Regulation of hypoxia-inducible factors by small ubiquitin-like modifiers

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Chapter 2

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Hypoxia in general

Oxygen is essential for the existence of complex, multicellular organisms. It is required as a substrate in order to utilize the highly efficient method of generating high-energy ATP molecules, necessary to drive many cellular processes. At the same time, utilizing oxygen presents a danger as reactive oxygen species are formed as a byproduct. These highly reactive molecules have the potential to damage proteins and nucleic acids. Damage done to the DNA could give rise to mutations and lead to apoptosis or uncontrolled proliferation of the cell. Therefore, the oxygen level in tissues and cells needs to be very carefully regulated and balanced. Insufficient oxygen leads to a shift in the cellular energy production to less efficient, anaerobic processes and eventually cell death, while an excess of oxygen might lead to the formation of dangerous reactive oxygen species (1).

The concentration of oxygen in ambient air, about 21%, is too high for most mammalian tissues. Instead, most tissues are kept at oxygen levels of about 2-9%. In this physiologically normoxic state, enough oxygen is available for cells to maintain normal metabolism. Hypoxia, on the other hand, is a state where the oxygen level is insufficient to allow for a normal metabolism. During hypoxia cells switch to the more inefficient process of glycolysis for ATP production. What constitutes as hypoxia can differ greatly between tissues. Physiological normoxia for poorly vascularized tissues such as bone constitutes a far lower oxygen concentration than physiological normoxia for highly vascularized tissue such as brain tissue (2).

Hypoxia is an important factor in embryonic development as the driving force behind the development of the cardiovascular system. As the embryo increases in size and complexity, it needs a way to deliver oxygen and nutrients to all developing tissues. Pro-angiogenic factors, such as Vascular Endothelial Growth Factor (VEGF), produced by oxygen-deprived tissues are thought to guide the sprouting of blood vessels throughout the growing embryo towards these sites of localized hypoxia. In addition, changes in oxygen concentration over time or distance (in the form of a gradient) also provide cues for cells to change their proliferation rate or to start differentiating (3-5). Human Embryonic Stem (ES) cells, for example, have been shown to proliferate at comparable rates when cultured at

'typical' *in vitro* levels of oxygen (21%) compared to levels closer to those encountered during normal embryonic development (1-3%). However, the appearance of patches of differentiated cells was much more profound in the ES cells cultured at the high oxygen concentration. A low oxygen environment, therefore, seems crucial for maintaining the full differentiation potential of ES cells (3).

In higher organisms, hypoxia occurs in pathologies such as tissue ischemia and inflammation and is also important during the development of solid tumors. These tumors can quickly outgrow the capacity of the local vasculature to supply sufficient nutrients and oxygen. Through the production of angiogenic stimulators, such as VEGF and Erythropoietin (EPO), solid tumors can stimulate not only the formation of additional blood vessels but also increase the resistance of the existing tumor vasculature to radiation therapy (6). Solid tumor hypoxia has been associated with poor prognosis, has been shown to render various anti-cancer treatments less effective and to create an environment that induces and selects for a more genetically unstable and aggressive tumor cell phenotype (promoting genetic instability (7), selection for p53 mutants (8) and up regulation of the multidrug resistance gene MDR1 (9,10)).

2.1 Hypoxia-Inducible Factors

Hypoxia-Inducible Factor-1 (HIF-1) was discovered during a study of a hypoxia-dependent transcriptional enhancer sequence in the flanking region of the Erythropoietin gene (11). Semenza and Wang identified a protein complex that specifically recognized and bound to a small sequence within the enhancer in an oxygen-dependent manner. This complex required de novo protein synthesis after oxygen depletion to be able to bind to DNA and was thus termed hypoxia-inducible factor.

