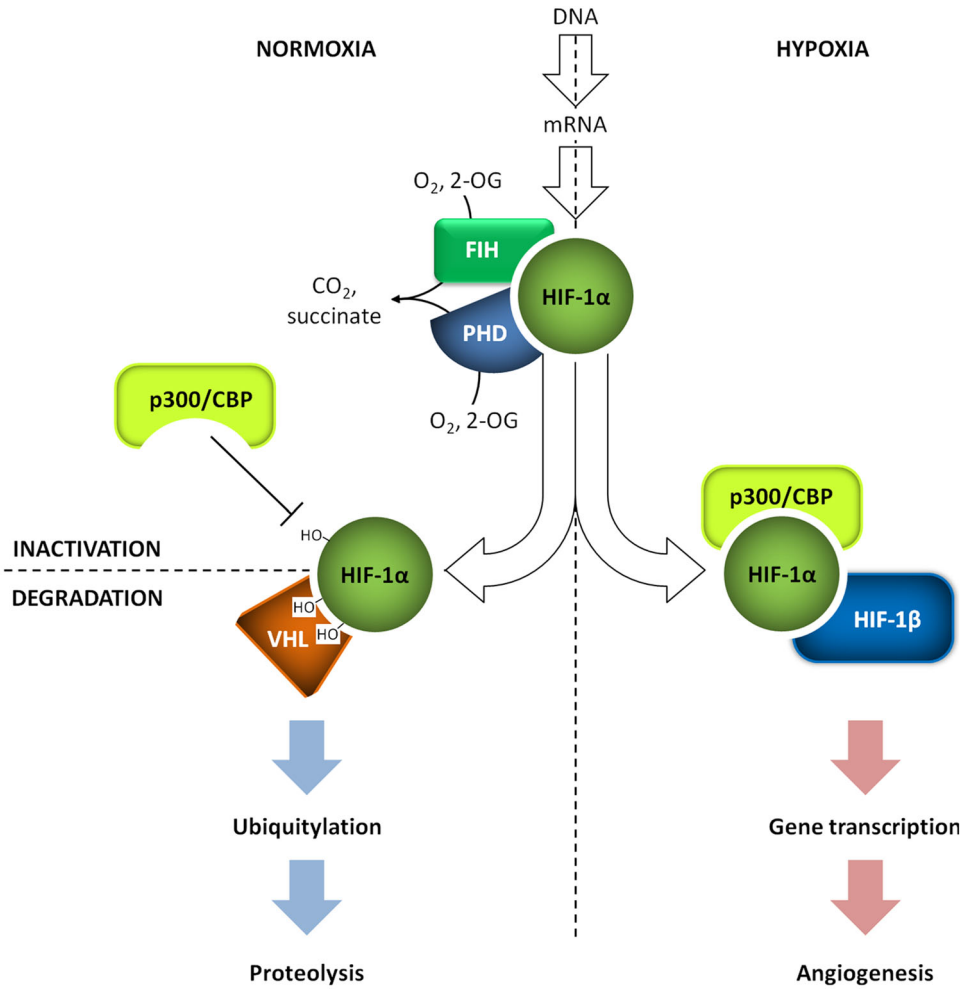

Fig. 1 HIF-α fate under normoxic (*left*) or hypoxic (*right*) conditions

disadvantages. For both cell injection and xenotransplantation, immunosuppressed mice must be used to avoid the rejection of the human cells or tissue. Knock-out mice have the advantage of being non-immunocompromised animals, so the behavior of the induced tumor is expected to be similar to that found in spontaneous tumors in humans. To avoid deleterious consequences, genetically modified mouse models usually carry a conditional (floxed) allele that can be eliminated through recombination to obtain the knock-out animal [25]. Combinations of Cre-*loxP* systems, specific promoters, and inducible systems (i.e., 4OHT) allow tissue- and time-specific inactivation of the desired protein [26, 27].

Here we discuss the role of the hypoxia pathway in cancer and review the GEMMs (knock-out and transgenic) available to study the hypoxia pathway with an emphasis on the central proteins of this pathway (Hif-α, Hif-1β, Fih, pVhl, and Phds).

Hypoxia-induced factor α

Hif-α can be considered the key regulator of hypoxia. As mentioned above, it is constitutively expressed in cells but is post-translationally modified and inactivated under normoxic conditions by an asparaginyl hydroxylase and three different prolyl hydroxylases that label the protein for degradation [14, 15]. As a consequence, its half-life is very short under normoxic conditions, being limited to only 5 min in growing cells [16, 28]. However, only 2 min after exposing cells to hypoxic conditions, Hif-α is stabilized and detectable in the cell nuclei, where it interacts with Hif-1β and the p300/CSP complex, leading to gene transcription through association with hypoxia response elements (HREs) [5, 29, 30].

All Hif-α isoforms are closely related. Hif-1α and Hif-2α share the same domain architecture (Fig. 2) and are capable of interacting with Hif-1β/Arnt [31]. Both proteins have an oxygen-dependent degradation domain (ODDD), with two highly conserved proline residues (402 and 564) [4, 31]. Both domains are in the central region of the protein and are preceded by bHLH and PAS domains [6]. In addition, there are two transactivation domains, an internal activation domain (NAD) and a carboxy-terminal activation domain (CAD). The NAD overlaps with the ODDD and is involved in gene transcription under hypoxic conditions [4]. The CAD is important because it interacts with the CH-1 (cysteine/histidine) domain of p300, a coactivator that interacts with Hif-1/2α and enhances gene transcription [30, 31]. However, each protein is regulated by different mechanisms, for example, Hsp70/CHIP-dependent ubiquitination of Hif-1α and iron-dependent translation of Hif-2α mRNA [31]. In addition, it has been suggested that Hif-2α binding to DNA may be regulated by redox conditions, while Hif-1α is not sensitive to redox conditions [33]. These results suggest that different physiological conditions of hypoxia may lead to selective responses induced by the specific activation of each isoform. Both Hif-1α and Hif-2α activate the expression of Vegf, Epo (erythropoietin), and proteins related to anaerobic metabolism; however, the Hif-1α/1β complex activates a subset of genes related to glycolytic enzymes that are not induced by the Hif-2α/1β complex [32]. Unlike Hif-1α, Hif-2α is expressed only in a subset of tissues and organs (endothelium, kidney, liver, lung, and brain).

