

# Direct phosphorylation events involved in HIF- $\alpha$ regulation: the role of GSK-3 $\beta$

Daniela Mennerich\*  
Elitsa Y Dimova\*  
Thomas Kietzmann

Faculty of Biochemistry and  
Molecular Medicine and Biocenter  
Oulu, University of Oulu, Oulu,  
Finland

\*These authors contributed  
equally to this work

**Abstract:** Hypoxia-inducible factors (HIFs), consisting of  $\alpha$ - and  $\beta$ -subunits, are critical regulators of the transcriptional response to hypoxia under both physiological and pathological conditions. To a large extent, the protein stability and the recruitment of coactivators to the C-terminal transactivation domain of the HIF  $\alpha$ -subunits determine overall HIF activity. The regulation of HIF  $\alpha$ -subunit protein stability and coactivator recruitment is mainly achieved by oxygen-dependent posttranslational hydroxylation of conserved proline and asparagine residues, respectively. Under hypoxia, the hydroxylation events are inhibited and HIF  $\alpha$ -subunits stabilize, translocate to the nucleus, dimerize with the  $\beta$ -subunits, and trigger a transcriptional response. However, under normal oxygen conditions, HIF  $\alpha$ -subunits can be activated by various growth and coagulation factors, hormones, cytokines, or stress factors implicating the involvement of different kinase pathways in their regulation, thereby making HIF- $\alpha$ -regulating kinases attractive therapeutic targets. From the kinases known to regulate HIF  $\alpha$ -subunits, only a few phosphorylate HIF- $\alpha$  directly. Here, we review the direct phosphorylation of HIF- $\alpha$  with an emphasis on the role of glycogen synthase kinase-3 $\beta$  and the consequences for HIF-1 $\alpha$  function.

**Keywords:** HIF-1, phosphorylation, GSK-3 $\beta$ , kinase, hypoxia, ubiquitinylation, tumor suppressor

## Introduction

Aerobic life is dependent on an adequate supply of oxygen. The ability of mammals to respond to an inadequate O<sub>2</sub> supply, commonly termed hypoxia, is crucial for their survival. Although a proper response to changed O<sub>2</sub> tensions triggers adaptation, a number of pathological conditions or failures in the O<sub>2</sub> response are associated with various diseases such as anemia, myocardial infarction, thrombosis, atherosclerosis, or cancer.

When exposed to hypoxia or even anoxic conditions, mammalian organisms initiate a variety of responses in different organs, aiming to increase the delivery of oxygen to the tissues. In addition to the switch from an aerobic to an anaerobic metabolism and the suppression of energy-using reactions, the carotid body chemoreceptor cells stimulate the brain stem center controlling the respiratory and cardiovascular systems to enhance ventilation, heart rate, and blood pressure (reviewed by Prabhakar<sup>1</sup>). In addition, neuroepithelial cells in the lung contribute to adjusting pulmonary perfusion and gas exchange. Moreover, organs and cells switch their gene expression profile: the kidneys produce erythropoietin, which increases red blood cell production in the bone marrow, and vascular cells produce vascular endothelial growth factor to promote angiogenesis and flow of enhanced blood volume (reviewed by Semenza<sup>2</sup>). In addition to the expression of erythropoietin and vascular endothelial growth factor,

Correspondence: Daniela Mennerich  
Faculty of Biochemistry and Molecular  
Medicine and Biocenter Oulu,  
University of Oulu, PO Box 3000,  
Oulu FI-90014, Finland  
Tel +358 294 487 714  
Fax +358 8 553 1141  
Email daniela.mennerich@oulu.fi

the expression of more than 500 genes, products of which are involved in glycolysis, angiogenesis, erythropoiesis, cell death, and differentiation, is also changed on exposure to hypoxia (reviewed by Wenger and Stiehl<sup>3</sup> and Semenza<sup>4</sup>).

In mammals, the hypoxia-dependent changes on the level of gene expression are mainly mediated by the  $\alpha$ -subunits of hypoxia-inducible transcription factors (HIFs). HIF  $\alpha$ -subunits are tightly regulated, and post-translational hydroxylations in response to hypoxia appear to be of major importance. In addition to hypoxia, HIF  $\alpha$ -subunits were also found to respond to various growth and coagulation factors, hormones, cytokines, or stress factors already under normoxia. These signals are often mediated by different protein kinases. Indeed, different kinases, among them glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ), have been identified to directly phosphorylate HIF- $\alpha$  proteins. This review discusses the regulation of HIF- $\alpha$  by GSK-3 $\beta$  and compares it with hydroxylase-dependent HIF- $\alpha$  protein regulation.