Using DNA affinity chromatography they were able to purify HIF-1 from cells and found it to be a heterodimeric complex, consisting of a 120 kDa HIF-1α and a 91 kDa HIF-1β subunit. Further analysis of both subunits revealed that they contained basic helix-loop-helix (bHLH) and Per-ARNT-SIM (PAS) domains, required for the interaction with DNA and for dimerization with other PAS domain-containing proteins. Both subunits are constitutively expressed. However, while the β subunit is intrinsically stable, the α subunit is under tight control of oxygen-sensitive degradation machinery. The stability and transcriptional activity of HIF-α is greatly reduced during normal oxygen conditions but is strongly and rapidly increased in response to hypoxia (12-15).

HIF-1 is a transcriptional activator and functions as a master regulator of oxygen homeostasis in cells and tissues. In oxygen-deprived cells, it controls the expression of genes such as VEGF and EPO that contain a hypoxia-responsive enhancer element in their promoter sequences. Genes that are up regulated by HIF-1 during hypoxia include pro-angiogenic factors, such as VEGF and EPO, survival and growth factors and proteins involved in glucose metabolism (12,16-18).

Besides HIF-1α, two additional HIF-α factors have been described: HIF-2α, also known as Endothelial PAS domain protein 1 (EPAS1), and HIF-3α. HIF-1α and -2α are 48% identical (19) and share many of the mechanisms regulating their stability and activity. Knock-out studies in mice have shown that HIF-1α and -2α both play essential and similar roles in fetal development and tumor angiogenesis, but they are not redundant. The majority of solid tumors show a high level of expression of HIF-1α and/or HIF-2α (20,21).

Disruption of HIF-1α expression in mouse embryos led to multiple defects in the cardiovascular development. While the initial formation of blood vessels did not seem to be impaired in the HIF-1α -/- embryos, the organization of the vessels was severely disrupted and embryos died early during development (E11.5) (17,22). Several different groups have generated HIF-2α -/- mice and observed somewhat different phenotypes. These differences could arise from differences in genetic background of the mice strains used to create them. Phenotypes observed include defective vascular remodeling with local hemorrhage (23), defective fetal catecholamine production (19) or altered lung maturation secondary to impaired surfactant secretion by alveolar type 2 cells (24). Little is known about the role of HIF-3α, although it is thought to be a hypoxia-dependent repressor.

Targeted deletion of HIF-1α specifically in the myeloid cell lineage has also uncovered an essential role for HIF-1α in the innate immune response (25-27). Sites of bacterial infection are characterized by localized ischemia and cells of the innate immune system can quickly adapt to this hypoxic environment by switching to glycolysis for their energy production (28,29). HIF-1α is a key regulator of glycolytic metabolism (17,30) and deletion of this transcription factor in myeloid cells greatly impaired their anti-bacterial function (25-27). Besides hypoxia, Nitric Oxide (NO) also stabilizes and activates HIF-1α, both directly by S-nitrosylation of cysteine residues found in HIF-1α (26,31) and indirectly by inhibiting the prolyl hydroxylases required for HIF-1α degradation (32). NO is utilized by myeloid cells for its vasodilatory and anti-bacterial properties, produced by the enzyme inducible Nitric Oxide Synthase (iNOS) which is itself a HIF-1α target gene (33,34), thus

creating an amplification loop. More recently, Li *et al* demonstrated that NO released by tumor-associated macrophages was able to stabilize HIF-1α in surrounding cells and thereby contributed to tumor radioresistance (26).

2.2 HIFs as drug targets

HIF has a central role in many of the pathways required for tumor development and growth and is highly expressed in the majority of them (20,21). That makes HIF an attractive factor to target in anti-cancer therapies. Studies using human cancer cell lines and tumors subcutaneously implanted in immunodeficient mice have shown that impairing HIF activity can indeed lead to a reduction in tumor growth (35,36). Therefore, a lot of effort is being put into finding ways of modulating HIF activity using a pharmacological approach, gene therapy or a combination of both. Screening studies have come up with several small molecules that inhibited HIF-1α when tested on cancer cell lines and in tumor xenograft studies in mice. A number of HIF-1α-modulating agents is already used in the clinic or undergoing clinical trials (35-37).

