Review

HIF-1 regulation: not so easy come, easy go

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The hypoxia-inducible factor-1 (HIF-1) is the master regulator of the cellular response to hypoxia and its expression levels are tightly controlled through synthesis and degradation. It is widely accepted that HIF-1α protein accumulation during hypoxia results from inhibition of its oxygen-dependent degradation by the von Hippel Lindau protein (pVHL) pathway. However, recent data describe new pVHL- or oxygen-independent mechanisms for HIF-1α degradation. Furthermore, the hypoxia-induced increase in HIF-1α levels is facilitated by the continued translation of HIF-1α during hypoxia despite the global inhibition of protein translation. Recent work has contributed to an increased understanding of the mechanisms that control the translation and degradation of HIF-1α under both normoxic and hypoxic conditions.

The physiology of hypoxia

Hypoxia is a state of reduced oxygen pressure below a critical threshold, which restricts the function of organs, tissues or cells [1]. Normal oxygen-partial-pressure (pO_2) levels range from 150 mm Hg in the upper airway to ~5 mm Hg in the retina, and a pO_2 <40 mm Hg in arterial blood constitutes hypoxia. Hypoxia can be caused by a reduction in oxygen supply, for example at increased altitude or by localized ischemia caused by the disruption of blood flow to a given area. Many solid tumors also contain hypoxic regions (pO_2 <5 mm Hg), owing to the inability of the local vasculature to supply sufficient oxygen to the rapidly growing tumor and because of the severe structural abnormality of tumor microvessels [2]. However, hypoxia also has an important and beneficial role in mammalian physiology; indeed, its presence is crucial for proper embryogenesis. At the cellular level, the response to hypoxia includes a switch from aerobic metabolism to anaerobic glycolysis and the expression of a variety of stress proteins regulating cell death or survival (Box 1). Further adaptations that occur at the tissue level to increase oxygen delivery include the induction of erythropoiesis and angiogenesis.

The hypoxia-inducible factor-1 (HIF-1) is recognized as the master regulator of the hypoxic response, activating the transcription of >100 genes crucial for adaptation to hypoxia [3]. HIF-1 also contributes to tumor growth and its increased expression has been correlated with poor patient prognosis [4]. The involvement of HIF-1 in pathophysiological conditions such as ischemia and cancer, and its value as a therapeutic target, has placed considerable interest on the understanding of HIF-1 regulation [5].

The HIF-1 transcription factor is a heterodimer of the regulated HIF-α subunit and the constitutively expressed HIF-1β subunit [6]. To date, three HIF-α isoforms (HIF-1α, HIF-2α and HIF-3α) have been described, and HIF-1α and HIF-2α are the best characterized. HIF-1α is expressed ubiquitously, whereas HIF-2α displays tissue-specific expression [7]. HIF-3α can also dimerize with HIF-1β to activate transcription [8]. HIF-3α has multiple splice variants, including the best known, inhibitory PAS domain protein, which is a truncated protein that functions as a dominant-negative inhibitor of HIF-1 [9]. The HIF-1 heterodimer binds to a conserved HIF-binding sequence within the hypoxia-responsive element in the promoter or enhancer regions of target genes, thereby eliciting their transactivation and an adaptive hypoxic response [10].

The induction of HIF-1 activity during hypoxia can be attributed to a variety of factors. HIF-1α is continuously transcribed and translated in hypoxia despite an overall decrease in global protein translation. Additionally, several mechanisms regulate the stability and activity of HIF-1α protein in an oxygen-dependent manner. Recent years have seen great advances in the field of HIF-1α regulation,

Glossary

Cap-dependent translation: a mechanism of translation initiation that requires the assembly of the eIF4F complex at the 5′ mRNA 7-methylguanosine (m^7G) cap structure. The eIF4F complex consists of three proteins: eIF4E, the cap-binding protein, eIF4G, a scaffolding protein, and eIF4A, an ATP-dependent helicase.

Cap-independent translation: a translation mechanism that is mediated by binding of ribosomes to internal ribosomes entry site (IRES) located in the 5′ UTR of mRNA; it does not require the mRNA cap.

SUMO: small ubiquitin-like modifier (SUMO) proteins possess only limited primary sequence homology to ubiquitin (18%) but share the characteristic ubiquitin-fold tertiary structure. SUMO exists as three isoforms (SUMO1, SUMO2 and SUMO3) and, like ubiquitin, can polymerize into poly-SUMO chains.

SUMOylation: a process analogous to ubiquitylation catalyzed by SUMO-specific ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) and substrate-specific ubiquitin-protein ligases (E3s) [82]. Modification of proteins by SUMOylation elicits diverse downstream effects such as alterations in protein interactions, translocation or degradation.

Ubiquitin: a highly conserved, 76-amino acid eukaryotic protein that can be covalently attached as repetitive chains to target proteins.

Ubiquitylation: a process involving a cascade of enzymes – an E1, an E2 and an E3 that recognizes and recruits specific target proteins [83]. The nature of the ubiquitin chain determines the fate of the substrate molecules: Lys48-linked polyubiquitin-tagged proteins (most commonly observed) are rapidly recognized and degraded by the 26S proteasome; other linkages such as the Lys63-linked polyubiquitin tag directs signal transduction and protein trafficking. De-ubiquitylation is a process by which the ubiquitin tag is removed from substrate proteins by specific proteases termed de-ubiquitylating enzymes (DUBs). DUBs play an important part in regulating the ubiquitin-proteasome system.

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Box 1. Role of HIF-1 in the response to hypoxia

Poorly vascularized regions are associated with hypoxia and nutrient deprivation, thereby limiting ATP production, a requirement for cell proliferation. Hypoxia induces HIF-1 activation, which activates a variety of genes that regulate the cellular response to hypoxia. These are summarized below.

