Hydroxylation of HIF-1: Oxygen Sensing at the Molecular Level

Gregg L. Semenza

Physiology 19:176-182, 2004. doi:10.1152/physiol.00001.2004

Physiology (formerly published as News in Physiological Science) publishes brief review articles on major physiological developments. It is published bimonthly in February, April, June, August, October, and December by the American Physiological Society, 9650 Rockville Pike, Bethesda MD 20814-3991.
 ISSN: 1548-9213, ESSN: 1548-9221. Visit our website at http://www.the-aps.org/.

PHYSIOLOGY 19: 176–182, 2004; 10.1152/physiol.00001.2004

Hydroxylation of HIF-1: Oxygen Sensing at the Molecular Level

Gregg L. Semenza
Program in Vascular Cell Engineering; Departments of Pediatrics, Medicine, Oncology, and Radiation Oncology; and Institute of Genetic Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205
gsemenza@jhmi.edu

The ability to sense and respond to changes in oxygenation represents a fundamental property of all metazoan cells. The discovery of the transcription factor HIF-1 has led to the identification of protein hydroxylation as a mechanism by which changes in Po_2 are transduced to effect changes in gene expression.

Multicellular life on Earth is based on the use of O_2 for the efficient generation of high-energy compounds, and O_2 consumption increases with the mass and metabolic activity of the organism. However, exposure to O_2 must be limited due to the damaging effects of reactive oxygen species (ROS) on cellular macromolecules. Thus all of the major physiological systems of mammals participate in complex homeostatic mechanisms that are designed to maintain the O_2 concentration to which each cell is exposed within a narrow range (FIGURE 1). The study of these systems has occupied physiologists for centuries. During the course of the past century, these studies have been extended to the cellular level. Finally, research over the past decade has produced dramatic insights into the molecular mechanisms underlying oxygen homeostasis during both prenatal and postnatal life.

Control of Oxygen-Regulated Gene Expression by HIF-1

Physiological responses involve changes in gene expression. The blood O_2-carrying capacity is maintained by the O_2-regulated production of erythropoietin (EPO), which stimulates the proliferation and survival of red blood cell progenitors. Analysis of *cis*-acting sequences required for increased transcription of the *EPO* gene in response to hypoxia led to the identification (70), biochemical purification (81), and molecular cloning (79) of hypoxia-inducible factor-1 (HIF-1). EPO is produced primarily within a rare cell type in the kidney. However, HIF-1 is expressed in all cell types and functions as a master regulator of oxygen homeostasis by playing critical roles in both embryonic development and postnatal physiology. HIF-1 has been identified in all metazoan species that have been analyzed from *Caenorhabditis elegans* to *Homo sapiens* (organisms whose cell numbers differ by more than 10 orders of magnitude), suggesting that the appearance of HIF-1 represented an adaptation that was essential to metazoan evolution.

The expression of over 70 genes is known to be activated at the transcriptional level by HIF-1, and specific HIF-1 binding sites have been identified for many of these genes. Although the list of HIF-1 target genes is extensive (FIGURE 2), it probably underestimates the total number of genes regulated by HIF-1 by at least an order of magnitude. The battery of genes regulated by HIF-1 is different in each cell type, and, for some genes, expression can be induced or repressed by HIF-1 depending on the cell type (34). Among the critical physiological processes regulated by HIF-1 target genes are erythropoiesis, angiogenesis, and glycolysis, which are examples of systemic, local tissue, and intracellular adaptive responses to hypoxia, respectively (30).

HIF-1 is a heterodimeric protein that is composed of HIF-1α and HIF-1β subunits. The amino-terminal half of each subunit consists of basic helix-loop-helix and Per-ARNT-Sim (PAS) domains that mediate heterodimerization and DNA binding. The carboxy-terminal half of HIF-1α contains two transactivation domains that mediate interactions with coactivators such as CREB binding protein (CBP) and p300 (30, 31, 59). Coactivators interact with both sequence-specific DNA binding proteins such as HIF-1 and with the general transcription factors associated with RNA Polymerase II (reviewed in Ref. 69). Coactivators also have histone acetyltransferase activity that is required to make the DNA embedded in chromatin accessible to the polymerase complex for transcription into RNA.