The other isoform, Hif-3α, shares less homology with Hif-1/2α, approximately 55 % [34], and its function and regulation are less understood. Hif-3α undergoes different alternative splicing events and has seven different described variants [10]. One of these spliced forms is the inhibitory domain PAS protein (IPAS), a truncated Hif-3α isoform with no transactivation domain [35]. This isoform interacts with Hif-1/2α, blocking Hif-α-mediated transcription. However, another alternative splicing form of Hif-3α has been associated with maximal transcription of some hypoxia-related genes [10].

When a growing tumor reaches a critical size, the cancer cells might lie so far from blood vessels that it may result in low-oxygen conditions. Under these conditions, Hif-1α and Hif-2α are not hydroxylated and thus are able to activate the hypoxia response. To study the role of Hif-α in cancer formation, both isoforms have been knocked-out in vivo to study the behavior of induced tumors in these animals. Theoretically, the absence of these genes should block gene activation downstream of the formation of the Hif-α/1β complex. However, homozygotic *Hif-1α*$^{-/-}$ or *Hif-2α*$^{-/-}$ embryos are not viable and die during development or shortly after birth [36–38], thus requiring the use of conditional *Cre* knock-out mice to analyze the role of these factors in adult animals.

Hif-1α

Conditional Hif-1α mice were analyzed in the seminal study by Ryan et al. [39]. To obtain a *Hif-1α* null mouse, they flanked the second *Hif-1α* exon with two *loxP* sequences. As this exon encodes the Hif-1β/Arnt-interacting domain of Hif-1α, the resulting *Hif-1α* gene would be deleted upon *loxP* recombination and it could not interact with its partner. They induced the transient expression of Cre in embryonic MEFs that were homozygous for the *Hif-1α* conditional allele to obtain *Hif-1α*-null cells. As a consequence, the cells exhibited no hypoxia-dependent Hif-1α response, lower levels of Vegf, and no changes in the expression of glycolytic enzymes compared with Hif-1α-positive cells. Tumors generated from these *HIF-1α*$^{-/-}$

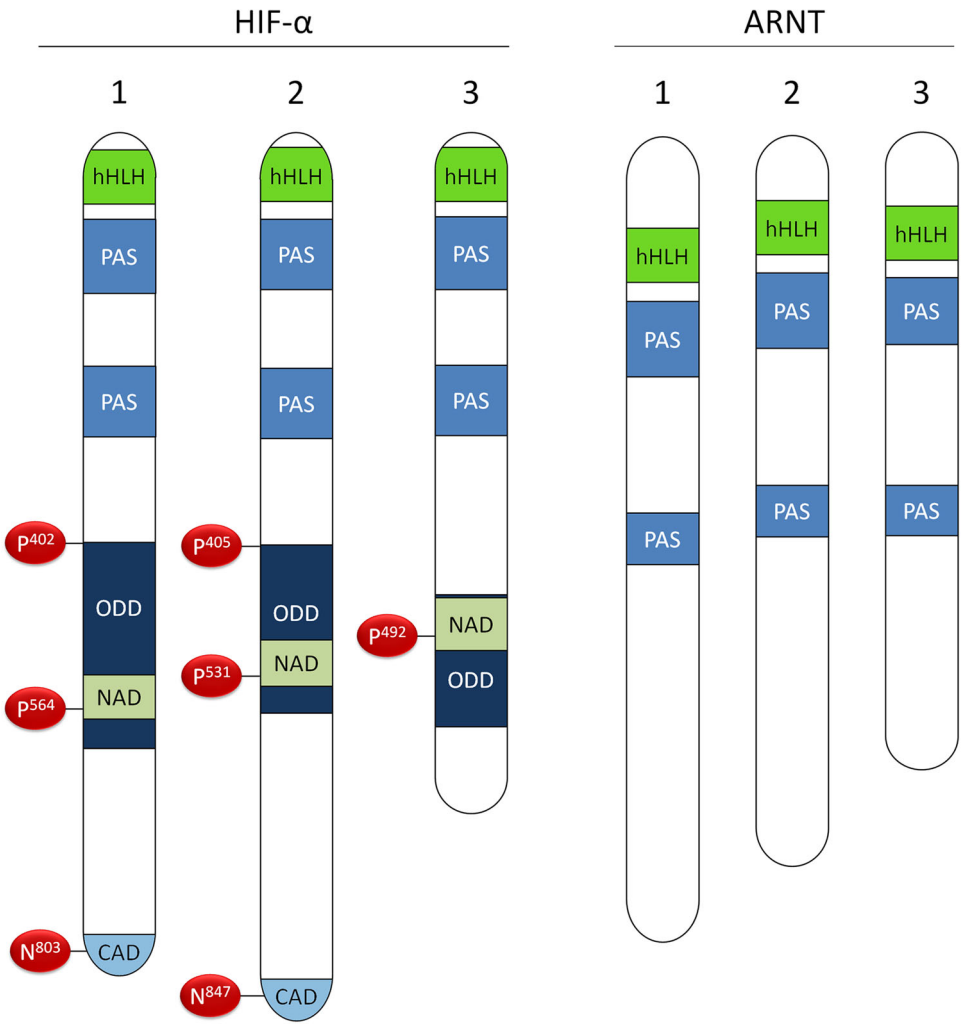

Fig. 2 Domain structures of the HIF-α (*left*) and ARNT (*right*) isoforms

cells in immunocompromised mice showed decreased tumor mass because the angiogenesis process was blocked.