Anaerobic metabolism

HIF-1 promotes both the uptake and metabolism of glucose through anaerobic glycolysis by upregulating the expression of glucose transporters (GLUT1 and GLUT3) and of glycolytic enzymes (6-phosphofructo-2-kinase and fructose-2,6-biphosphatase). To maintain the metabolic flux through glycolysis, HIF-1 activation also leads to the inhibition of the Kreb's cycle by upregulating pyruvate dehydrogenase kinase 1, which decreases the availability of pyruvate and lactate dehydrogenase A, thus, increasing the conversion of pyruvate into lactate. This activity tips the balance towards lactic acid production and away from the mitochondrial Kreb's cycle and oxidative phosphorylation, both of which require oxygen [84]. This shift from aerobic to anaerobic metabolism is frequently observed in cancer cells, even in normoxia, and is known as the Warburg effect.

pH regulation

The metabolic shift towards anaerobic glycolysis results in potentially toxic intracellular acidosis owing to the increased production of lactic acid and CO_2. To counter this, hypoxia-induced HIF-1 also upregulates the expression of monocarboxylate transporter 4, which mediates lactic acid efflux, and of membrane-bound carbonic anhydrase IX, which catalyses the conversion of extracellular CO_2 to carbonic acid (H_2CO_3). The latter contributes to the acidification of the extracellular space and enables an increase in intracellular pH through the subsequent uptake of HCO_3^- (a weak base).

Angiogenesis

HIF-1 directly activates the expression of several pro-angiogenic factors, the best characterized of which is the vascular endothelial growth factor. This event promotes the formation of new blood vessels, thus restoring the supply of oxygen and nutrients. Increased angiogenesis is one of the key HIF-1-dependent pro-tumorigenic events that enable continued tumor growth.

Other responses

In addition to the roles mentioned above, HIF-1 increases oxygen transport by promoting erythropoiesis and has been linked to changes in cell proliferation and survival through its effects on c-Myc and on components of the cycle cell and cell-death machinery. In solid tumors, HIF-1 is important in the promotion of metastasis and in maintaining cells in an undifferentiated state via the Notch pathway [85].

providing a clearer understanding of established pathways and also introducing new and sometimes controversial findings involving novel regulators and mechanisms controlling HIF-1α levels. Here, we aim to summarize the most recent information involving HIF-1 protein regulation by focusing on two important aspects governing the availability of the HIF-1α subunit: its proteasomal degradation, a well-characterized field that has recently seen a wealth of new information, and its translation, a still developing and, at times, controversial field.

Regulation by degradation

To enable the rapid response to changes in oxygen levels, cells have evolved a highly sophisticated mechanism for both sensing and adapting to hypoxia. The oxygen-dependent regulation of the HIF-α subunit through its ubiquitin-proteasomal degradation (ubiquitin; see Glossary) by the von Hippel Lindau protein (pVHL) pathway has been well studied, focusing mainly on HIF-1α, although HIF-2α is believed to be regulated in a similar manner.

The pVHL–HIF-1α degradation pathway

Under aerobic conditions, HIF-1α is hydroxylated by specific prolyl hydroxylases (PHD1, PHD2 and PHD3) at two conserved proline residues (Pro402 and Pro564) situated within its oxygen-dependent degradation (ODD) domain. This reaction requires oxygen, 2-oxoglutarate and ascorbate [11]. Under hypoxic conditions (<5% O_2), PHD activity is inhibited, resulting in HIF-1α stabilization. In addition to the enzymatic inhibition of the PHDs, hypoxia causes perturbations in the mitochondrial electron-transport chain, thus, increasing the levels of cytoplasmic reactive-oxygen species (ROS), which alters the oxidation state of Fe^{2+} (a cofactor for PHD activity) to Fe^{3+}, which cannot be utilized. This alteration inhibits PHD activity and promotes HIF-1α stabilization. Thus, the disruption of mitochondrial function using either pharmacological or genetic inhibition or knockout of the mitochondrial electron-transport chain convincingly prevents HIF-1α stabilization during hypoxia [12,13]. In addition, the essential role of mitochondria in HIF-1α regulation is highlighted by the exclusive enrichment of mitochondrial inhibitors from a library of >600 000 diverse compounds by using a HIF-1-reporter assay [14]. However, the role of ROS in hypoxia and HIF-1 regulation remains controversial owing to discrepancies in different model systems, a lack of tools for accurate detection of ROS and variability in the severity and length of hypoxia applied. It is believed that future studies will provide a clearer role for the mitochondria in HIF-1α regulation. In addition to mitochondrial-dependent mechanisms, the PHDs are subject to regulation by other factors including intracellular calcium concentrations [15] and the seven in absentia homologs 1 and 2 (Siah1 and Siah2) E3 ubiquitin ligases [16].

HIF-1α hydroxylation facilitates binding of pVHL to the HIF-1α ODD [17]. pVHL forms the substrate-recognition module of an E3 ubiquitin ligase complex comprising elongin C, elongin B, cullin-2 and ring-box 1, which directs HIF-1α poly-ubiquitylation and proteasomal degradation. Recognition of HIF-1α by pVHL is further facilitated by HIF-1α acetylation at Lys532 by arrest-defective-1 (ARD1) *N*-acetyltransferase, which functions mainly under normoxic conditions [18]. It should be noted, however, that the acetylation of HIF-1α by ARD1 and its importance have been disputed [19,20]. The central role of pVHL in HIF-1α regulation is manifest in von Hippel Lindau (VHL) disease where the inactivation of the *VHL* gene results in the development of highly vascularized tumors of the kidney, retina and central nervous system [21]. In addition to pVHL, human double minute 2 (Hdm2), the E3 ligase that binds to and degrades the p53 tumor-suppressor protein, can also induce HIF-1α proteasomal degradation in an oxygen-independent manner via p53–HIF-1α binding [22].