## HIFs: basic aspects

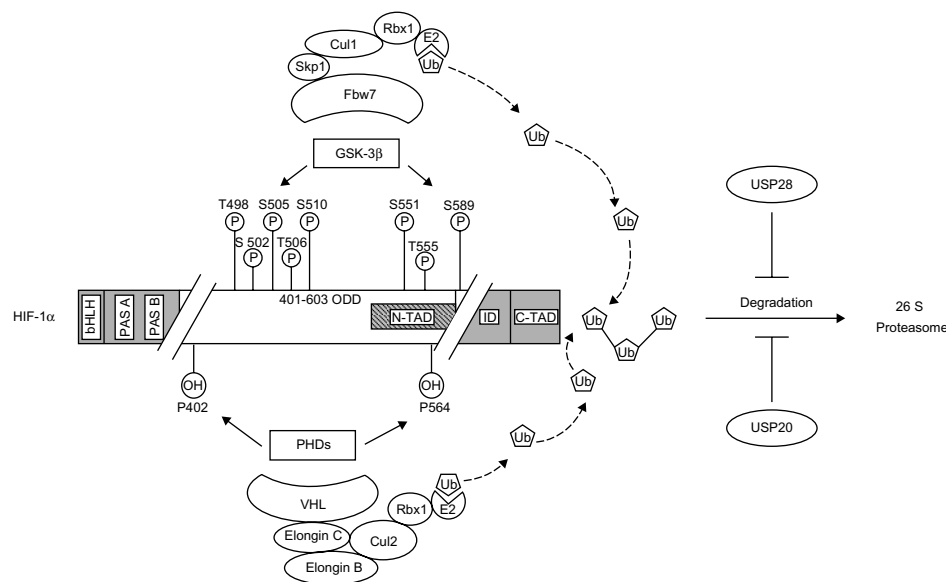
In their active form, HIFs are heterodimeric transcription factors consisting of an  $\alpha$ - and  $\beta$ -subunit. The HIF  $\beta$ -subunit represents the stable nuclear subunit primarily represented by the ubiquitously found ARNT (arylhydrocarbon receptor-nuclear translocator) protein; however, ARNT2 or ARTN3, although to a lesser extent, also appear to be able to take part in the formation of HIF dimers (reviewed by Semenza<sup>5</sup>). In contrast, the  $\alpha$ -subunits represent the O<sub>2</sub>-sensitive dimerization partner. So far, three  $\alpha$ -subunit proteins, HIF-1 $\alpha$ , HIF-2 $\alpha$  (also known as EPAS,<sup>6</sup> HLF,<sup>7</sup> HRF,<sup>8</sup> or MOP2<sup>9</sup>), and HIF-3 $\alpha$  have been identified. Together, the different HIF  $\alpha$ - and  $\beta$ -subunits may give rise to the formation of several combinations of HIF dimers.<sup>5,10</sup> HIF-1 $\alpha$  and HIF-2 $\alpha$  are the best-studied HIF- $\alpha$  isoforms. Although they share structural and functional similarities, it appears that differences in the cell-type expression pattern, the target genes, the embryonic deletion phenotypes, and the effects on tumorigenesis exist between HIF-1 $\alpha$  and HIF-2 $\alpha$ .<sup>11–14</sup> The function of HIF-3 $\alpha$ , from which several splice variants exist in humans,<sup>15,16</sup> is largely unknown, although some human HIF-3 $\alpha$  variants and a mouse splice variant termed inhibitory PAS protein (IPAS) appear to act as negative regulators of the hypoxic response.<sup>16–19</sup>

Similar to the ARNT proteins, the HIF  $\alpha$ -proteins belong to the basic helix-loop-helix PAS (Per-ARNT-Sim) protein family. In particular, HIF-1 $\alpha$  and HIF-2 $\alpha$  show the highest degree of sequence identity in the basic

helix-loop-helix (85%), PAS-A (68%), and PAS-B (73%) domains. Both also contain two nuclear localization sequences responsible for translocation to the nucleus under hypoxia; they are localized in the N terminus (amino acids 17–33 in HIF-1 $\alpha$  and amino acids 1–50 in HIF-2 $\alpha$ ) and in the C terminus (amino acids 718–721 in HIF-1 $\alpha$  and amino acids 689–870 in HIF-2 $\alpha$ ).<sup>20,21</sup> With the exception of HIF-3 $\alpha$ , which does not contain a C-terminal transactivation domain (C-TAD),<sup>22,23</sup> HIF  $\alpha$ -subunits also contain N- and C-terminal transcriptional activation domains (N-TAD and C-TAD). A unique oxygen-dependent degradation domain (ODDD, amino acids 401–603 in HIF-1 $\alpha$  and amino acids 517–682 in HIF-2 $\alpha$ ) overlaps N-TAD. The residues between N-TAD and C-TAD represent an inhibitory domain (amino acids 604–785 in HIF-1 $\alpha$  and amino acids 683–825 in HIF-2 $\alpha$ ).<sup>24,25</sup>