A different approach to analyze the effects of Hif-1α in cancer is gene silencing. Méndez et al. [40] obtained a murine glioma GL261-derived cell line with *Hif-1α* knocked-down by shRNA. These cells were then injected into the brain of mice. No differences in survival rate and tumor size were observed between animals injected with the shRNA knocked-down GL261 cells and those injected with the parental GL261 cells, However, *Hif*-1α silencing diminished invasiveness of tumors.

Based on these studies, Hif-1α deletion can be considered anti-tumorigenic because the reduction in invasion observed in *Hif-1α*$^{-/-}$ cells. These results show that Hif-1α is necessary for tumor properties such as cell invasion. This notion is also supported by studies of Takeda et al. [41] using RIP1-Tag2 mice, a strain that spontaneously develops pancreatic solid tumors in islets [42]. Conditionally, deletion of *Hif-1α* only in insulin-secreting cells, did not have an effect on lifespan or tumor formation in RIP1-Tag2/*Hif-1α*$^{fl/fl}$ (RIP-Tag2/HKO) mice. However, conditional inactivation of VEGF in RIP1-Tag2/*Vegf*$^{fl/fl}$ mice (RIP-Tag2/VKO) led to a significant extension of lifespan. The double conditional mouse, RIP-Tag2/*Vegf*$^{fl/fl}$/*Hif-1α*$^{fl/fl}$ (RIP-Tag2/DKO) had an intermediate lifespan between that of each single conditional mouse. Interestingly, the absence of Hif-1α did not affect Vegf expression levels. This maintenance of Vegf expression may occur through Hif-2α, although the authors did not test whether this isoform was expressed. Loss of Vegf led to decreased tumor size in both RIP1-Tag2/VKO and RIP1-Tag2/DKO mice because of the inhibition of angiogenesis. Nevertheless, they observed higher cellular proliferation rates in RIP1-Tag2/DKO mice compared to RIP1-Tag2/VKO mice. This effect may be explained because Hif-1α expression suppresses cellular proliferation. They also observed that the absence of Hif-1α correlated with normal expression of adhesion molecules. In addition, *Hif-1α*$^{-/-}$ cells exhibited

a reduced invasive phenotype. Taken together, these data suggest that Hif-2α may compensate for Hif-1α deficiency. However, Hif-1α might have a threshold of expression that suppresses cell proliferation (low oxygen tension) and promotes invasion (higher oxygen tension). The authors hypothesized that ROS production due to oxidative phosphorylation may stabilize Hif-1α.

Another strategy to analyze the role of the HIF pathway in cancer is to study the effects of a constitutively activation of the pathway in transgenic mice. Animal models in which Hif-α is constitutively expressed have shown that its expression is not protumorigenic by itself [43]. For this approach, Fu and colleagues generated a mouse model that resembles Von Hippel–Lindau (VHL) kidney disease [26]. As a result of this disease, the probability of developing cancer is highly increased [44]. One of the most common cancers developed by VHL disease patients is clear cell renal cell carcinoma (ccRCC), which is characterized by elevated expression of Hif-1α and/or Hif-2α and "clear" cells with a large vacuole surrounding the nucleus [26]. This big vacuole accumulates lipids or glycogen. Although VHL disease is caused by mutations of the *Vhl* tumor suppressor gene, the authors suggested that it can be mimicked by Hif-1α overexpression. They obtained transgenic mice expressing Hif-1α mutated at the three key residues for hydroxylation: Pro^{402}, Pro^{562}, and Asn^{803}. Thus, the ability of Phds and Fih to inactivate Hif-α is blocked, and hypoxia genes are transcribed. In addition, to better mimic VHL disease, this Hif-1α triple mutant (mHif-1α) was expressed from a kidney-specific promoter, allowing the specific expression of mHif-1α only in the kidney. As a result, they obtained mice with a phenotype very similar to ccRCC with "clear" cell clusters as early as 3 months after birth. These cells spontaneously evolved to carcinoma beginning at 14 months of age. The animals exhibited abnormal vascularization and increased expression of CA-IX and Vegf, both related to ccRCC. These effects were restricted to the kidney, no abnormalities in other organs were observed. Therefore, the authors produced a good animal model that resembles the early and middle stages of ccRCC, although no invasion or metastasis to other tissues was observed.

Genetic activation of Hif has also been achieved in transgenic mice that express Hif-1α P402A/P564A (mHIF-1α) under the control of the dispensable ROSA26 locus, allowing ubiquitous expression [43]. However, in this case, ccRCC was not observed in the transgenic mice. As stated by the authors, this result could be due to the use of a non-physiological promoter. However, another possible explanation is that Hif-1α was inhibited by hydroxylation of the unmutated asparagine residue at position 803, blocking p300/CSP co-activation.

HIF-2α

Hif-2α, also known as HLF (Hif-1α-like factor) or EPAS1 (Endothelial PAS domain-containing protein 1), is also associated with angiogenesis and is important for vascularization during embryonic development [45]. Under hypoxic conditions, Hif-2α also interacts with Arnt, forming a complex that activates gene transcription [31].

Hif-2α has been successfully deleted in the germline of mice by different groups [45–47], although it has also been reported to be a lethal deletion [37, 48]. The surviving mice, however, showed multiple defects, including smaller size, and numerous syndromes related to the circulatory system. *Hif-2α* knock-in was tested by Covello et al. [49]. They targeted a cDNA encoding *Hif-2α* to the *Hif-1α* locus to drive Hif-2α expression under regulatory control of the *Hif-1α* locus. Nevertheless, the homozygous *Hif-2α* knock-in mice died early during development, clearly indicating non-redundant functions for Hif-1α and Hif-2α. Indeed, previous results have shown that the expression of Hif-2α is required in $Vhl^{-/-}$ renal carcinomas, making it the most oncogenic Hif protein [50]. In addition, there is also a correlation between decreased survival and Hif-2α overexpression, regardless of Hif-1α [51]. However, an antitumorigenic role for Hif-2α has also been described. In rat glioma tumors, Hif-2α overexpression increased tumor cell apoptosis, leading to reduced tumor growth [52].