New regulators of the pVHL–HIF-1α degradation pathway

pVHL itself is subject to tight regulation by mechanisms that modulate its stability or its affinity for HIF-1α and other components of the pVHL E3 ubiquitin ligase com-

plex. One such regulator is E2-endemic pemphigus foliaceus (EPF) ubiquitin carrier protein (UCP), a member of the E2 enzyme family [23]. Although a cognate E3 ligase for UCP has not been identified, UCP can specifically ubiquitylate pVHL leading to its degradation [24]. Hence, UCP overexpression causes the proteasomal-dependent degradation of pVHL, resulting in the accumulation of HIF-1α in normoxia and in increased tumor growth and metastasis, whereas UCP knockdown increases pVHL levels, thereby decreasing HIF-1α levels and inhibiting tumor growth [24]. Additionally, UCP expression correlates with decreased pVHL and increased HIF-1α expression in a panel of cell lines and primary and metastatic tumors [24]. UCP is highly expressed in many human cancers, including ovarian cancer [25]; this finding could provide an explanation for the elevated levels of HIF-1α that are observed in non-hypoxic regions of such tumors [26].

Other novel regulators of HIF-1α include osteosarcoma-9 (OS-9) and spermidine/spermine-N^1-acetyltransferase (SSAT)2, which were both identified as HIF-1α binding partners in a yeast two-hybrid screen [27,28]. OS-9 overexpression results in the marked reduction of HIF-1α protein levels under both normoxia and hypoxia by promoting pVHL-dependent HIF-1α degradation. OS-9 forms a ternary complex with HIF-1α and PHD2 or PHD3, thereby promoting HIF-1α proline hydroxylation and directing pVHL binding and subsequent proteasomal degradation [27]. Paradoxically, OS-9 is overexpressed in osteosarcomas [29], which also express HIF-1α, so its importance in HIF-1α regulation in cancer remains unclear.

SSAT2 simultaneously binds to pVHL and elongin C, thereby stabilizing their interaction and promoting HIF-1α ubiquitylation. Accordingly, SSAT2 overexpression decreases HIF-1α levels, whereas SSAT2 knockdown increases HIF-1α levels under both normoxia and hypoxia [28]. SSAT2 might be a necessary component of the pVHL E3 ligase complex because it is required for the decrease in HIF-1α levels mediated by overexpression of either PHD2 or pVHL. SSAT2 acetylates thialysine (*S*-[2-aminoethyl]-L-cysteine), a naturally occurring modified amino acid [30], and mutant SSAT2 defective in thialysine acetyltransferase activity, although still able to interact with HIF-1α, is substantially less effective at reducing HIF-1α protein levels compared with wild-type SSAT2. Hence, the inhibitory activity of SSAT2 on HIF-1α is believed to result from the combined effect of its acetyltransferase activity and its physical interactions with HIF-1α, pVHL and elongin C.

Regulation by de-ubiquitylation

The pVHL-interacting de-ubiquitylating enzyme (VDU2; also called USP20) is the sole HIF-1α de-ubiquitylating enzyme (DUB) identified to date [31]. VDU2 itself can be ubiquitylated and degraded by the pVHL E3 ligase complex [32]. VDU2 binds and de-ubiquitylates HIF-1α in a pVHL-dependent manner, hence salvaging it from proteasomal degradation. The region of pVHL, which is commonly altered in VHL disease, harbors the binding sites for both VDU2 and HIF-1α [33]. Because pVHL can ubiquitylate both HIF-1α and VDU2, cellular HIF-1α levels might be determined by the balance between the pVHL-mediated ubiquitylation and VDU2-mediated de-ubiquitylation. The relationship between pVHL, VDU2 and HIF-1α in cancer remains to be determined.

Regulation by SUMOylation

Hypoxia induces small ubiquitin-like modifier (SUMO)-1 expression [34] and increases HIF-1α SUMOylation, a process that has been shown to lead to its stabilization [35]. However, new evidence indicates that HIF-1α SUMOylation can also lead to HIF-1α degradation. Hypoxia-induced HIF-1α SUMOylation can promote hydroxyproline-independent HIF-1α–pVHL E3 ligase complex binding, thus leading to HIF-1α ubiquitylation and proteasomal degradation [36]. These findings provide evidence for an alternative signal for pVHL binding in the absence of proline hydroxylation. SENP1 (SUMO1/sentrin specific peptidase 1) is a nuclear SUMO protease that deconjugates SUMOylated HIF-1α, enabling it to escape pVHL-mediated degradation during hypoxia. Accordingly, SENP1 knockdown decreases HIF-1α expression in a pVHL-dependent manner. Furthermore, *SENP1*$^{-/-}$ embryos exhibit severe fetal anemia owing to deficient erythropoietin production (they die mid-gestation), thus providing strong evidence for a physiological role of SENP1 in regulating HIF-1α [36]. By contrast, the RWD-containing SUMOylation enhancer (RSUME), which increases overall SUMO conjugation by interacting with the SUMO E2 enzyme Ubc9, interacts with and increases HIF-1α SUMOylation resulting in increased HIF-1α protein levels and transactivation [37]. RSUME is induced by hypoxia in tumors and is expressed in the necrotic zone of gliomas, underscoring its importance during hypoxia and in tumor maintenance and growth.

Hence, hypoxia-induced HIF-1α SUMOylation can promote either its stabilization or pVHL-dependent degradation. Further characterization of the type (SUMO 1–3) or nature of HIF-1α SUMO conjugation during hypoxia should shed light on this newly identified mechanism for HIF-1α regulation.

pVHL-independent pathways for HIF-1α degradation

Increasing evidence indicates that mechanisms other than pVHL-dependent HIF-1α degradation have an important role in controlling HIF-1α levels. Compared with pVHL, the regulation of these new pathways seems to be less dependent on oxygen availability and more on specific cellular conditions such as calcium or the presence of growth factors.