The HIF-1β subunit is constitutively expressed, whereas the expression and activity of the HIF-1α subunit are precisely regulated by the cellular O_2

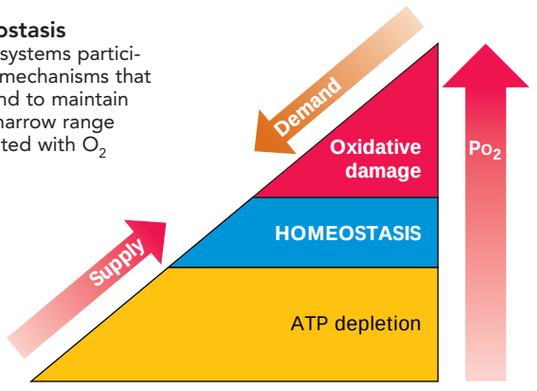

FIGURE 1. Oxygen homeostasis
All of the major physiological systems participate in complex homeostatic mechanisms that regulate O_2 supply and demand to maintain cellular oxygenation within a narrow range that balances the risks associated with O_2 deficiency and excess.

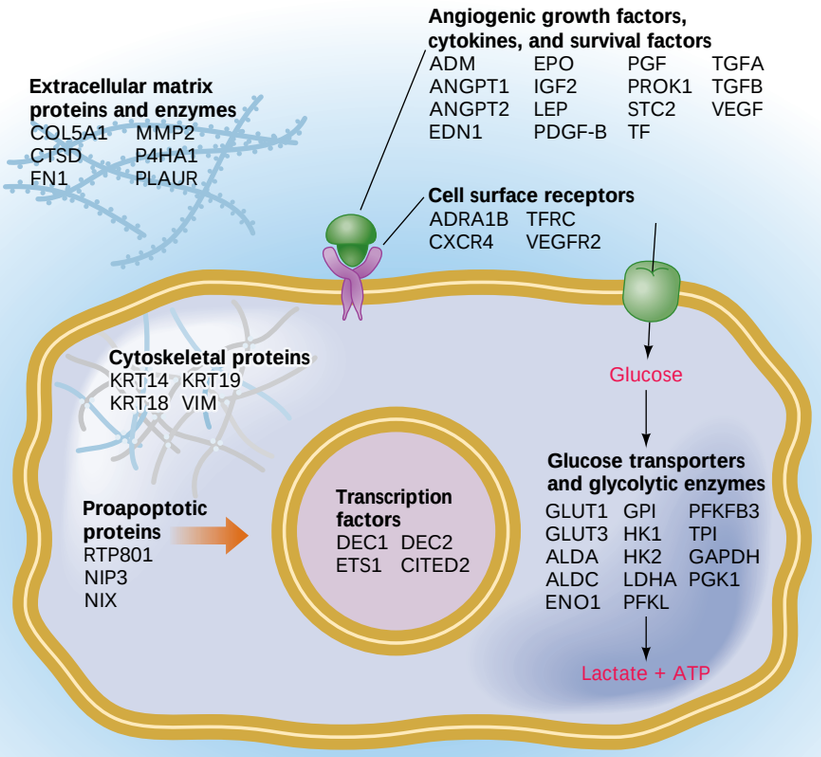

FIGURE 2. Representative HIF-1 target genes
Hypoxia inducible factor-1 (HIF-1) activates the transcription of genes encoding secreted signaling proteins, including angiogenic growth factors and survival factors, cell surface receptors, extracellular matrix proteins and modifying enzymes, transcription factors, cytoskeletal proteins, proapoptotic proteins, and glucose transporters and glycolytic enzymes. ADM, adrenomedullin; ADRA1B, α_{1B}-adrenergic receptor; ALD, aldolase; ANGPT, angiopoietin; CITED, CREB binding protein (CBP)/p300-interacting transactivator; COL5A1, collagen V α1-subunit; CTSD, cathepsin D; CXCR, chemokine receptor; DEC, differentiated embryo chondrocyte expressed; EDN, endothelin; ENO, enolase; EPO, erythropoietin; ETS, erythroblastosis virus transforming sequence; FN, fibronectin; GLUT, glucose transporter; GPI, glucose phosphate isomerase; HK, hexokinase; KRT, keratin; LDHA, lactate dehydrogenase A; LEP, leptin; MMP, matrix metalloproteinase; NIP, BCL2/adenovirus E1B 19-kDa-interacting protein; NIX, NIP3-like; P4HA1, prolyl-4-hydroxylase α1-subunit; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3; PFKL, phosphofructokinase L; PGF, placental growth factor; PGK, phosphoglycerate kinase; PLAUR, urokinase-type plasminogen activator receptor; PROK, prokineticin (endocrine gland-derived VEGF); STC, stanniocalcin; TF, transferrin; TFRC, transferrin receptor; TGFA and TGFB, transforming growth factor-α and -β; TPI, triose phosphate isomerase; VEGFR, VEGF receptor; VIM, vimentin.

concentration. HIF-1α accumulates instantaneously under hypoxic conditions and on reoxygenation is rapidly degraded, with a half-life of <5 min in posthypoxia tissue culture cells (28, 79). This represents an overestimate of the half-life, because it includes the time required for O_2 to diffuse out of the culture medium. In an isolated, perfused, and ventilated lung preparation subjected to hypoxia and reoxygenation, the half-life of HIF-1α is <1 min (85). No protein has been shown to have a shorter half-life.