To avoid problems associated to global *Hif-2α* deletion, conditional knock-out mice should be obtained to study the effects of specific *Hif-2α* deletion. Skuli et al. [53] generated a $Hif\text{-}2\alpha^{fl/\Delta}$ Cre mouse with an endothelial-specific deletion of *Hif-2α*. Like the Hif-1α conditional mice, *Hif-2α* cKO mice developed normally and exhibited efficient deletion of *Hif-2α* from endothelial cells. The KO mice exhibited acute vessel permeability. The authors subjected these KO mice to subcutaneous injection of Lewis lung carcinoma (LLC) cells. As a result, the KO mice developed smaller tumors than the control mice and increased tumor cell apoptosis due to Hif-2α deletion. These results are consistent with a smaller tumor vascularization in the KO mice even though the xenograft cells are not *Hif-α* defective.

Other studies have combined a conditional $Hif\text{-}2\alpha^{fl/fl}$ allele with conditional alleles of other genes important for tumor formations such as *Kras* [54]. The latter has been extensively described as an oncogenic protein due to a Gly-to-Asp substitution in position 12 that permanently activates the protein [55]. The effects of conditional Hif-2α overexpression in the liver in $Hif\text{-}2\alpha^{fl/fl}$ mice were previously described using Pro-to-Ala (in positions 405 and 531) substitutions to avoid the recognition of Hif-2α by Phds and its subsequent degradation [39]. Mutated Hif-2α

expression led to the same vascular lesions that occurred upon pVhl inactivation; however, no spontaneous cancer development was observed. When $Kras^{G12D}$ expression was combined with Hif-2α expression in mice, a decrease in lifespan was found compared with mice that only expressed $Kras^{G12D}$. In addition, although the tumor multiplicity was similar for both mouse strains, increased size of lung tumors was observed in Hif-2α overexpressing mice. Mice expressing both $Kras^{G12D}$ and Hif-2α also had increased lung tumor burdens compared with mice expressing only $Kras^{G12D}$. Additionally, tumor vascularity and invasiveness were increased in the double conditional mice. This result may suggest a direct role for Hif-2α in carcinogenesis. However, *Hif-2α* deletion in the same $Kras^{G12D}$ mouse model also increased lung tumorigenesis [56]. Both conditions (overactivation and inactivation of Hif-2α) are associated with changes in gene expression that promote tumor development.

Hif-2α appears to act as a tumor suppressor gene in the pancreas. Loss of *Hif-2α* promotes Kras-induced pancreatic neoplasia in mice through a mechanism that may involve Wnt signaling [57].

Similar to the experiments performed for Hif-1α, Kim et al. [46] tested the effects of expression of a non-degradable Hif-2α protein in mice under the same non-physiological promoter. Mice expressing $Hif\text{-}2\alpha^{P405A/P531A}$ (mHif-2α) exhibit skin abnormalities (erythema, partial alopecia), epidermal hyperplasia, and a high number of microvessels, all of which are similar to the changes observed with pVhl loss. mHif-2α expression in the liver decreased lifespan to 6–8 weeks and increased tumor proliferation. However, consistent with the results obtained with mHif-1α, mHif-2α expression by itself did not lead to tumorigenesis. Studies with nude mice showed that Hif-2α downregulation in $Vhl^{-/-}$ cells could suppress tumor growth, suggesting Hif-2α can act as a tumor promoter in cells with other mutations that lead to cancer [58].

Altogether, these results show that Hif-2α may act as a tumor suppressor or a tumor promoter depending on its gene targets and its corresponding activation threshold.

Hif-3α

The third member of the Hif family is also the least well known. Although Hif-3α splicing variants (IPAS, Hif-3α4) have been shown to act as negative regulators of Hif-α target genes [35, 59] and to be down-regulated in renal cell carcinoma [59], no Hif-3α mouse models have been described.

The c-Myc/Hif connection

c-Myc is a proto-oncogene that has been connected to hypoxia through Hif-α regulation. However, Hif-1α and Hif-2α have opposite roles in relation to c-Myc. Hif-1α acts as a negative regulator of c-Myc activity, while Hif-2α enhances c-Myc transcriptional activity [60, 61]. c-Myc deletion is lethal during embryogenesis [62], which makes it necessary to use a conditional gene deletion to study c-Myc, as with Hif-α. C-Myc constitutive expression has been linked to carcinogenesis [63], suggesting that Hif-2α is more important in tumor development than Hif-1α.

Hypoxia-induced factor β

Hypoxia-induced factor β (Hif-1β), also known as Arnt (Aryl hydrocarbon Receptor Nuclear Translocator), has two paralogs, Arnt2 and Arnt3. Arnt is usually the paralog involved in the hypoxia response, while Arnt2 is involved in neuronal development and synapse plasticity, and Arnt3 is involved in circadian rhythms [64]. However, Arnt2 can also form heterodimers with Hif-α during development and in kidney and neural cells [65, 66]. Arnt forms heterodimers not only with Hif-α but also with Ahr (Aryl Hydrocarbon Receptor), which is an important transcription factor activated by xenobiotic binding [67]. Arnt and Arnt2 are close structural homologs with 63 % identity [68]. They also have structural similarities with Hif-α, although they contain no ODDD. In both cases, genetic ablation in the mouse is lethal, although there are developmental differences. $Arnt^{-/-}$ embryos exhibit aberrant development, while $Arnt2^{-/-}$ embryos exhibit normal development and die perinatally [65]. Therefore, as with Hif-1/2α or Phd2, conditional mice must be used to avoid lethality. In conditional systems, $Arnt^{fl/fl}$ mice exhibited loss of Hif-dependent gene activation [69].