HIF-1α protein stability is regulated through an oxygen-independent pathway involving the molecular chaperone 90 kDa heat-shock protein (HSP90) and receptor of activated protein kinase (PK)C (RACK1), which compete for binding to HIF-1α. RACK1 homodimerizes and recruits elongin C and other components of the E3 ligase complex to HIF-1α, leading to HIF-1α ubiquitylation and degradation in a manner mechanistically similar to the pVHL pathway. Thus, Hsp90 inhibitors such as 17-(allylamino)-17-demethoxygeldanamycin (17AAG), cause oxygen- and pVHL-independent HIF-1α degradation [38].

RACK1–HIF-1α binding is dependent upon the presence of SSAT1, which stabilizes the RACK1–HIF-1α interaction [39]. It is intriguing that both SSAT1 and SSAT2 (described earlier) bind to and promote HIF-1α ubiquitylation by completely different mechanisms: SSAT2 promotes oxygen and pVHL-dependent HIF-1α degradation, whereas SSAT1 promotes oxygen-independent, RACK1-dependent HIF-1α degradation. Similar to SSAT2, studies using spermidine/spermine-N^1-acetyltransferase-inactive SSAT1 mutants demonstrate that SSAT1 acetyltransferase activity is required for it to promote HIF-1α degradation.

The RACK1 pathway can also be regulated by calcium through the activity of calcineurin, a calcium- and calmodulin-dependent and serine/threonine-specific protein phosphatase. Calcineurin A dephosphorylates RACK1 in a calcium-dependent manner, thus blocking RACK1 dimerization and inhibiting RACK1-mediated HIF-1α degradation [40].

Phosphatidylinositol 3-kinase–Akt (PtdIns3K–Akt) signaling activates a variety of signaling cascades with diverse outcomes, including cell survival and death [41]. The PtdIns3K–Akt pathway is also intricately linked to HIF-1 regulation, not only by inducing HIF-1α translation in response to growth factors (see later) but also through the regulation of HIF-1α protein degradation. However, the modulation of the PtdIns3K–Akt pathway and its role in HIF-1α regulation during hypoxia remains controversial and is highly context dependent. It has been suggested that the PtdIns3K pathway might be activated by short-term hypoxia but inhibited by prolonged hypoxia [42–44].

Glycogen synthase kinase 3 (GSK3), which consists of two isoforms (α and β), is phosphorylated and inactivated by Akt. GSK3β overexpression results in prolyl-hydroxylation- and pVHL-independent HIF-1α ubiquitylation and proteasomal degradation via GSK3β-mediated HIF-1α phosphorylation [45]. Similarly, overexpression of forkhead box (FOX)O4 or of constitutively active FOXO3a,