## Oxygen-dependent regulation of HIF $\alpha$ -subunits: role of hydroxylation

HIF  $\alpha$ -subunit activation under hypoxia is mainly the result of an increased protein stability and coactivator recruitment, although transcriptional and translational mechanisms also were shown to be involved in HIF  $\alpha$ -subunit activation.<sup>22,26–30</sup> As a result, HIF- $\alpha$  proteins accumulate, translocate to the nucleus, and dimerize with HIF- $\beta$  to form a functional transcription factor.<sup>31</sup> Thus, in the presence of oxygen (ie, normoxia), HIF- $\alpha$  proteins become degraded. This is primarily achieved by oxygen-dependent hydroxylations at the ODDD.<sup>32</sup> Under normoxia, prolyl hydroxylase domain proteins (PHDs),<sup>33,34</sup> in particular PHD2, hydroxylate two crucial residues in the ODDD of HIF  $\alpha$ -subunits (P402 and P564 in HIF-1 $\alpha$  and P405 and P531 in HIF-2 $\alpha$ ).<sup>25,32,35</sup> Prolyl hydroxylation is required for binding the von Hippel-Lindau protein (VHL),<sup>36,37</sup> which represents the substrate recognition subunit of an E3 ubiquitin-protein ligase consisting of elongin C, elongin B, RING box 1, cullin 2, and an E2 ubiquitin-conjugating enzyme (Figure 1). The prolyl hydroxylation and ubiquitination can be further promoted by the binding of PHD2 to OS9<sup>38</sup> and that of HIF-1 $\alpha$ , VHL, and elongin C to SSAT2, respectively.<sup>39,40</sup> In addition to prolyl hydroxylation, a conserved asparagine residue (N803 in HIF-1 $\alpha$  and N852 in HIF-2 $\alpha$ ) in the C-TAD is hydroxylated by the factor-inhibiting HIF in an oxygen-dependent manner. This hydroxylation prevents interaction with the coactivator proteins CBP/p300.<sup>41–44</sup> Thus, the major posttranslational modification appears to be the oxygen-dependent hydroxylation.<sup>36,37</sup>



**Figure 1** Phosphorylation- and hydroxylation-mediated proteasomal degradation of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ).

**Notes:** HIF-1 $\alpha$  is phosphorylated on specific residues (T498, S502, S505, T506, and S510 or S551, T555, and S589) by glycogen synthase kinase 3 $\beta$ , leading to interaction with Fbw7 that serves as the recognition component of an E3 ubiquitin ligase complex also containing Skp1, Cul1, and Rbx1 and results in degradation of HIF-1 $\alpha$  via the ubiquitin proteasome pathway. This degradation pathway is blocked by the ubiquitin-specific protease 28 (USP28). In contrast, HIF-1 $\alpha$  is hydroxylated on specific residues (P402 and P564) by a family of prolyl hydroxylases (PHDs), leading to interaction with the VHL ubiquitin ligase complex, containing VHL, elongin C, elongin B, Cul2, and Rbx1, and results also in the proteasomal degradation of HIF-1 $\alpha$ . In this case, the HIF-1 $\alpha$  degradation could be blocked by the ubiquitin-specific protease 20 (USP20).

**Abbreviations:** bHLH, basic helix-loop-helix; PAS A, Per-ARNT-Sim A domain; PAS B, Per-ARNT-Sim B domain; ODD, oxygen-dependent degradation domain; N-TAD, N-terminal transactivation domain; ID, inhibitory domain; C-TAD, C-terminal transactivation domain; Fbw7, F-box and WD-40 protein 7; VHL, von Hippel-Lindau protein; Cul1, Cullin 1; Cul2, Cullin 2; Skp1, S phase kinase associated protein 1; Rbx1, Ring box 1; E2, E2 ubiquitin-conjugating enzyme; Ub – ubiquitin.

## Regulation of HIF $\alpha$ -subunits by phosphorylation

In addition to hydroxylation, HIF- $\alpha$  transcriptional activity and protein stability appear also to be dynamically regulated by other posttranslational modifications such as acetylation, S-nitrosylation, SUMOylation, and phosphorylation (for review, see Dimova and Kietzmann<sup>45</sup>). Phosphorylation appears to be of special importance under normoxic conditions, mediating the response of HIF- $\alpha$  to various growth and coagulation factors, hormones, cytokines, or stress factors (reviewed by Dimova et al<sup>46</sup>) under normoxia. Indeed, a panel of protein kinases is reported to be involved in HIF-1 $\alpha$  phosphorylation, either directly (Table 1) or indirectly.<sup>47–52</sup> Although the individual action of certain kinases on HIF-1 $\alpha$  regulation was mainly studied *in vitro* (Table 1), the *in vivo* mechanisms are likely much more complex. At least the extent to which the kinases can be involved in HIF- $\alpha$  phosphorylation may vary according to the signal, cell type, or tissue. Given the different developmental and/or differentiation status of a cell or tissue, the expression of various growth factors, their receptors, and respective signaling components and the composition of the extracellular surroundings differ. Thus, it seems not to be surprising that phosphorylation of HIF- $\alpha$  by different kinases or after modulation of signaling pathways may be a highly cell type-specific event. Although direct proof is currently lacking, it is plausible that the phosphorylation

pattern of HIF- $\alpha$  in a certain cell may be explained by different layers of regulations that affect kinases depending on the cellular context.