The dimerization of Hif-α/Arnt is inhibited by acriflavine (ACF), a mixture of flavines that have been shown to inhibit cancer development in xenograft cancer models by blocking Hif-α/Arnt dimerization. [70]. In this way, $Arnt^{fl/fl}$ mice were used to study the effect of ACF. Macrophages purified from $Arnt^{fl/fl}$ mice did not show any response to ACF, while in $Arnt^{\Delta/+}$ macrophages, the expression of Hif target genes was affected. However, this study did not analyze the effect of ACR in $Arnt^{fl/fl}$ mice, although there would possibly be no effects.

The metabolism of xenobiotic substances has a demonstrated role in cancer, mainly through the activity of cytochrome P450 enzymes [71], and the Arnt:Ahr complex is involved in the transcription of these enzymes. Based on these observations, an $Arnt^{fl/fl}$ mouse model with conditional Arnt expression in the skin was used to study the effect of the xenobiotic benzo[a]pyrene (B[a]P) on tumor development [73]. B[a]P acts as a skin tumor initiator, where it is metabolized to B[a]P-7,8-diol-9,10-epoxide by two cytochrome P450 enzymes, CYP1A1 and CYP1B1.

However, in all cases, *Arnt* deletion blocked the appearance of the tumors. Due to its dual role as an inducer of the hypoxic response and xenobiotic metabolism, the authors suggested that Arnt, and not Hif-1α, should be the target of anticancer therapies [73].

Von Hippel–Lindau

VHL disease is an autosomal dominant, hereditary cancer syndrome characterized by an increased risk of clear cell renal carcinoma, retinal and central nervous system hemangioblastomas and pheochromocytomas. VHL patients are VHL heterozygotes, harboring one wild-type allele and one defective allele. Tumors or cysts develop when the remaining wild-type *Vhl* allele is somatically inactivated or lost. Biallelic *Vhl* inactivation is also common in both sporadic hemangioblastomas and clear cell renal carcinomas [73–75]. *Vhl* inactivation has been reported in 86.6 % of clear cell renal carcinoma cases as a result of sequence alterations or promoter methylation in tumor DNA [76]. The *Vhl* tumor suppressor is a component of the E3 ubiquitin ligase complex that contains elongin B, elongin C, Cul2, and Rbx1.

The Vhl protein (pVhl) is a pivotal negative regulator of Hif activity. In the presence of oxygen and iron, specific proline residues of Hif-α become hydroxylated. These hydroxylated prolines are recognized by pVhl, which leads to the ubiquitination and proteasomal degradation of Hif-α. Vhl inactivation results in the stabilization of Hif-α and overproduction of hypoxia-inducible mRNAs. Downregulation of Hif-α by Vhl explains some of the phenotypes of *Vhl*-defective clear cell renal carcinomas, which are highly vascularized tumors due, at least in part, to Vegf overproduction. However, it has been suggested that deregulation of Hif-α is not sufficient for tumorigenesis and that loss of Hif-independent function(s) of Vhl could play a critical role in tumorigenesis [77].

Vhl also has some Hif-independent functions that might be relevant to tumor biology. Roe et al. [78] found that pVhl directly associates with p53 and enhances its stabilization. pVhl promotes an interaction between p53 and p300 upon genotoxic stress, leading to the acetylation of p53, which increases p53 transcriptional activity and p53-mediated cell cycle arrest and apoptosis. Moreover, pVhl was found to associate with Atm and increase Ser-15 phosphorylation of p53, a result that was further confirmed by the finding that pVhl suppresses the Mdm2-mediated ubiquitination and nuclear export of p53. Because Mdm2 binds to the NH2-terminus of p53, it is unlikely that pVhl directly blocks the Mdm2–p53 interaction. Alternatively, pVhl might indirectly block the Mdm2-mediated degradation of p53 by recruiting Atm and mediating the Atm-dependent Ser-15 phosphorylation of p53. It has been suggested that pVhl is key in the formation of the Atm-p53 complex, based on the observation that p53 stabilization is drastically reduced in *Vhl*-deficient RCC cells in response to genotoxic stress. Altogether, these findings point to a new and interesting function for the tumor suppressor pVhl, namely the upregulation of p53 during tumorigenesis.

To study the role of pVHL in vivo, mouse models deficient for *Vhlh* (the murine homolog of *VHL*) have been generated. In 1997, Gnarra et al. [79] developed a mouse line defective for one *Vhlh* allele by targeted homologous recombination. The heterozygous $Vhlh^{+/-}$ mice appeared phenotypically normal and survived beyond 15 months without any evidence of spontaneous disease. However, the homozygous $Vhlh^{-/-}$ mice developed placental lesions at approximately 9.5–10.5 days of gestation (E9.5 to 10.5) and died in utero between E10.5 and E12.5 because of defective placental vasculogenesis. To circumvent the embryonic lethality of the conventional *Vhlh* deletion, Haase et al. [80] generated a conditional VHL-null allele (2-*lox* allele) and a *Vhlh*-null allele (1-*lox* allele) using Cre-*loxP* technology. Contrary to what was observed in the conventional heterozygous *Vhlh* mice, mice heterozygous for the 1-*lox* allele developed vascular tumors in the liver. Liver-specific deletion of Vhl using Albumin-*Cre* transgenic mice resulted in severe steatosis and formation of blood-filled vascular cavities as well as foci of increased vascularization within the hepatic parenchyma. Molecular analysis of $Vhl^{-/-}$ hepatocytes revealed increased expression levels of Vegf, Glut-1 and Epo, as well as stabilization of Hif-2α. This seminal paper proved that targeted inactivation of *Vhlh* in mice could recapitulate the clinical features of VHL disease; thus, this mouse strain could be used as a model for the disease. However, it remains unclear why heterozygous *Vhlh* mice develop vascular tumors in the liver but not in other organs as is commonly observed in *Vhlh*.