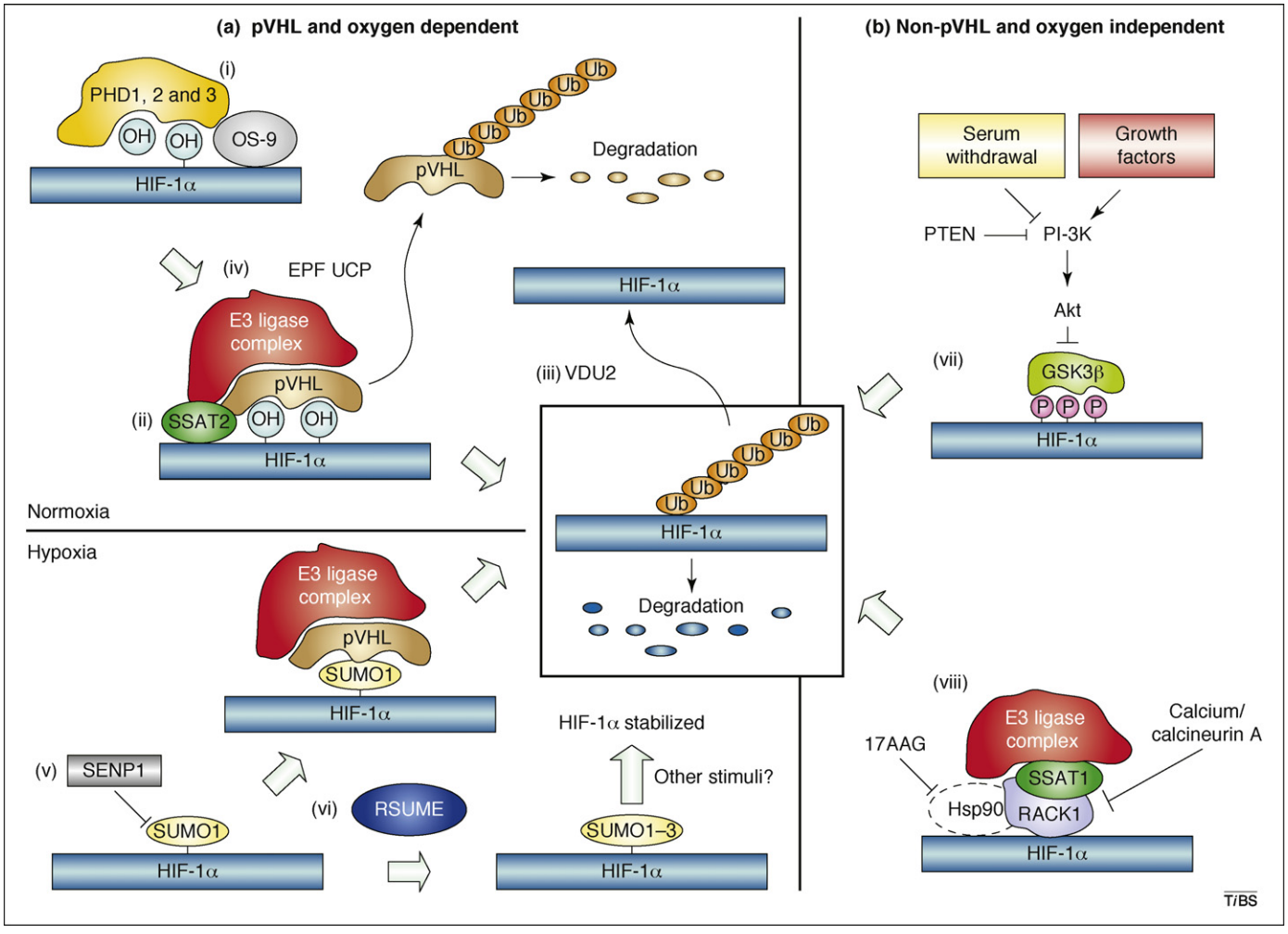

Figure 1. Important pathways regulating HIF-1α degradation. HIF-1α, the oxygen-dependent subunit of the HIF-1 transcription factor, is regulated through degradation by multiple pathways that are either dependent **(a)** or independent **(b)** of oxygen and pVHL. (a) Under normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylases (PHDs), a process facilitated by OS-9 (i). This event leads to the recruitment of the pVHL E3 ligase complex to HIF-1α, a process facilitated by SSAT2 (ii), which binds to HIF-1α, pVHL and elongin C. The pVHL E3 ligase complex ubiquitylates HIF-1α, leading to its degradation. However, ubiquitylated HIF-1α can be rescued from degradation by VDU2-mediated de-ubiquitylation (iii). Alternatively, pVHL can be ubiquitylated and degraded by EPF UCP (iv). Hypoxia results in HIF-1α SUMOylation, which can facilitate the recognition of HIF-1α by the pVHL E3 ligase complex and lead to HIF-1α degradation. HIF-1α SUMOylation can be reversed by SENP1 (v), resulting in stabilization. However, hypoxia-induced RSUME-mediated SUMOylation (RSUME) (vi) can increase HIF-1α stability indicating that the role of SUMOylation in HIF-1α regulation is still unclear. (b) Oxygen-independent regulators of HIF-1 include (vii) GSK3β, which phosphorylates HIF-1α leading to its ubiquitylation, and (viii) RACK1, which binds to HIF-1α as a dimer when Hsp90 is inhibited (such as by 17AAG) and recruits components of the E3 ligase complex through a process facilitated by SSAT1. GSK3β is inactivated by the PtdIns3K pathway, which is itself activated by growth factors and inhibited by serum withdrawal or the phosphatase and tensin homolog (PTEN). Calcineurin A inhibits RACK1-dependent degradation of HIF-1α in a calcium-dependent manner by inhibiting RACK1 dimerization. Abbreviations: Ub, ubiquitin.

both of which are also negatively regulated by Akt, also represses HIF-1α, the former by inducing pVHL-independent HIF-1α ubiquitylation and degradation [46] and the latter by inhibiting HIF-1α transactivation in a p300-dependent manner [47]. Hence, it is possible that prolonged hypoxia might inhibit the PtdIns3K pathway, resulting in increased GSK3β activity and perhaps also FOXO4 and FOXO3a, which then results in decreased HIF-1α levels and activity [42].

In summary, the regulation of HIF-1α protein levels through degradation is complex, involving multiple pathways and regulatory factors, some yet to be fully characterized. This includes the constitutive proteasomal-dependent, ubiquitin-independent degradation of HIF-1α that has been recently been reported [48,49]. Some processes, such as those mediated by the pVHL pathway, are oxygen-regulated, but many of the newly described mechanisms such as RACK1 or GSK3β seem to be regulated by other physiological stimuli (Figure 1). It lies to future work to provide a clearer understanding of these pathways and, perhaps, unveil other novel players in the regulation of HIF-1α degradation.

Translational regulation of HIF-1α

Although much is known about HIF-1α degradation, knowledge about HIF-1α synthesis, especially during hypoxia, has lagged behind substantially. HIF-1α synthesis during hypoxia is largely regulated at the level of translation rather than transcription [50,51], and several mechanisms have been implicated in the process (Figure 2).

Mechanisms of HIF-1α translation under normoxia

A variety of oncoproteins, growth factors and cytokines regulate HIF-1α protein translation in normoxic conditions [10]. In certain contexts, HIF-1α protein induction is dependent on activation of the PtdIns3K–Akt mammalian target of rapamycin (mTOR) and the mitogen-activated PK (MAPK) pathways [50,52]. These pathways phosphorylate the translational repressors eukaryotic initiation factor (eIF)4E-binding proteins (4E-BP1, 4E-BP2 and 4E-BP3) and the ribosomal kinase S6K [53]. eIF4E is an mRNA cap-binding protein that mediates the binding of the eIF4F complex to the 5′ cap structures of mRNA. Hypophosphorylated 4E-BP1 binds to eIF4E with high affinity, thereby