In addition to being activated by a variety of extracellular signals, the PI3K/Akt cascade appeared also to be regulated by hypoxia, thus integrating hypoxia signaling with extracellular signals affecting multiple cellular processes such as apoptosis, metabolism, cell proliferation, and cell growth (for review, see Braccini et al<sup>53</sup>). The PI3K/Akt pathway is considered to control HIF-1 $\alpha$  within the cell via regulation of HIF-1 $\alpha$  protein synthesis and stability. However, it appeared that HIF- $\alpha$  proteins are not directly phosphorylated by PKB/Akt but, rather, by a PKB/Akt target. The PKB/Akt targets HDM2,<sup>54,55</sup> mammalian target of rapamycin (mTOR),<sup>56</sup> and GSK-3<sup>57</sup> were shown to contribute to changes in HIF- $\alpha$  protein levels; however, only GSK-3 was shown to do this directly, ie, by phosphorylating HIF- $\alpha$  proteins.

## GSK-3 and HIF- $\alpha$ regulation

GSK-3 is a serine/threonine kinase that was first identified as a negative regulator of glycogen synthesis; inhibition is achieved through phosphorylation of glycogen synthase.<sup>58,59</sup> Since its initial discovery, GSK-3 has been found to be involved in numerous signaling pathways initiated by diverse stimuli and to contribute to the regulation of cell proliferation, stem cell renewal, apoptosis, and development, which are processes

Table 1 HIF- $\alpha$  as a direct phosphorylation target

Signal	Kinase	Mapped phosphorylated domain or exact amino acids residue or residues	System	Consequences for HIF-1 function	Reference
N/A	ERK1/2	Not mapped	HeLa and in vitro translated HIF-1 $\alpha$ ; CCL39 cells stably expressing the Raf-1:ER chimera	Promotes HIF-1-mediated transcriptional activation	123
Hypoxia	ERK1	Within the TAD-C of HIF-1 $\alpha$	HMEC-1 and COS-7 cells; in vitro kinase assays with overexpressed and immunoprecipitated HIF-1 $\alpha$ and ERK1	Induces HIF-1 $\alpha$ transcriptional activity during hypoxia but not the stabilization of the protein	124
Kaposi's sarcoma-herpes virus	MAPK (PD98059), p38 $\alpha$ , and p38 $\gamma$	Within the inhibitory domain of HIF-1 $\alpha$	COS-7 cells; in vitro kinase assays with HIF-1 $\alpha$ recombinant protein and overexpressed and immunoprecipitated kinases	Activates the transactivity of HIF-1 $\alpha$	125
N/A	GSK-3 $\beta$	Within the oxygen-dependent degradation domain	COS-7 cells; in vitro kinase assays with HIF-1 $\alpha$ recombinant protein and overexpressed and immunoprecipitated kinases	Impairs HIF-1 $\alpha$ protein stability	86
Hypoxia	MEK-1/p42/p44 MAPK pathway	522–649 and 650–822 amino acids of the TAD-C of HIF-1 $\alpha$	Hep3B cells; in vitro transactivation and kinase assays with HIF-1 $\alpha$ recombinant protein	Induces hypoxia-induced HIF-1 $\alpha$ transactivation	126
N/A	Not identified	Thr-796 in HIF-1 $\alpha$ and Thr-844 in endothelial Per-ary/hydrocarbon receptor-nuclear translocator-Sim domain protein 1	HEK293 and HeLa cells; phosphorylation of overexpressed HIF-1 $\alpha$ protein	Necessary for the interaction with CBP/p300	127
Ischemia (hypoxia and low glucose)	p38 $\alpha$ , p38 $\beta$ , p38 $\delta$ , and p38 $\gamma$	Not mapped	MiaPaCa-2 cells; in vitro kinase assays with in vitro translated HIF-1 $\alpha$ and overexpressed, immunoprecipitated kinases	Prevents the interaction between VHL and HIF-1 $\alpha$	128
N/A	ERK2	Ser-641/Ser-643	HIF-1 $\alpha$ recombinant protein and HeLa extracts; mass spectroscopy analysis	Enhances the nuclear accumulation and activity of HIF-1 $\alpha$ by blocking its CRM1-mediated nuclear export	129
Intermittent hypoxia	Protein kinase A	Not mapped	EAh926 endothelial cells, HMEC-1 cells; inhibitory studies	Influences the HIF-1 $\alpha$ transcriptional activity	130
N/A	GSK-3 $\beta$	Ser-551, Thr-555 and Ser-589	HepG2 cells; in vitro kinase assays with recombinant HIF-1 $\alpha$ and GSK-3 $\beta$	HIF-1 $\alpha$ degradation in a VHL-independent manner	85
N/A	ERK1/2	CRM1-dependent phosphorylation sensitive NES in HIF-1 $\alpha$ amino acids 616–658	HIF-1 $\alpha$ recombinant protein and HeLa extracts	Nuclear localization	131
N/A	CK1 $\delta$	Ser-247	HeLa and Huh7 cells; HIF-1 $\alpha$ recombinant protein and HeLa extracts	Inhibits HIF-1 $\alpha$ heterodimerization with arylhydrocarbon receptor-nuclear translocator	132
Hypoxia	Polo-like kinase 3	Ser-576/Ser-657	Wild-type and <i>PLK3</i> <sup>-/-</sup> MEFs; in vitro kinase assay with recombinant HIF-1 $\alpha$ and kinase; mass spectrometric analyses	HIF-1 $\alpha$ destabilization	133
Hypoxia	Ataxia telangiectasia mutated kinase	Ser-696	NBS1-1LB cells, in vitro kinase assay using overexpressed and immunoprecipitated kinase and recombinant HIF-1 $\alpha$ , followed by liquid chromatography-tandem mass spectrometry analysis	Necessary for HIF-1 $\alpha$ stabilization	134