In addition to HIF-1α, a structurally and functionally related protein designated HIF-2α, which is the product of the *EPAS1* gene, can also heterodimerize with HIF-1β (77). HIF-1α:HIF-1β and HIF-2α:HIF-1β heterodimers appear to have overlapping but distinct target gene specificities (22, 73). Unlike HIF-1α, HIF-2α is not expressed in all cell types, and when expressed it can be inactive as a result of cytoplasmic sequestration (56). A third protein, designated HIF-3α, has also been identified (18). Its role has not been well defined, although a splice variant, designated IPAS, has been shown to bind to HIF-1α and inhibit its activity (45, 46).

Molecular Mechanisms of Oxygen Sensing

The mechanism underlying the dramatic regulation of HIF-1α protein expression was a source of great debate, with several models proposed that invoked, for example, the functioning of an O_2-binding hemoprotein or an ROS-generating NADPH oxidase as central to the oxygen sensing that determined HIF-1α levels (reviewed in Ref. 66). Among the observations used to support these models was the finding that HIF-1 DNA binding activity and target gene expression were induced in cells exposed to the iron chelator desferrioxamine or to cobalt chloride (80). Remarkably, HIF-1α transactivation domain function is also induced in cells exposed to hypoxia, iron chelation, or cobalt chloride (30, 31, 59), suggesting a common mechanism for regulating both HIF-1α expression and activity.

The O_2-dependent degradation of HIF-1α involves ubiquitination and degradation by the 26S proteasome (23, 32, 61). The von Hippel-Lindau tumor suppressor protein (VHL) is required for this process (FIGURE 3), because renal carcinoma cells lacking functional VHL constitutively express HIF-1α and HIF-1 target genes under nonhypoxic conditions (6, 49). VHL forms a complex with elongin B, elongin C, cullin 2, and RBX1 to form an E3 ubiquitin-protein ligase capable of functioning with E1 ubiquitin-activating and E2 ubiquitin-conjugating enzymes to mediate the ubiquitination of HIF-1α (33).