Different phenotypes were observed in different studies using HIF-1α inhibition as a potential for cancer therapy, presumably because of differences in tumor cell origin (36). Inhibition in tumor vascularization was not always the primary cause for the observed reduction in tumor growth, indicating that HIF-1α expression confers additional benefits to tumors besides promoting angiogenesis (6,38,39).

Tumor cells rely heavily on glycolysis for their energy metabolism, even under normoxic circumstances. This is known as the Warburg effect. As HIF-1α plays a key role in the regulation of glycolytic enzymes as well as angiogenic factors, inhibition of HIF in tumors impairs their metabolic adaptation (38). Xu *et al* have used a shRNA-mediated approach to downregulate HIF-1α expression in tumors implanted in immunodeficient mice. They observed decreased tumor growth, metastasis and invasion after localized treatment with a HIF-1α shRNA-encoding plasmid through intratumoral injection. Furthermore, treated tumors showed an increase in apoptosis which was dependent on the caspase cascade reaction (39). Another effect of inactivating HIF-1α may be an increase in the effectiveness of radiation therapy. Inhibition of HIF-1α was shown to increase the radiosensitivity of the vasculature surrounding the tumor, caused by the inhibition of protective cytokine expression (6). Loss of this vasculature would contribute greatly to killing tumor cells by radiation therapy.