N/A	GSK-3 $\beta$	Thr-498, Ser-502, Ser-505, Thr-506, and Ser-510	SK-OV3 human ovarian cancer cells; in vitro kinase assays using purified GSK-3 $\beta$ and overexpressed and immunoprecipitated HIF-1 $\alpha$	HIF-1 $\alpha$ degradation in a VHL-independent manner under hypoxia	87
N/A	CDK1	Ser-668	HCT116 cells; in vivo interaction; in vitro kinase assays with peptides from HIF-1 $\alpha$ and purified CDK1	HIF-1 $\alpha$ stabilization under normoxia via inhibition of the HIF-1 $\alpha$ proteasomal degradation	135

**Abbreviations:** HIF, hypoxia-inducible factor; TAD-C, C-terminal transactivation domain; GSK-3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; CCL39, Chinese hamster fibroblast cell line; COS-7, African green monkey kidney fibroblast-like cell line; Hep3B, human liver hepatocellular cell line, containing hepatitis B; HepG2, human liver hepatocellular cell line; HMEC, human mammary epithelial cell; MEFs, mouse embryonic fibroblasts; NBSJ-LB, SV40-transformed Nijmegen Breakage syndrome fibroblasts; SK-OV3, human ovarian cancer cells; HeLa, human epithelial cervix adenocarcinoma cell line; HEK293, human embryonic kidney cell line; MiaPaCa-2, human epithelial pancreas carcinoma cell line; Huh7, human hepatocarcinoma cell line; HCT116, human colorectal carcinoma cell line; CRM1, exportin 1; MAPK, mitogen-activated protein kinase family; CDK1, cyclin-dependent kinase 1; CK1 $\delta$ , casein kinase 1 $\delta$ .

often associated with hypoxia. Because of these multiple involvements, dysregulation of GSK-3 has been implicated in the pathogenesis of human diseases, including type 2 diabetes, bipolar disorders, inflammation, Alzheimer's disease, and cancer (reviewed by Frame and Cohen,<sup>60</sup> Grimes and Jope,<sup>61</sup> and Woodgett<sup>62</sup>). Two isoforms, GSK-3 $\alpha$  (51 kDa) and GSK-3 $\beta$  (47 kDa), have been identified in mammals. Despite their homology in the catalytic domain (98%), they significantly differ in their N- and C-terminal parts<sup>63,64</sup> and do not have entirely overlapping roles in metabolism (reviewed in Force and Woodgett<sup>65</sup>). Moreover, GSK-3 $\beta$  (GSK-3 $\beta^{-/-}$ ) homozygous knockout mice showed an embryonic lethal phenotype around day 16 because of hepatic apoptosis or a cardiac pattern defect,<sup>66,67</sup> whereas homozygous GSK-3 $\alpha$  (GSK-3 $\alpha^{-/-}$ ) knockout mice are viable and fertile.<sup>68,69</sup> GSK-3 is a target of PKB/Akt, which can phosphorylate both GSK-3 isoforms (serine 21 of GSK-3 $\alpha$  and serine 9 of GSK-3 $\beta$ ), leading to an inhibition of GSK-3 activity.<sup>70</sup> Interestingly, these serine residues can also be phosphorylated by other kinases such as ERK1/2,<sup>71</sup> p70 ribosomal S6 kinase 1,<sup>72</sup> cAMP (cyclic adenosine monophosphate)-dependent protein kinase A (PKA),<sup>73</sup> and protein kinase C (PKC).<sup>74</sup> In contrast, an autophosphorylation event leading to phosphorylation of tyrosine 279 in GSK-3 $\alpha$  and of tyrosine 216 in GSK-3 $\beta$  increases GSK-3 activity.<sup>75,76</sup> Neurons seem to possess a spliced GSK-3 $\beta$  variant called GSK-3 $\beta$ 2 that contains a 13 amino acid residue insert within the kinase domain, leading to reduced kinase activity.<sup>77,78</sup>