A region of HIF-1α encompassing amino acid residues 400–600 is necessary and sufficient for O_2-regulated ubiquitination and degradation (23, 32,

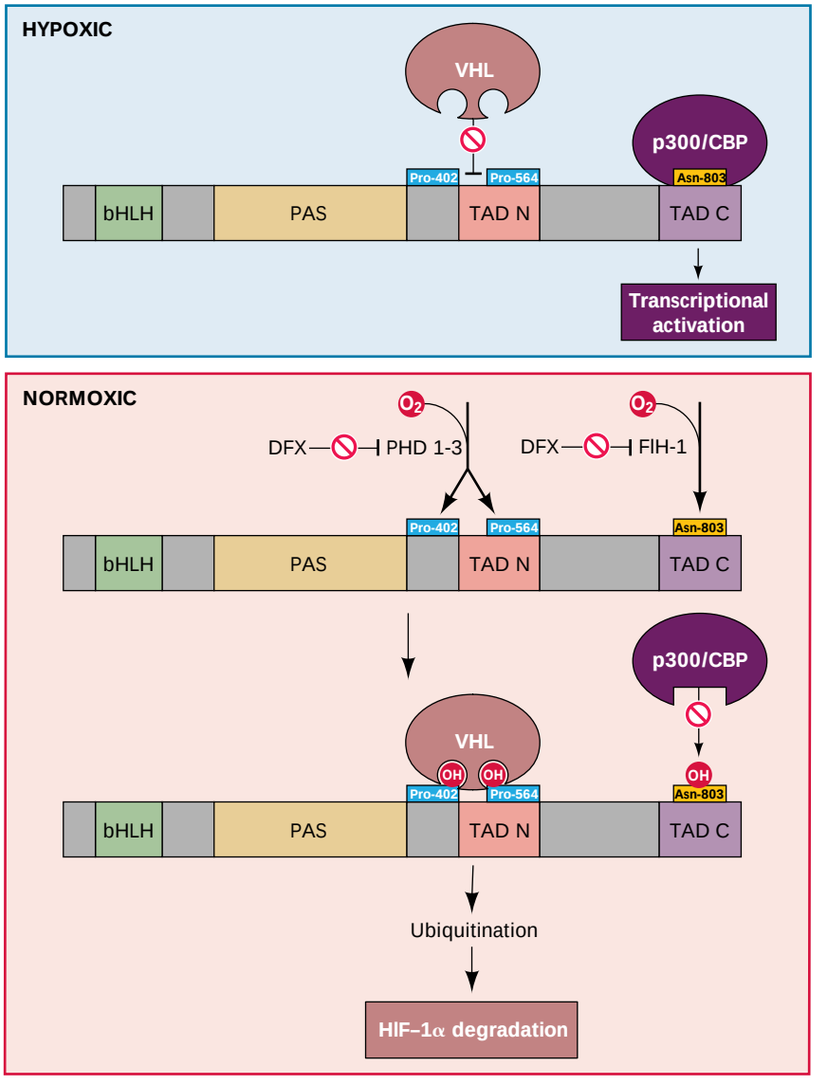

FIGURE 3. Oxygen sensing by hydroxylation of HIF-1α
The amino-terminal half of HIF-1α consists of basic helix-loop-helix (bHLH) and Per-ARNT-Sim homology (PAS) domains. The carboxy-terminal half contains the transactivation domains (TAD-N and TAD-C). The HIF-1α prolyl hydroxylases (HPH)/prolyl hydroxylase domain proteins (PHD) 1–3 hydroxylate Pro-402 and Pro-564. Factor inhibiting HIF-1 (FIH-1) hydroxylates Asn-803. Proline hydroxylation is required for the interaction of HIF-1α with the von Hippel-Lindau tumor-suppressor protein (VHL), which is the recognition component of an E3 ubiquitin-protein ligase that targets HIF-1α for proteasomal degradation. Asparagine hydroxylation prevents the interaction of HIF-1α with the coactivators CBP and p300. The enzymes, which contain Fe(II) at the active site, can be inactivated by desferrioxamine (DFX) and other iron chelators. O_2 appears to be a rate-limiting substrate for the hydroxylases under physiological conditions, thus providing a mechanism for the direct regulation of the stability and activity of HIF-1α as a function of the cellular O_2 concentration.