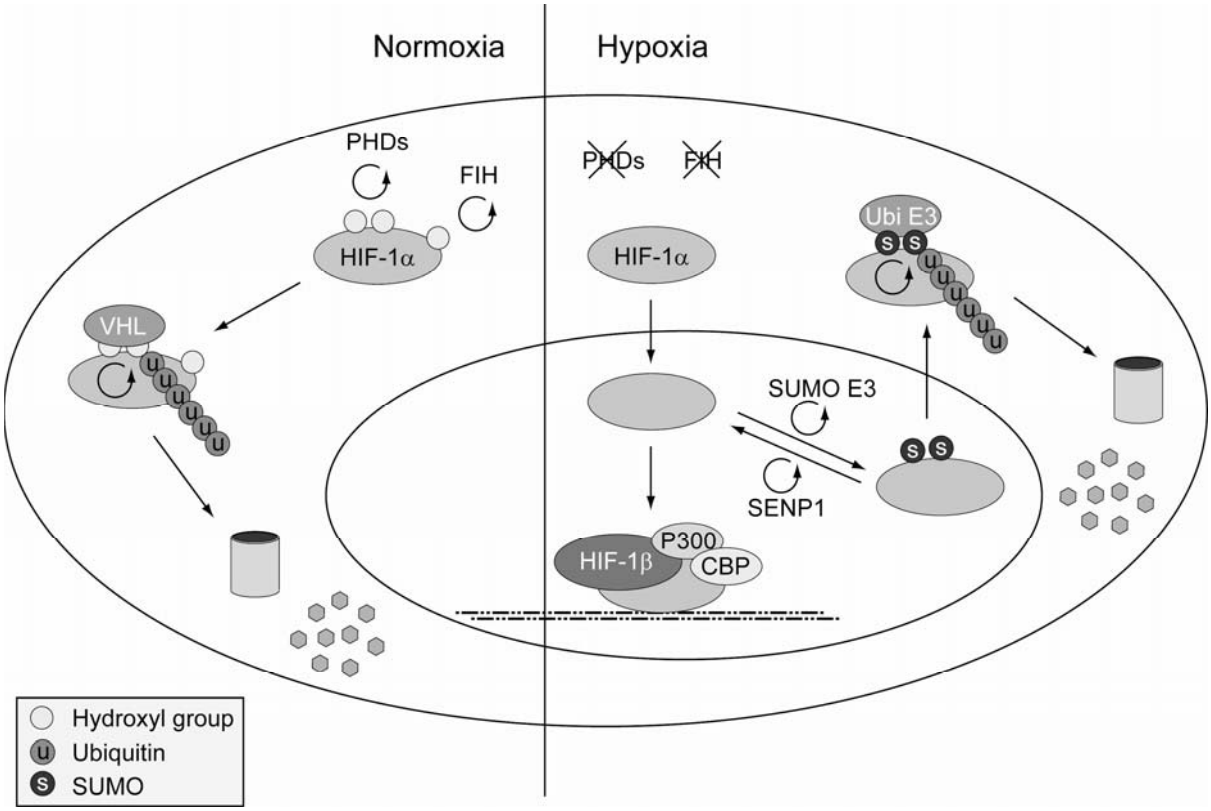

Figure 3: HIF-1α regulation by post-translational modifications during normoxia and hypoxia

When sufficient oxygen is available to cells, continuously expressed HIF-1α is quickly inactivated and degraded after synthesis. HIF-1α is inhibited in its transcriptional activity by hydroxylation of a C-terminal asparagine residue. Hydroxylation by PHDs of two proline residues present in the ODD domain of HIF-1α allows recognition by VHL, followed by poly-ubiquitination and proteasomal degradation. During hypoxia, the PHD proteins and FIH are unable to function. HIF-1α is then free to interact with HIF-1β, DNA and other transcriptional coactivators. An oxygen-independent pathway for HIF-1α degradation is provided through sumoylation. Sumoylation of HIF-1α by as of yet unknown E3 factors allows for recognition by VHL independent of HIF-1α's hydroxylation status. This in turn leads to poly-ubiquitination and proteasomal degradation of HIF-1α.

2.3 Regulation of HIFs during normoxia by post-translational modifications

The stability and activity of the HIF proteins is tightly regulated by a wide range of post-translational modifications (Figure 3). The modifications that regulate HIF-α stability all occur on residues that reside in the Oxygen-Dependent Degradation (ODD) domain. This domain contains two conserved proline residues that are hydroxylated by specific Prolyl Hydroxylases (PHDs). The PHD enzymes require oxygen and Fe^{2+} for their enzymatic activity and are therefore regarded as the oxygen sensors of the cell (40,41). Insufficient

oxygen in the cell will inhibit the activity of the PDH enzymes, leading to a stabilization of HIF-α.

Hydroxylation of HIF-α by the PDH enzymes allows subsequent recognition by the Von Hippel Lindau (VHL) protein (42-44), part of an E3 ubiquitin ligase complex (45). The E3 complex binds to the ODD domain and mediates poly-ubiquitination on the Lys-532 residue of HIF-1α, marking the protein for proteasomal degradation. Impaired VHL activity characterizes the Von Hippel Lindau disease, a hereditary syndrome where patients suffer from highly vascularized tumors as a result of constitutive HIF-1 activity (46).

Once ubiquitinated, HIF-1α can be rescued from proteasomal degradation by the VHL-interacting De-Ubiquitinating (VDU2) enzyme. VDU2 is itself a target for VHL-mediated ubiquitination and degradation, leading to the possibility that HIF-α protein levels are finely regulated by a balance between VHL and VDU2 levels (47,48).

Besides regulating the activity of HIF-α during normoxia through degradation, its activity is also directly modulated by other post-translational modifications in a stability-independent manner. Factor Inhibiting HIF (FIH) hydroxylates an asparagine residue located in the C-terminal Transactivation Domain (C-TAD) which prevents HIF-α from binding the transcriptional cofactors P300/CBP (49).