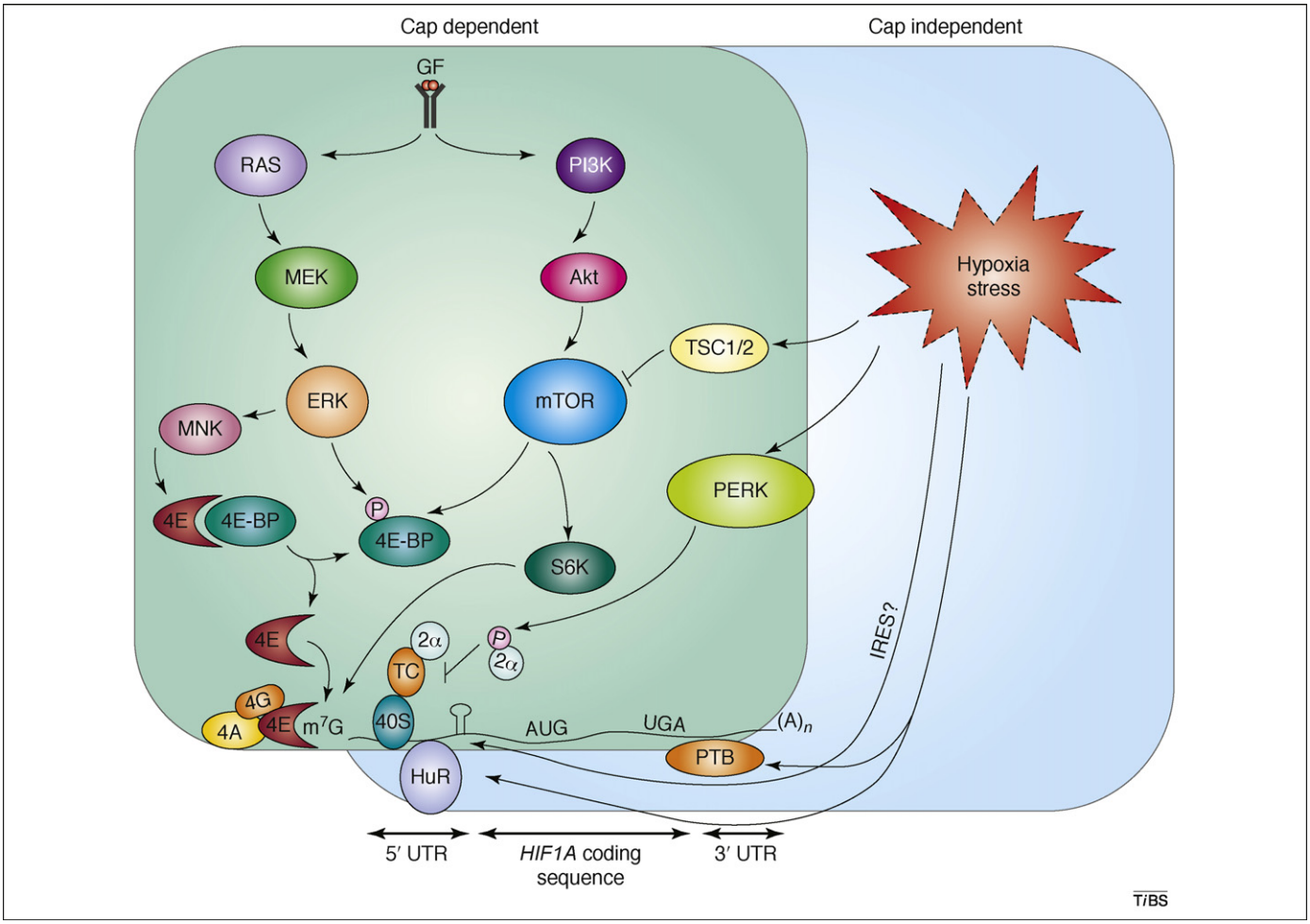

Figure 2. Important pathways regulating HIF-1α translation. During normoxic conditions *HIF1A* mRNA is translated by cap-dependent mechanisms (green background). Under certain cellular contexts, HIF-1α translation can be stimulated by growth factors (GFs), oncoproteins or cytokines that activate the PtdIns3K–Akt–mTOR and the MAPK (RAS–MEK–ERK) pathways. Cellular stress such as hypoxia or the absence of nutrients can inhibit cap-dependent translation by two mechanisms. In the first, the ER kinase PERK phosphorylates eIF2-α (2α) and prevents its assembly with the ternary complex (TC). In the second, TSC1- and/or TSC2-mediated mTOR inhibition results in 4E-BP hypophosphorylation leading to its interaction with eIF4E (4E) and blocking eIF4F-complex formation and subsequent translation initiation. mTOR inhibition also prevents S6K activation and the regulation of downstream translation components. It has been suggested that during hypoxia HIF-1α is also translated via a cap-independent mechanism (blue background), possibly through the IRES. The RNA-binding proteins PTB and HuR have been proposed to bind to *HIF1A* mRNA at the 3′ UTR and 5′ UTR, respectively, and enhance HIF-1α translation in response to the hypoxia mimetic $CoCl_2$. Abbreviations: PI3K, phosphatidylinositol 3-kinase.

preventing eIF4F-complex formation and translation initiation. Activation of mTOR and extracellular signal-regulated kinase (ERK) promotes protein synthesis by phosphorylating 4E-BP1 on several sites, hence decreasing its affinity for eIF4E and enabling the formation of the eIF4F complex and subsequent cap-dependent translation [54]. Phosphorylation of a second substrate, S6K, by mTOR and ERK controls translation by phosphorylating components of the translational machinery including the ribosomal protein S6, eIF4B and eukaryotic elongation factor 2 kinase (eEF2K) [55]. In addition, the MAPK pathway can also activate the MAPK signal-integrating kinases (MNK) that phosphorylate eIF4E, although the role of eIF4E phosphorylation is not well understood.

It was originally believed that phosphorylation of S6 by S6K stimulates translation by increasing the affinity of ribosomes for the 5′-terminal oligopyrimidine tract (5′ TOP) motif in certain mRNAs. The 5′ TOP motif begins with a C residue at the cap site followed by an uninterrupted stretch of pyrimidines and is located at the 5′ terminus of an mRNA. The putative presence of a 5′ TOP motif downstream of nucleotide +32 in the 5′ untranslated region (UTR) of HIF1-α was proposed to be the mechanism by which mTOR and S6K drive HIF1-α translation [56,57]. However, it is now believed that the 5′ TOP motif is not present in *HIF1A* mRNA, and both S6K and S6 are dispensable for the translational activation of TOP mRNAs by growth factors [55]. Hence, the mechanism for S6K-mediated translational regulation of HIF-1α remains unclear.