Conditional inactivation of *Vhlh* has been achieved in several other organs including kidney, bone, pancreas, and bone narrow [27, 81–83]. Specific inactivation of *Vhlh* in the kidney results in the development of renal cysts that express markers of multiple nephron segments and show evidence of increased proliferation and dedifferentiation. Interestingly, the development of these renal cysts seems to be a Hif-1α independent process [81]. Lack of *Vhlh* in osteoblasts results in the upregulation of Hif-α, which leads to increased Vegf expression and the development of extremely dense, highly vascularized long bones [82]. Specific deletion of *Vhlh* in the pancreas results in the formation of highly vascularized, microcystic adenomas similar to those found in VHL patients [84]. Interestingly, *Vhlh* inactivation in pancreatic β-cells results in defects in

glucose homeostasis, indicating an important and previously unappreciated role for pVhl in β-cell function [83]. More recently, mice deficient for *Vhlh* in myeloid cells have been shown to exhibit erythema, enhanced neovascularization in matrigel plugs, and increased production of Vegf in the bone marrow, all of which were completely abrogated by either genetic or pharmacological inactivation of Hif-1 [27].

Thus, the *Vhlh* mouse models might be useful models to help advance our understanding of the Vhl/Hif pathway and define the molecular events that are involved in the formation of Vhl-associated tumors. However, it is important to note that although these models display, to some extent, the clinical features of Vhl for a particular organ, no mouse model exists that completely recapitulates VHL disease; none of these models develop retinal hemangioblastomas or pheochromocytomas, which are common manifestations of VHL disease.

Factor-inhibiting hypoxia-inducible factor

Factor-inhibiting hypoxia-inducible factor (Fih) catalyzes the post-translational hydroxylation of asparaginyl residues [85]. Fih is an asparaginyl hydroxylase ubiquitously expressed and predominantly localized in the cytoplasm that modifies Hif-α through Asn^{803} (in Hif-1α) or Asn^{853} (in Hif-2α) hydroxylation [17, 86]. This post-translational modification hinders the interaction between Hif-α and the coactivator proteins p300/CBP due to steric impedance. As a consequence, gene activation downstream of Hif-α is inhibited under normoxic conditions. Under severe hypoxia (<1 % O_2), Fih can no longer hydroxylate Hif-α, and the hypoxia-dependent genes are transcribed [17].

Fih expression has been observed in *Vhl*-defective ccRCC cells, in which Fih acts as a partial suppressor of Hif-α activity [87]. While studies of *Fih* knock-out mice revealed a relationship between Fih and metabolism [15], to the best of our knowledge, no studies of $Fih^{-/-}$ animals in cancer have been performed.

However, another interesting possibility is the modification of expression of other gene(s) that may act on the HIF pathway. For example, microRNAs that modify protein transcription have been proposed. miR-31 and miR-135b lower Fih expression, allowing the activation of Hif, as observed in head and neck squamous cell carcinoma (HNSCC) [88]. miR-135b expression was tested in conditional knock-out mice, *Tgfbr1*/*Pten* 2cKO (K14-$CreER^{tam}$; $Tgfbr1^{fl/fl}$; $Pten^{fl/fl}$), that develop HNSCC spontaneously after treatment with DMBA and tamoxifen [63]. Higher levels of miR-135b correlated with lower expression of Fih mRNA and a subsequent decrease in protein expression. This was followed by upregulation of Hif-1α mRNA expression. Due to the higher Hif-1α levels in the *Tgfbr1*/*Pten* 2cKO mice, the animals exhibited increased cancer cell proliferation, migration, and angiogenesis, probably as result of the high Vegf expression. Deletion of the Transforming Growth Factor-Beta Receptor (*Tgfbr1*) in mice leads to an increase in miR-135b levels, which reveals a correlation between Tgfbr1 and the hypoxia pathway.

Prolyl hydroxylase domain-containing proteins

Prolyl hydroxylase domain-containing proteins (PHDs) catalyze prolyl 4-hydroxylation of Hif-1/2α only in the presence of oxygen. These proteins belong to a 2-oxoglutarate (2OG)-dependent dioxygenase superfamily [11, 14]. In addition to 2-OG, these enzymes need Fe^{2+}, O_2, and ascorbate to correctly modify Hif-1/2α. In mammals, four different isoforms have been identified: 1, 2, 3, and 4 [14]. As for Hif-α, Phd isoforms 1 and 2 are very similar (407 and 426 residues, respectively), while isoform 3 is shorter (only 239 amino acids) [14]. The final isoform, Phd-4, has been found attached to the membrane with its active site directed to the lumen of the ER [89]. Although this isoform is not cytoplasmic, modification of Phd-4 levels in cultured cells led to changes in Hif-α protein level [90, 91]. This isoform is larger, with 502 amino acids and a transmembrane domain [90, 91].

As with all 2-OG-dependent dioxygenases, the Phds possess a double-stranded beta-helix core fold where Fe^{2+} is bound that constitutes the catalytic center [91, 92]. Phd2 is expressed ubiquitously, while the other two isoforms, Phd1, and Phd3, are mainly expressed in the placenta and heart, respectively [14]. Expression of the three soluble isoforms has been described in the kidney, but in different cell types [93]. Hif-α can be hydroxylated in vitro by all soluble Phds [94], although only *Phd2* knockdown results in Hif-1α accumulation under normoxia [95].