An oxygen-independent way of regulating HIF-1α stability is provided by Heat-Shock Protein 90 (HSP90) and Receptor of Activated Protein C kinase (RACK1). They compete for HIF-1α binding. HSP90 binds HIF-1α in the cytoplasm and protects it from proteasomal degradation. Upon HSP90 dissociation, HIF-1α can be bound by RACK1, which then functions similar to VHL in recruiting an E3 ubiquitin ligase complex (50).

2.4 Regulation of HIFs during hypoxia by post-translational modifications

The oxygen-dependent enzymes mediating HIF-α degradation and inhibition cannot function under hypoxic conditions, resulting in HIF-α stabilization and rapid accumulation. Nuclear translocation of HIF-1α, required for transcriptional activity, is facilitated by phosphorylation of two serine residues, Ser-641 and Ser-643, by p42 Mitogen-Activated Protein Kinase (MAPK). Rather than stimulating nuclear import, phosphorylation of these residues facilitates nuclear accumulation by inhibiting nuclear export by CRM1/Exportin1 (51). Another phosphorylation event takes place on a threonine residue (Thr-796 in HIF-1α) located in the C-TAD and is mediated by Casein Kinase 2 (CK2). *In vitro* studies have demonstrated that

phosphorylation on this residue inhibits hydroxylation of HIF-α by FIH, thereby allowing interaction with P300/CBP (49,52,53).

S-nitrosylation takes place on two cysteine residues in HIF-1α, Cys-520 in the ODD domain and Cys-800 in the C-TAD. Not surprisingly given their location, modification of these residues modulates HIF-1α stability and activity, respectively. Modification of Cys-520 was shown to stabilize HIF-1α by inhibiting the interaction with VHL (26), whereas modification of Cys-800 increased transcriptional activity by stimulating the recruitment of P300 (31).

HIFs are also controlled by sumoylation as discussed in the next section.

2.5 Regulation of HIF-1α by SUMOs

Sumoylation adds another layer of HIF-1α regulation by post-translational modifications to an already very highly-regulated protein. Mutational analysis has shown that sumoylation takes place on two lysine residues of HIF-1α, Lys-391 and Lys-477 (54). The functional consequences of HIF-1α sumoylation are still somewhat unclear, as conflicting observations have been reported.

Carbia-Nagashima *et al* proposed a stabilizing and activating role for HIF-1α sumoylation. They characterized a small RWD domain-containing Sumoylation Enhancer (RSUME) that is induced by various cellular stresses, including hypoxia. RSUME interacts directly with Ubc9, the E2 enzyme required for sumoylation, and functions as a general enhancer of sumoylation. RSUME also promoted HIF-1α sumoylation, leading to an increase in stability and transcriptional activity (55).

In contrast, Cheng *et al* reported that HIF-1α sumoylation during hypoxia promotes proteasomal degradation. Their SENP1 deficient mice died early during embryonic development due to defective erythropoiesis, caused by a lack of EPO production in these embryos. This in turn, was caused by excessive HIF-1α degradation. Further study indicated that SUMO conjugation of HIF-1α during hypoxia could provide a hydroxyproline-independent way for VHL to recognize HIF-1α and mark it for degradation. Desumoylation by SENP1 was required for HIF-1α stabilization during hypoxia (56).

Finally, Berta *et al* also published data supporting a role for sumoylation in inhibiting HIF-1α transcriptional activity. They showed that HIF-1α pointmutants deficient for SUMO conjugation exhibited greater transcriptional activity than wild-type HIF-1α. This increase

could not be attributed to a difference in protein stability as wild-type and mutant forms of HIF-1α showed a similar expression level and half-life (57).

Taken together, these data on the role of HIF-1α sumoylation are difficult to reconcile. However, it is important to note that both SENP1 and RSUME have much broader roles in regulating sumoylation in cells (55,58). Depletion of RSUME and SENP1 from cells would have a great effect on many other proteins besides HIF-1. We have set out to further investigate the role that sumoylation plays in the regulation of HIFs. We have focused on HIF-1α and HIF-2α mutants that lack SUMO acceptor lysines and compared these mutants to wild-type HIF-1α and HIF-2α. Our results also indicate that HIF-1α and HIF-2α are inhibited by sumoylation, in agreement with Cheng *et al* and Berta *et al*.