Although GSK-3 is mostly known in the insulin field as a regulator of glycogen synthesis, it has been shown that early hypoxia enhanced PI3K/Akt activity and increased HIF-1 $\alpha$  protein levels.<sup>57</sup> Similarly, hypoxia was capable of inhibiting GSK-3 $\beta$  by phosphorylation in different cell types, such as PC-12 (rat pheochromocytoma cell line) cells,<sup>79</sup> HT1080 (human fibrosarcoma cell line) cells,<sup>80</sup> and HepG2 (human liver hepatocellular cell line) cells,<sup>57</sup> as well as in vivo.<sup>81</sup> Although this effect was not observed in other cell types, including some breast cancer cell lines,<sup>82</sup> PC-3 prostate cancer cells,<sup>83</sup> and 3T3 cells,<sup>84</sup> it was considered to have a cell type-specific component. However, the findings that GSK-3 inhibition<sup>57</sup> and small interfering RNA-mediated depletion induced HIF-1 $\alpha$ , whereas GSK-3 $\beta$  overexpression reduced HIF-1 $\alpha$  protein levels,<sup>85</sup> suggested that HIF-1 $\alpha$  is a direct target of GSK-3 $\beta$ .

Indeed, the ODDD<sup>86</sup> and three sites, S-551, T-555, and S-589, located within the ODDD overlapping the N-TAD of HIF-1 $\alpha$  were found to be directly phosphorylated by GSK-3 $\beta$ .<sup>85</sup> Another study reported five sites, T-498, S-502, S-505, T-506, and S-510, within the N-TAD of HIF-1 $\alpha$  as GSK-3 $\beta$  phosphorylation sites.<sup>87</sup> The disparity of the different

phosphorylation sites is difficult to explain, but the different oxygen concentrations (8% O<sub>2</sub> compared with 2% O<sub>2</sub>) used in these studies may contribute to the differences. It is possible that different oxygen levels may induce variable signaling pathways that have unequal effects on HIF-1 $\alpha$  and its ability to act as a substrate for GSK-3 $\beta$ . Another possibility could be the different cell types (HepG2 compared with SK-OV-3 [human ovarian cancer cells]) that were used in the studies. Despite the differences in the phosphorylation sites, both studies demonstrated that the regulation of HIF-1 $\alpha$  by GSK-3 $\beta$  is independent of O<sub>2</sub>, hydroxylation, and recruitment of the VHL-containing E3 ubiquitin ligase. Experiments with VHL-deficient cells showed that GSK-3 $\beta$ -dependent HIF-1 $\alpha$  degradation occurred independent of VHL, indicating that the phosphorylation of HIF-1 $\alpha$  by GSK-3 $\beta$  target HIF-1 $\alpha$  for proteasomal degradation in an oxygen-independent manner.<sup>85</sup> This suggested involvement and recruitment of another so-far-unknown E3 ubiquitin ligase to GSK-3 $\beta$ -phosphorylated HIF-1 $\alpha$ .

Indeed, two groups demonstrated that the F-box and WD protein Fbw7 (also known as hCdc4 in yeast, hSel10 in *Caenorhabditis elegans*, or Ago in *Drosophila*) acted as the substrate-recognition component of a multisubunit E3 ubiquitin ligase, which was crucial for the proteasomal degradation of GSK-3 $\beta$  phosphorylated HIF-1 $\alpha$ .<sup>85,87</sup> In this E3 ligase, Fbw7 interacts with SKP1 (S-phase kinase-associated protein 1), CUL1 (cullin 1), and RBX1, forming the so-called SCF complex. Similar to VHL, Fbw7 is considered to serve as a tumor suppressor, and three Fbw7 isoforms (Fbw7 $\alpha$ , Fbw7 $\beta$ , and Fbw7 $\gamma$ ) are known to be produced by alternative splicing. They are found in the nucleoplasm, cytoplasm, and nucleolus, respectively.<sup>88</sup> In addition to HIF-1 $\alpha$ , Fbw7 was shown to be involved in the degradation of various oncogenic proteins, including cyclin E,<sup>89</sup> c-Myc,<sup>90,91</sup> c-Jun,<sup>92,93</sup> and Notch.<sup>94</sup>