When hypoxia gene transcription is activated, transcription of the *Phd2* and *Phd3* genes increases [96, 97]. With low oxygen levels, Phds cannot hydroxylate Hif-α, but the cell is ready for rapid Hif-1α clearance when hypoxic conditions end. While Hif-1α regulates the transcription of Phd2 and Phd3, Hif-2α has been shown to induce the transcription of Phd3 [97].

The role of Phds in cancer is controversial; both pro- and anti-tumorigenic effects have been described [98]. Mice with genetic ablation of *Phd2* are not viable because of severe defects in the placenta, while *Phd1* or *Phd3* deletion has no effect on mouse development or survival [77].

Phd2

Phd2 has generally been annotated as a tumor suppressor gene because its expression has been associated with a

decrease in Hif-1/2α levels [5, 11, 14, 96, 97]. However, Phd2 deficiency has also been associated with decreased cell invasion and metastasis [99, 100]. Therefore, it should also be considered a tumor-promoting gene because Phd2 overexpression has been detected in pancreatic tumors [101, 102].

Because *Phd2*$^{-/-}$ is lethal, studies of this gene have used heterozygous or conditional *Cre Phd2*$^{fl/fl}$ mice [103]. A decrease in Phd2 expression in heterozygous Phd2 mice (*Phd2*$^{+/-}$) induced endothelial normalization in tumors and more mature xenograft tumor blood vessels [99]. The 50 % decrease in Phd2 expression was offset by an increase in the Phd1 and Phd3 levels in *Phd2*$^{+/-}$ cells. However, in all studied oxygen conditions, Hif-α was detected at higher levels in *Phd2*$^{+/-}$ cells, along with increased Vegf receptor-1 and VE-cadherin expression [99]. Although tumors in *Phd2*$^{+/-}$ mice grew normally, their metastatic ability was reduced, most likely because of improved tumor oxygenation [99]. Due to the lack of Phd2, Hif-α was not hydroxylated, increasing erythropoietin production in the kidney [68, 94]. This is a common feature of Phd2 deficient cells, as erythropoietin is also detected at higher levels in non-tumorigenic cells [94, 104]. In addition, *Phd2* null mice recover better after myocardial infarction, with more capillaries than *Phd2*-expressing mice [105]. However, it must be noted that, in this case, the animals were doubly treated with shPhd2 and shFih to reduce Hif-α hydroxylases; therefore, the observed effect may not be only due to *Phd2* knockdown. In vivo experiments with xenograft tumors showed that shPhd2 treatment reduced tumor growth, which has been related to its effect on Tgf-β [100, 106], which is also transcribed under hypoxic conditions. In addition, the treatment of tumor cells with antibodies against Tgf-β also inhibited cell growth [100]. The authors suggested a possible connection between Phd2 and the Tgf-β pathway, concluding that Phd2 inhibition could change the role of Tgf-β from a tumor promoter to a tumor suppressor. In hematopoietic cells, Phd2 ablation also led to a reduction in tumor growth [107]. Overall, reduction of Phd2 expression had no effects on tumor growth, but partially blocked metastatic ability. However, Phd2 ablation leads to reduced tumor growth, as opposed to a more malignant cancer phenotype, due to hypoxia pathway activation and, most likely, crosstalk between the hypoxia and Tgf-β pathways. Again, Hif-α expression alone (caused by *Phd2* ablation) is not protumorigenic.

Other groups have described a different phenotype. In human cancer cell lines, decreased expression of Phd2 has been connected to an increased ability to form tumors [108, 109]. For example, *Phd2* knockdown cells xenografted into mice increased tumor growth compared with control cells [109]. In contrast, in non-small-cell lung carcinoma (NSCLC) cells, Phd2 has been detected at higher levels in patients with a poor prognosis [110]. The reasons for these discrepancies are currently unknown.

Phd1

The deletion of Phd1 in mice (*Phd1*$^{-/-}$) has been reported to produce hypoxia tolerance, a characteristic attributed to cancer malignancy [111]. *Phd1* deletion induces a global change in basal metabolism that allows the cells to survive under a limited oxygen supply [112]. In *Phd1*$^{-/-}$ animals, Pdk4 is upregulated, partially blocking the TCA cycle through a reduction in the amount of pyruvate that enters the cycle. This blockage allows the maintenance of mitochondrial metabolism with lower oxygen requirements. Under ischemic conditions, ROS production is increased, blocking mitochondrial oxidative phosphorylation [113]. Due to the role of hypoxia as a ROS inducer, *Phd1*$^{-/-}$ cells are protected because of their lower oxygen requirements, as has been shown for both muscle and hepatic cells [112, 114]. *Phd1* deletion increased Hif-2α expression in muscle under ischemic conditions with no changes in Hif-1α levels, suggesting that Hif-2α is a Phd1 target. In cultured cells, Phd1 hydroxylates the oncogenic RNA polymerase II subunit Rpb1 on Pro1465 [115]. In summary, *Phd1* deletion has a double protective effect: it protects against ROS production during ischemia and suppresses the oncogenic properties of hydroxylated Rpb1.

In mice, experiments with Phd1 have been mainly carried out using nude mouse models. HCT116 colon carcinoma cells overexpressing Phd1 were subcutaneously injected into nude mice [116] and tumor growth was reduced, mainly due to necrosis and poorer oxygenation ability. Due to Phd1 overexpression, Hif-1α was inactivated, suppressing downstream gene activation, which explained why the observed phenotype was similar to that of *Hif-1α*$^{-/-}$ cells. However, the difference between non-tumorigenic *Phd1*$^{-/-}$ cells and colon carcinoma cells overexpressing Phd1 must be noted; the main target for Phd1 in "normal" muscle cells was Hif-2α, while in HCT116 colon cells, it was Hif-1α.