References

1. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2007) *Molecular Biology of the Cell*. Garland Science.

2. Ivanovic Z (2009) Hypoxia or in situ normoxia: The stem cell paradigm. *J. Cell Physiol.*, **219**, 271-275.

3. Ezashi T, Das P and Roberts RM (2005) Low O2 tensions and the prevention of differentiation of hES cells. *Proc. Natl. Acad. Sci. U. S. A.*, **102**, 4783-4788.

4. Genbacev O, Joslin R, Damsky CH, Polliotti BM and Fisher SJ (1996) Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia. *J. Clin. Invest.*, **97**, 540-550.

5. Genbacev O, Zhou Y, Ludlow JW and Fisher SJ (1997) Regulation of human placental development by oxygen tension. *Science*, **277**, 1669-1672.

6. Moeller BJ, Cao Y, Li CY and Dewhirst MW (2004) Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules. *Cancer Cell*, **5**, 429-441.

7. Koshiji M, To KK, Hammer S, Kumamoto K, Harris AL, Modrich P and Huang LE (2005) HIF-1alpha induces genetic instability by transcriptionally downregulating MutSalpha expression. *Mol. Cell*, **17**, 793-803.

8. Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW and Giaccia AJ (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature*, **379**, 88-91.

9. Comerford KM, Wallace TJ, Karhausen J, Louis NA, Montalto MC and Colgan SP (2002) Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. *Cancer Res.*, **62**, 3387-3394.

10. Wartenberg M, Ling FC, Muschen M, Klein F, Acker H, Gassmann M, Petrat K, Putz V, Hescheler J and Sauer H (2003) Regulation of the multidrug resistance transporter P-glycoprotein in multicellular tumor spheroids by hypoxia-inducible factor (HIF-1) and reactive oxygen species. *FASEB J.*, **17**, 503-505.

11. Semenza GL and Wang GL (1992) A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol. Cell Biol.*, **12**, 5447-5454.

12. Jiang BH, Rue E, Wang GL, Roe R and Semenza GL (1996) Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *J. Biol. Chem.*, **271**, 17771-17778.

13. Wang GL and Semenza GL (1993) General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc. Natl. Acad. Sci. U. S. A.*, **90**, 4304-4308.

14. Wang GL, Jiang BH, Rue EA and Semenza GL (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc. Natl. Acad. Sci. U. S. A.*, **92**, 5510-5514.

15. Wang GL and Semenza GL (1995) Purification and characterization of hypoxia-inducible factor 1. *J. Biol. Chem.*, **270**, 1230-1237.

16. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD and Semenza GL (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell Biol.*, **16**, 4604-4613.

17. Ryan HE, Lo J and Johnson RS (1998) HIF-1 alpha is required for solid tumor formation and embryonic vascularization. *EMBO J.*, **17**, 3005-3015.

18. Semenza GL, Roth PH, Fang HM and Wang GL (1994) Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J. Biol. Chem.*, **269**, 23757-23763.

19. Tian H, McKnight SL and Russell DW (1997) Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev.*, **11**, 72-82.

20. Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ and Harris AL (2000) The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. *Am. J. Pathol.*, **157**, 411-421.

21. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL and Simons JW (1999) Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res.*, **59**, 5830-5835.

22. Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY *et al.* (1998) Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev.*, **12**, 149-162.

23. Peng J, Zhang L, Drysdale L and Fong GH (2000) The transcription factor EPAS-1/hypoxia-inducible factor 2alpha plays an important role in vascular remodeling. *Proc. Natl. Acad. Sci. U. S. A.*, **97**, 8386-8391.