Several studies have shown that loss of the *fbw7* gene is associated with malignant transformation, especially in ovarian cells and T cells,<sup>95</sup> in breast cancer cells,<sup>96</sup> and later also in human colorectal cancers,<sup>97</sup> which leads then to chromosomal instability and some types of malignancy. Furthermore, investigation of more than 1,500 human tumors revealed that approximately 6% of those tumors showed mutations in the Fbw7 coding region. Specifically, cholangiocarcinomas (35%), T-cell acute lymphocytic leukemia (31%), and endometrial (9%), colon (9%), and stomach (6%) cancer<sup>98</sup> had the highest mutation rates. Strikingly, nearly half (43%) of these were missense mutations that resulted in amino acid substitutions within the WD40 domain (Arg465 and Arg479), which

are shared by all three Fbw7 isoforms, suggesting that all Fbw isoforms might collectively contribute to the tumor-suppressor function.<sup>98</sup>

With respect to HIF-1 $\alpha$ , all three Fbw7 isoforms were able to induce HIF-1 $\alpha$  degradation, and the loss of the Fbw7 WD domain abolishes GSK-3-initiated degradation, leading to higher HIF-1 $\alpha$  levels, which has been found to be associated with several tumors.<sup>99–101</sup> The finding that HIF  $\alpha$  subunits can be targeted for degradation by two different E3 substrate recognition proteins indicates that the system is highly dynamic.

Important in this context is that ubiquitinylation of proteins is a reversible posttranslational modification. The removal of ubiquitin is mediated by a family of deubiquitylating enzymes. The human genome encodes nearly 100 deubiquitylating enzymes that are predicted to be active and that oppose the function of around 600 E3 ligases.<sup>102,103</sup> Similar to E3s, deubiquitylating enzymes have a central role in cell cycle regulation and DNA damage response and, depending on the context, can act either as a tumor promoter or suppressor (see references in Love et al<sup>104</sup>). With respect to VHL, two different deubiquitinating enzymes, VDU1 (USP33) and VDU2 (USP20), were suggested to oppose the VHL-E3 ubiquitin ligase.<sup>105,106</sup> Later, it was shown that VDU2 but not VDU1 can interact with HIF-1 $\alpha$ .<sup>107</sup> Experiments with cycloheximide and hypoxia showed that the half-life of HIF-1 $\alpha$  was significantly increased upon overexpression of VDU2, whereas a catalytic inactive VDU2 C154A mutant had no effect. In addition, it was shown that only VDU2, not VDU1, deubiquitinated HIF-1 $\alpha$ , resulting in the stabilization of HIF-1 $\alpha$  protein<sup>107</sup> (Figure 1). Experiments with GSK-3 $\beta$ - and Fbw7-deficient cells revealed that GSK-3 $\beta$ - and Fbw7-dependent HIF-1 $\alpha$  degradation can be antagonized by ubiquitin-specific protease 28 (Figure 1).<sup>99</sup> These findings suggest that the GSK-3 $\beta$ -dependent degradation of HIF-1 $\alpha$  is not limited by the presence of oxygen and is therefore independent of VHL. Together, these results demonstrate that HIF-1 $\alpha$  protein stability is regulated in a dynamic manner involving different ubiquitin ligases and deubiquitinases. As such, the hydroxylation- and VHL-dependent ubiquitination and degradation of HIF-1 $\alpha$  under normoxia is opposed by the deubiquitinase VDU2. In contrast, the oxygen-independent but phosphorylation-dependent ubiquitination of HIF-1 $\alpha$  is counteracted by ubiquitin-specific protease 28-mediated deubiquitination. The latter process allows the integration of the HIF system into the cellular response to various physiologic and pathophysiologic signals independent of the oxygen tension.