Phd3

In contrast with the other two soluble isoforms, Phd3 acts mainly on the C-terminal hydroxylation site, while the other isoforms also hydroxylate the N-terminal site [2, 98]. Alterations in Phd3 expression have been associated with cancer, although the effects are contradictory: upregulation in pancreatic cancer cells and downregulation in colorectal cancer cells [117, 118]. Only nude mice injected with human cells have been used to study pancreatic cancer cells, whereas transgenic *Phd3*$^{-/-}$ mice have only been used to study the feedback loop between Hif-α and Phd3,

Table 1 Hypoxia animal models

Gene	Isoform	Animal model	Tissue/cells	Expression		References
HIF-α	HIF-1α	Mice	MEFs *Hif-1α*$^{fl/fl}$ cells	Knock-out	Decreased tumor mass in immunocompromised mice	Ryan et al. [39]
		Murine	GL261 *Hif-1α* shRNA cells	Knock-down	Diminished invasiveness of tumors	Mendez et al. [40]
		Mice	RIP1-Tag2/*Hif-1α*$^{fl/fl}$ mice	Insulin-secreting cells Knock-out	No changes in tumor spontaneous formation	Takeda et al. [41]
		Mice	RIP-Tag2/*Vegf*$^{fl/fl}$/*Hif-1α*$^{fl/fl}$ mice	Insulin-secreting cells Knock-out	Decreased tumor size. High proliferation rate	Takeda et al. [41]
		Mice	Triple mutated *Hif-1α* mice	Kidney knock-in	ccRCC spontaneous formation	Fu et al. [26]
		Mice	MEFs double mutated *Hif-1α*	Ubiquitous under ROSA26 locus	Microvesicular steatosis	Kim et al. [43]
	HIF-2α	Mice	Hif-2α$^{-/-}$	Knock-out	Lethal	Peng et al. [45]; Steenhard et al. [46]; Scortegagna et al. [47]
		Mice		Knock-in *Hif-2α* under *Hif-1α* promoted	Homozygous lethality	Covello et al. [49]
		Rat	Glioma tumors cells	Overexpression	Reduced tumor growth	Acker et al. [52]
		Mice	*Hif-2α*$^{fl/\Delta}$ mice	Endothelial knock-out deletion	Reduced tumor growth after injection of LLC cells	Skuli et al. [53]
		Mice	*Kras*^{G12D}/Double mutated *Hif-2α* mice	Lung knock-in	Increased tumor vascularity and invasiveness	Kim et al. [54]
		Mice	*Kras*^{G12D}/*Hif-2α*$^{-/-}$ mice	Lung knock-out	Increased lung tumorigenesis	Kim et al. [54]
		Mice	Double mutated *Hif-2α* mice	Liver expression	Increased tumor proliferation	Kim et al. [54]
Hif-β/Arnt	Arnt	Mice	*Arnt*$^{fl/fl}$ mice		Loss of Hif-dependent gene activation	Tomita et al. [69]
		Mice	*Arnt*$^{\Delta/+}$ macrophages		Altered Hif target genes expression	Lee et al. [70]
		Mice	*Arnt*$^{fl/fl}$ mice	Skin knock-out	Blocked tumor formation	Shi et al. [72]
Vhl		Mice	RIP-Tag2/*Vegf*$^{fl/fl}$ mice	Insulin-secreting cells Knock-out	Decreased tumor size	Takeda et al. [41]
		Mice	Vhlh$^{fl/fl}$ mice	Liver knock-out	Severe steatosis and increased vascularization	Haase et al. [80]
		Mice	Vhlh$^{fl/fl}$ mice	Kidney knock-out	Increased proliferation and dedifferentiation	Rankin et al. [81]
		Mice	Vhlh$^{fl/fl}$ mice	Osteoblasts knock-out	Upregulation of Hif-1α	Wang et al. [82]
		Mice	Vhlh$^{fl/fl}$ mice	Pancreas knock-out	Formation of highly vascularized adenomas	Shen et al. [84]
Fih		Mice	*Tgfbr1*$^{fl/fl}$/*Pten*$^{fl/fl}$	Fih microRNA	*Hif-1α* upregulation. Increased cancer cell proliferation	Zhang et al. [88]
Phd	Phd2	Mice	*Phd2*$^{+/-}$ mice	Reduced Phd2 expression	Metastatic ability reduced	Mazzone et al. [99]
		Mice	Xenograft mice	shPhd2	Reduced tumor growth	Klotzsche-von Ameln et al. [100]
	Phd1	Mice	*Phd1*$^{-/-}$		Hypoxia tolerance *Hif-2α* increased expression	Wouters et al. [111]
		Mice	Xenograft mice	Phd1 overexpression	*Hif-1α* inactivation	Erez et al. [116]
	Phd3	Mice	*Phd2*$^{fl/fl}$/*Phd3*$^{-/-}$	Knock-out	Hepatic steatosis, dilated cardiomyopathy, premature mortality	Minamishima et al. [119]

along with a conditional Phd2 gene ($Phd2^{fl/fl}$) [119]. However, the phenotype of these mice—hepatic steatosis, dilated cardiomyopathy, and premature mortality—may be due to the combined effects of Phd2 and Phd3 loss.

Future directions

Despite the large number of experiments performed in the field of hypoxia, the use of animal models to uncover the physiological roles in the cancer context for each protein in the pathway remains minimal (see Table 1). Most studies still focus on the biochemical implications of knocking down one specific protein. Regarding cancer, addressing the necessity of one specific hypoxia molecule in the development of specific tumors is only the beginning. Genetically modified mouse models of cancer that develop tumors in specific tissues that highly resemble human tumors (i.e., *Kras* v12 knock-in, Egfr-mut transgenic, *Pten* KO) need to be combined with transgenic Hif-1α (or Phd, or Arnt) backgrounds and analyzed in depth to fully understand the contribution of hypoxia to tumor growth in different tissue and organ contexts.

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Conflict of interest None.

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