24. Compernolle V, Brusselmans K, Acker T, Hoet P, Tjwa M, Beck H, Plaisance S, Dor Y, Keshet E, Lupu F *et al.* (2002) Loss of HIF-2alpha and inhibition of VEGF impair fetal lung

maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. *Nat. Med.*, **8**, 702-710.

25. Cramer T, Yamanishi Y, Clausen BE, Forster I, Pawlinski R, Mackman N, Haase VH, Jaenisch R, Corr M, Nizet V *et al.* (2003) HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell*, **112**, 645-657.

26. Li F, Sonveaux P, Rabbani ZN, Liu S, Yan B, Huang Q, Vujaskovic Z, Dewhirst MW and Li CY (2007) Regulation of HIF-1alpha stability through S-nitrosylation. *Mol. Cell*, **26**, 63-74.

27. Peyssonnaux C, Datta V, Cramer T, Doedens A, Theodorakis EA, Gallo RL, Hurtado-Ziola N, Nizet V and Johnson RS (2005) HIF-1alpha expression regulates the bactericidal capacity of phagocytes. *J. Clin. Invest.*, **115**, 1806-1815.

28. Lewis JS, Lee JA, Underwood JC, Harris AL and Lewis CE (1999) Macrophage responses to hypoxia: relevance to disease mechanisms. *J. Leukoc. Biol.*, **66**, 889-900.

29. Sbarra AJ and Karnovsky ML (1959) The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. *J. Biol. Chem.*, **234**, 1355-1362.

30. Seagroves TN, Ryan HE, Lu H, Wouters BG, Knapp M, Thibault P, Laderoute K and Johnson RS (2001) Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells. *Mol. Cell Biol.*, **21**, 3436-3444.

31. Yasinska IM and Sumbayev VV (2003) S-nitrosation of Cys-800 of HIF-1alpha protein activates its interaction with p300 and stimulates its transcriptional activity. *FEBS Lett.*, **549**, 105-109.

32. Metzen E, Zhou J, Jelkmann W, Fandrey J and Brune B (2003) Nitric oxide impairs normoxic degradation of HIF-1alpha by inhibition of prolyl hydroxylases. *Mol. Biol. Cell*, **14**, 3470-3481.

33. Jung F, Palmer LA, Zhou N and Johns RA (2000) Hypoxic regulation of inducible nitric oxide synthase via hypoxia inducible factor-1 in cardiac myocytes. *Circ. Res.*, **86**, 319-325.

34. Melillo G, Taylor LS, Brooks A, Musso T, Cox GW and Varesio L (1997) Functional requirement of the hypoxia-responsive element in the activation of the inducible nitric oxide synthase promoter by the iron chelator desferrioxamine. *J. Biol. Chem.*, **272**, 12236-12243.

35. Giaccia A, Siim BG and Johnson RS (2003) HIF-1 as a target for drug development. *Nat. Rev. Drug Discov.*, **2**, 803-811.

36. Semenza GL (2003) Targeting HIF-1 for cancer therapy. *Nat. Rev. Cancer*, **3**, 721-732.

37. Escuin D, Simons JW and Giannakakou P (2004) Exploitation of the HIF axis for cancer therapy. *Cancer Biol. Ther.*, **3**, 608-611.

38. Chen J, Zhao S, Nakada K, Kuge Y, Tamaki N, Okada F, Wang J, Shindo M, Higashino F, Takeda K *et al.* (2003) Dominant-negative hypoxia-inducible factor-1 alpha reduces tumorigenicity of pancreatic cancer cells through the suppression of glucose metabolism. *Am. J. Pathol.*, **162**, 1283-1291.

39. Xu K, Ding Q, Fang Z, Zheng J, Gao P, Lu Y and Zhang Y (2009) Silencing of HIF-1alpha suppresses tumorigenicity of renal cell carcinoma through induction of apoptosis. *Cancer Gene Ther.*, **17**, 212-222.