Mechanisms of HIF-1α translation under hypoxia

Hypoxia (0–3% oxygen) leads to an almost immediate shutdown of general protein translation as a means of decreasing energy consumption during stress. Translation inhibition during hypoxia is regulated by at least two separate pathways [58,59]. The first pathway, the unfolded protein response (UPR), is activated rapidly (1–2 h) at oxygen concentrations of <1% and stimulates the endoplasmic reticulum (ER) kinase PKR-like ER kinase (PERK), which phosphorylates a crucial regulator of translation initiation, eIF2α [60]. This modification prevents the assembly of the 40S ribosome-binding eIF2–GTP–met-tRNA ternary complex that is required for translation initiation. The second pathway, controlled by mTOR, is activated by prolonged hypoxia and inhibits translation by disrupting the eIF4F complex [61]. Under conditions of hypoxia or nutrient and energy stress, mTOR activity is inhibited through REDD1 (regulated in development and DNA-damage responses) and tuberous sclerosis (TSC)1–TSC2 complex resulting in 4E-BP1 hypophosphorylation, followed by subsequent dissociation of the eIF4F complex. Hypoxia also suppresses translation elongation through AMP-activated-protein-kinase- or mTOR-dependent phosphorylation of eEF2 kinase, which phosphorylates eEF2, thus, inhibiting ribosome binding and arresting translation [53]. Thus, the level and length of oxygen deprivation seems to determine the specific mechanism(s) for translation inhibition, each driving distinct gene-expression patterns.

Despite a decrease in global protein translation during hypoxia, a small group of proteins crucial for survival, including HIF-1α, continue to be translated. The evidence supporting the continued translation of HIF-1α was determined using several independent approaches including reporter assays with the HIF-1α 5′ UTR, ^{35}S labeling, translation and proteasomal inhibitors (cycloheximide and MG132) and polysome analysis [50,51,62–66]. However, how HIF-1α is selectively translated during periods of global translation inhibition remains incompletely understood. One suggested mechanism is through internal-ribosome-entry-site (IRES) elements, which are RNA sequences that form secondary or tertiary structures and direct ribosome binding without the need for the eIF4F cap-binding complex. Several studies have reported that the 5′ UTR of HIF-1α contains an IRES capable of promoting translation of a downstream reporter in bicistronic reporter assays [66–68]. However, recent work convincingly disputes the role of IRES in HIF1-α translation [62,69]. Moreover, whether an IRES-dependent mechanism contributes substantially to protein translation during hypoxia remains a subject of debate [70,71], and observations of protein translation in hypoxia owing to cryptic promoter activity rather than IRES-mediated translation have been reported [62,69]. It has also been suggested that, during hypoxia, the RNA-binding proteins polypyrimidine tract-binding protein (PTB) and HuR bind the HIF-1α 3′ UTR and 5′ UTR, respectively, thereby enhancing HIF-1α translation [51,68]. Recent work indicates that, in breast cancer, overexpressed 4E-BP1 and eIF4G function are a hypoxia-activated switch that facilitates cap-independent translation over cap-dependent translation of HIF-1α and other key pro-angiogenic and pro-survival mRNAs [63]. Thus, although translation of HIF-1α and a subset of other stress-survival proteins is maintained or even increased during hypoxia, despite the global decrease in cap-mediated protein translation, the exact mechanism remains unclear.

A role for calcium in the regulation of HIF-1α translation during hypoxia is receiving increased attention. Most cells respond to hypoxia with a sustained increase in cytoplasmic free calcium that results from the combined influx of extracellular calcium and the release of calcium present in the ER lumen [72]. Studies addressing the role of calcium in the regulation of HIF-1α levels have been inconsistent with regards to the effect (increase versus decrease) or the mechanism (e.g. dependent on ERK, c-Jun N-terminal protein kinases [JNKs], PKC-α or UPR) [15,40,64,73–78]. This controversy reflects the complexity of calcium-dependent signaling, which has wide-ranging effects including the regulation of HIF-1α degradation, translation and transcription. Further studies are needed to define the role of calcium in HIF-1α regulation.

Concluding remarks and future perspectives

The precise regulation of HIF-1α protein levels through synthesis and degradation under both normoxia and hypoxia is crucial for cell survival and continued proliferation. HIF-1α regulation via both oxygen-dependent and -independent pathways indicates an important role for HIF-1α outside of its established role in the hypoxic response. These mechanisms enable the control of HIF-1α not only in response to changes in oxygen tension but

also to other oxygen-independent stimuli. For example, the normoxic induction of HIF-1α synthesis by the PtdIns3K–Akt pathway might necessitate the activity of HIF-1α regulators such as RACK1 that can modulate HIF-1α levels irrespective of cellular oxygen tension. During hypoxia, this pathway complements the pVHL pathway, which might itself be fine-tuned by other regulators such as OS-9 and SSAT2 [28,39]. The existence of a variety of mediators and mechanisms for HIF-1α degradation might be required to cope with the complexity of physiological hypoxia, which can be transient or prolonged, mild or severe, and, hence, require a variety of adaptive responses.

It is also necessary to address the importance of the cellular localization of HIF-1α and its regulators. pVHL is predominantly cytoplasmic but engages in dynamic nuclear–cytoplasmic shuttling, a process essential for pVHL ubiquitylation of HIF-1α, which occurs both in the nucleus and cytoplasm [79]. However, recent work shows that the cellular compartmentalization of HIF-1α during hypoxia and reoxygenation differs in different cell types. Accordingly, the localization of HIF-1α proteasomal degradation is also cell-type specific; for example, in HepG2 (hepatocellular carcinoma) cells, HIF-1α is degraded mainly in the cytoplasm, whereas in mouse brain epithelial cells, HIF-1α is degraded both in the nucleus and the cytoplasm [80]. Hence, the various HIF-1α regulators might have roles of differing importance depending on their localization and cellular context.

The mechanism(s) responsible for preferential HIF-1α translation under conditions of stress when overall translation is decreased remains unclear. One potential mechanism for preferential HIF-1α translation involves recruitment of RNA-binding proteins such as PTB and HuR and conceivably additional RNA-binding proteins that will be identified in the future. Another attractive hypothesis is that HIF-1α translation might also be regulated by microRNAs (miRNA), a group of small, non-coding RNAs that regulate gene expression in different contexts, including hypoxia [81]. Future characterization of hypoxia-responsive miRNAs will determine their potential involvement in HIF-1α translational regulation. Finally, regulation of translation efficiency might be determined by the abundance of *HIF1A* mRNA and competition for binding to the translational machinery. This idea is supported by the finding that, during hypoxia, changes in newly synthesized HIF-1α protein levels parallel the downregulation of global protein synthesis [62]. Such a mechanism could be controlled by processes such as transcription and mRNA degradation and stabilization.