## Interconnection among the GSK-3, hypoxia/HIF- $\alpha$ , and Wnt/ $\beta$ -catenin pathways

The finding that GSK-3 $\beta$  is involved in the degradation of HIF-1 $\alpha$  indicated similarities with the destruction of  $\beta$ -catenin in the canonical Wnt signaling pathway. In this pathway, GSK-3 $\beta$  and  $\beta$ -catenin are part of a “destructive complex” in which binding of GSK-3 $\beta$  and  $\beta$ -catenin promotes phosphorylation of  $\beta$ -catenin by GSK-3 $\beta$ , which requires priming phosphorylation by casein kinase 1,  $\alpha$ -isoform. The phosphorylated  $\beta$ -catenin is recognized by the F-box/WD protein  $\beta$ -TrCP and subsequently ubiquitinated and targeted for proteasomal degradation (for review, see Cohen and Frame<sup>108</sup> and Metcalfe and Bienz<sup>109</sup>). When this phosphorylation event is blocked,  $\beta$ -catenin accumulates and binds to the T-cell-specific transcription factor/lymphoid enhancer-binding factor 1 family of transcriptional activators to activate numerous target genes (reviewed by Reya and Clevers<sup>110</sup>) contributing to embryonic development and adult tissue homeostasis (reviewed by Clevers<sup>111</sup>). Similarly, GSK-3 $\beta$ -mediated phosphorylation of HIF-1 $\alpha$  recruits Fbw7, and thus targets HIF-1 $\alpha$  for ubiquitination and proteasomal degradation.<sup>99</sup> Those very similar scenarios imply interference or interconnection of both the Wnt/ $\beta$ -catenin and hypoxia/HIF-1 signaling on the level of GSK-3. Actually, crosstalk between the hypoxia and/or HIF-1 $\alpha$  and Wnt/ $\beta$ -catenin pathway was reported and appears to be quite complex because of controversial and likely cell/tissue/differentiation-stage specific data.<sup>112–117</sup> Indeed, it was reported that hypoxia and/or HIF-1 $\alpha$  can inhibit Wnt/ $\beta$ -catenin signaling. Several mechanisms, such as binding of HIF-1 $\alpha$  to hARD1 (human arrest-defective-1 protein) with subsequent interference with acetylation of  $\beta$ -catenin,<sup>112</sup> blocking processing and secretion of Wnt proteins,<sup>113</sup> down-regulating  $\beta$ -catenin via p53-dependent activation of Siah-1 (seven in absentia homolog 1),<sup>118</sup> or direct interaction between HIF-1 $\alpha$  and  $\beta$ -catenin<sup>119,120</sup> were proposed to contribute to these effects.

In contrast, hypoxia was also shown to activate Wnt/ $\beta$ -catenin signaling in undifferentiated cells and *in vivo*.<sup>115,117</sup> In hypoxic embryonic stem cells, this occurred via HIF-1 $\alpha$ -mediated expression of lymphoid enhancer-binding factor 1 and T-cell-specific transcription factor, followed subsequently by increased interaction of  $\beta$ -catenin with lymphoid enhancer-binding factor 1/T-cell-specific transcription factor, and thus activating Wnt/ $\beta$ -catenin targets.<sup>115</sup> In addition, hypoxia was able to activate  $\beta$ -catenin via GSK-3 $\beta$  inactivation<sup>116,121</sup> in different human cell lines such as HT-29 (human colorectal adenocarcinoma cell line)

and HepG2; this activation contributed to an endothelial mesenchymal transition program, leading to significantly increased invasiveness,<sup>121</sup> and in renal tubular cells, this process impaired wound healing.<sup>116</sup> Together, the reported findings indicate that complex interconnections between hypoxia and/or the HIF-1 $\alpha$  and Wnt/ $\beta$ -catenin pathway exist and that cell-, tissue-, and differentiation-specific aspects contribute to their functional consequences.

## Conclusion

Hypoxia and HIFs play important roles in many critical aspects of physiological and pathological processes. Most solid cancers contain hypoxic areas, and clinical data demonstrate that overexpression of HIF-1 $\alpha$  is associated with an increased risk for patient mortality. In line, downregulation of HIFs interferes with tumor growth, vascularization, invasion, and metastasis, as well as radiation and chemotherapy. Activation of multiple oncogenic pathways including growth factor signaling coupled with enhanced kinase signaling is a common event in tumors, thus making it likely that kinases are involved in the modulation of HIF- $\alpha$  function. Because regulation of HIF- $\alpha$  protein stability is critical for its activation, identification of kinases contributing to HIF- $\alpha$  stability may provide a link explaining normoxic HIF- $\alpha$  stabilization by extracellular stimuli. In light of this, dysregulation of GSK-3 $\beta$  is thought to underlie the pathogenesis of various diseases that are also associated with hypoxia and changed HIF- $\alpha$  levels, such as type 2 diabetes mellitus, Alzheimer’s disease, mood disorders, cardiovascular diseases, and cancer.<sup>122</sup> Thus, given that GSK-3 upstream regulation leads to inhibition of GSK-3 and HIF-1 $\alpha$  accumulation, this raises the question whether it is an option to target GSK-3 in those diseases and disregard the adverse effects.

Although research from the last decade has demonstrated that a number of kinase pathways contribute to HIF-1 $\alpha$  regulation, data for HIF-2 $\alpha$  or HIF-3 $\alpha$  are limited. Taking into consideration the overlapping, but different, roles of the HIF- $\alpha$  proteins, more knowledge about the phosphorylation-dependent regulation of HIF-2 $\alpha$  and HIF-3 $\alpha$  is necessary to better understand both already-observed general and different effects.

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## Disclosure

The authors have no conflicts of interest in this work.

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