40. Bruick RK and McKnight SL (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. *Science*, **294**, 1337-1340.

41. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A *et al.* (2001) C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell*, **107**, 43-54.

42. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS and Kaelin WG, Jr. (2001) HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science*, **292**, 464-468.

43. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ *et al.* (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science*, **292**, 468-472.

44. Masson N, Willam C, Maxwell PH, Pugh CW and Ratcliffe PJ (2001) Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *EMBO J.*, **20**, 5197-5206.

45. Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, Maher ER, Pugh CW, Ratcliffe PJ and Maxwell PH (2000) Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J. Biol. Chem.*, **275**, 25733-25741.

46. Lonser RR, Glenn GM, Walther M, Chew EY, Libutti SK, Linehan WM and Oldfield EH (2003) von Hippel-Lindau disease. *Lancet*, **361**, 2059-2067.

47. Li Z, Na X, Wang D, Schoen SR, Messing EM and Wu G (2002) Ubiquitination of a novel deubiquitinating enzyme requires direct binding to von Hippel-Lindau tumor suppressor protein. *J. Biol. Chem.*, **277**, 4656-4662.

48. Li Z, Wang D, Messing EM and Wu G (2005) VHL protein-interacting deubiquitinating enzyme 2 deubiquitinates and stabilizes HIF-1alpha. *EMBO Rep.*, **6**, 373-378.

49. Cho H, Ahn DR, Park H and Yang EG (2007) Modulation of p300 binding by posttranslational modifications of the C-terminal activation domain of hypoxia-inducible factor-1alpha. *FEBS Lett.*, **581**, 1542-1548.

50. Liu YV and Semenza GL (2007) RACK1 vs. HSP90: competition for HIF-1 alpha degradation vs. stabilization. *Cell Cycle*, **6**, 656-659.

51. Mylonis I, Chachami G, Samiotaki M, Panayotou G, Paraskeva E, Kalousi A, Georgatsou E, Bonanou S and Simos G (2006) Identification of MAPK phosphorylation sites and their role in the localization and activity of hypoxia-inducible factor-1alpha. *J. Biol. Chem.*, **281**, 33095-33106.

52. Gradin K, Takasaki C, Fujii-Kuriyama Y and Sogawa K (2002) The transcriptional activation function of the HIF-like factor requires phosphorylation at a conserved threonine. *J. Biol. Chem.*, **277**, 23508-23514.

53. Lancaster DE, McNeill LA, McDonough MA, Aplin RT, Hewitson KS, Pugh CW, Ratcliffe PJ and Schofield CJ (2004) Disruption of dimerization and substrate phosphorylation inhibit factor inhibiting hypoxia-inducible factor (FIH) activity. *Biochem. J.*, **383**, 429-437.

54. Bae SH, Jeong JW, Park JA, Kim SH, Bae MK, Choi SJ and Kim KW (2004) Sumoylation increases HIF-1alpha stability and its transcriptional activity. *Biochem. Biophys. Res. Commun.*, **324**, 394-400.

55. Carbia-Nagashima A, Gerez J, Perez-Castro C, Paez-Pereda M, Silberstein S, Stalla GK, Holsboer F and Arzt E (2007) RSUME, a small RWD-containing protein, enhances SUMO conjugation and stabilizes HIF-1alpha during hypoxia. *Cell*, **131**, 309-323.

56. Cheng J, Kang X, Zhang S and Yeh ET (2007) SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. *Cell*, **131**, 584-595.

57. Berta MA, Mazure N, Hattab M, Pouyssegur J and Brahimi-Horn MC (2007) SUMOylation of hypoxia-inducible factor-1alpha reduces its transcriptional activity. *Biochem. Biophys. Res. Commun.*, **360**, 646-652.

58. Mukhopadhyay D and Dasso M (2007) Modification in reverse: the SUMO proteases. *Trends Biochem. Sci.*, **32**, 286-295.