Numerous observations link intracellular calcium levels to the regulation of HIF-1α protein levels. These include studies with chelating agents and ionophores and various

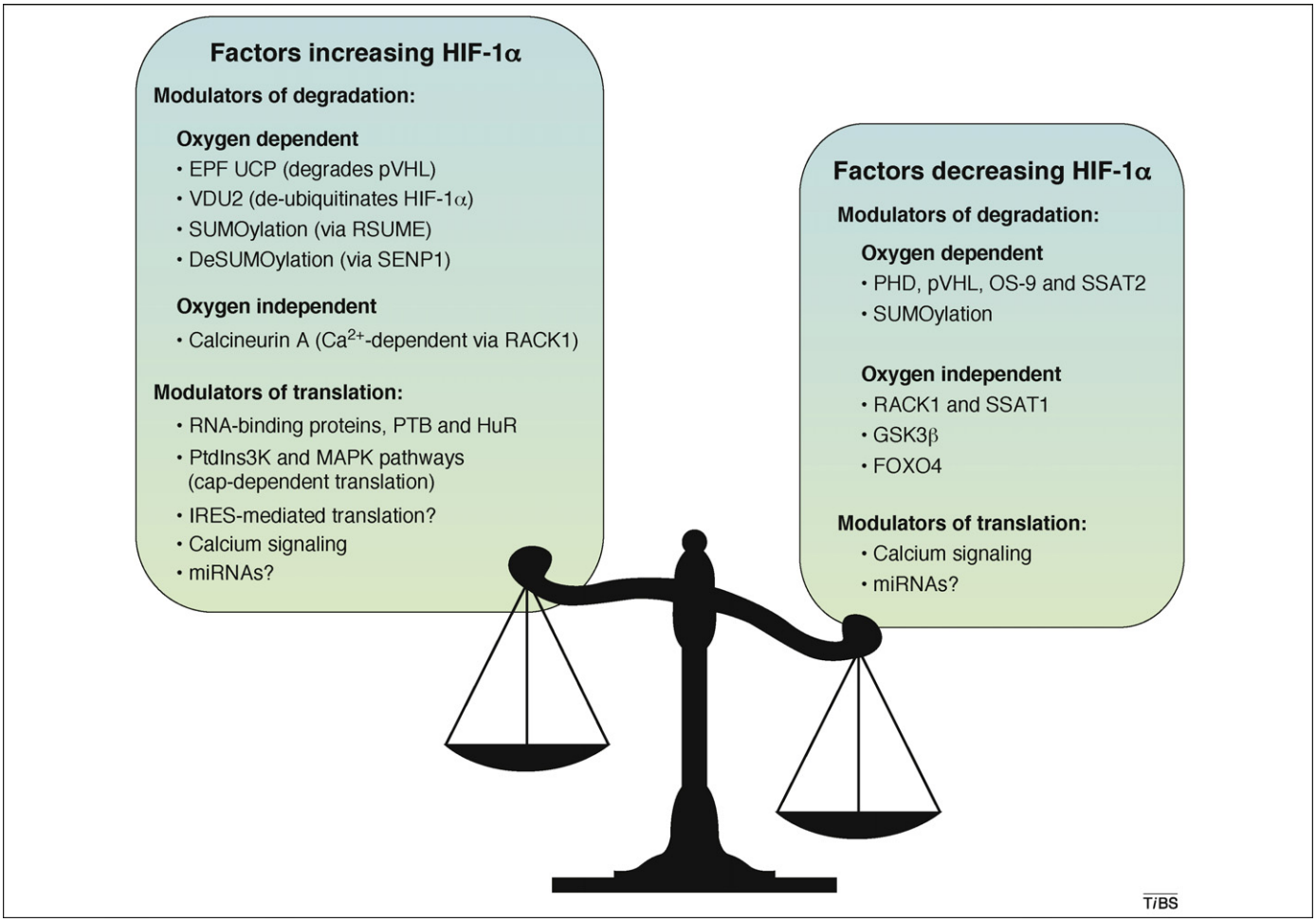

Figure 3. Levels of HIF-1α are determined by the balance between degradation and synthesis. Schematic showing the contributions of key modulators of HIF-1α degradation and translation in determining the total cellular HIF-1α levels. Some mechanisms including SUMOylation and the role of calcium remain unclear and can have contradictory outcomes under different cellular contexts.

potential mechanisms involving ERK, JNK, PKC-α or the UPR [15,40,64,73–78]. Another mechanism is the activation of calcineurin by calcium, which promotes HIF-1α expression by dephosphorylating RACK1 [40]. Hence it is possible that physiological changes in intracellular calcium, such as those mediated by hypoxia or by growth-factor-induced stimulation of calcium-signaling cascades, play an important part in controlling HIF-1α both at the levels of synthesis and degradation.

It is clear that HIF-1α levels are regulated by multiple pathways that are themselves activated or inactivated under diverse conditions and subject to self-regulation through feedback loops. Ultimately, as for all proteins, the level of HIF-1α expression is determined by the balance between the rates of synthesis (transcription and translation) and degradation (Figure 3). The mechanisms that regulate HIF-1α translation remain unclear, although various regulators have been proposed that require further investigation. The existence of multiple pathways for HIF-1α synthesis and degradation could be indicative of the need for both rapid and gradual responses to hypoxia and to other physiological stimuli. Regulated HIF-1α degradation might enable the rapid modulation of HIF-1α levels, thereby providing a quick response to a variety of stimuli, whereas translational regulation might ensure continued HIF-1α synthesis during extended periods of stress or hypoxia. Further work will demonstrate how and when these diverse pathways interact and synergize to promote the highly sophisticated regulation of HIF-1α.

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