74). VHL interacts, via its β-domain, with amino acid residues 532–585 of HIF-1α (55, 75). Because the ubiquitination and degradation of other key regulatory proteins such as IκB are regulated by phosphorylation, great effort was made to identify phosphorylatable (serine, threonine, tyrosine) residues of HIF-1α that were important for regulation of protein half-life, but to no avail. Instead, Pro-564 is hydroxylated in an O_2-dependent manner, and this modification is required for VHL binding (25, 27, 87). Pro-402 represents a second site of hydroxylation and VHL binding (48). Pro-402 and Pro-564 are each contained within a similar amino acid sequence (LXXLAP, where A is alanine, L is leucine, P is proline, and X is any amino acid). HIF-2α and HIF-3α expression are also regulated by prolyl hydroxylation and VHL binding (20, 49, 50).

Three prolyl hydroxylases were identified in mammalian cells and shown to use O_2 as a substrate to generate 4-hydroxyproline at residue 402 and/or 564 of HIF-1α (2, 13, 24). These proteins are homologues of EGL-9, which was identified as the HIF-1α prolyl hydroxylase in *C. elegans* by genetic studies (13). Alternative designations for the three mammalian homologues include EGL-9 homologue (EGLN), prolyl hydroxylase domain protein (PHD), and HIF-1α prolyl hydroxylase (HPH) 1–3. The hydroxylation reaction also requires 2-oxoglutarate (α-ketoglutarate) as a substrate and generates succinate as a side product. Ascorbate is required as a cofactor. The prolyl hydroxylase catalytic site contains an Fe(II) ion that is coordinated by two histidine and one aspartate residue. Unlike heme-containing proteins, the Fe(II) in 2-oxoglutarate-dependent oxygenases can be chelated or substituted by Co(II), rendering the enzyme inactive. Most importantly, these prolyl hydroxylases have a relatively high K_m for O_2 that is slightly above its atmospheric concentration, such that O_2 is rate limiting for enzymatic activity under physiological conditions (13, 20). As a result, changes in the cellular O_2 concentration are directly transduced into

changes in the rate at which HIF-1α is hydroxylated, ubiquitinated, and degraded. However, a thorough analysis of the relationship between O_2 concentration and enzyme activity for each of the PHDs in living cells, and a comparison with the corresponding dose-response curve for HIF-1α expression (29), has not yet been reported. In particular, the plot of HIF-1α protein levels as a function of O_2 concentration in HeLa cells yielded a sigmoidal curve suggestive of cooperativity (29), a finding that is not readily explained by the known biochemistry of the HIF-1α prolyl hydroxylases.

Remarkably, HIF-1α transactivation domain function is regulated by O_2-dependent hydroxylation of Asn-803, which blocks the binding of the coactivators CBP and p300 (41). Factor inhibiting HIF-1 (FIH-1), which was identified in a yeast two-hybrid screen as a protein that interacts with and inhibits the activity of the HIF-1α transactivation domain (44), functions as the asparaginyl hydroxylase (19, 40). As in the case of the prolyl hydroxylases, FIH-1 appears to use O_2 and 2-oxoglutarate and contain Fe(II) in its active site (11, 42, 51), although it has a K_m for O_2 that is three times lower than the prolyl hydroxylases (37).

Spectroscopic analyses of a peptide from the HIF-1α transactivation domain complexed with the interacting domain of CBP or p300 revealed that Asn-803 is present within an α-helix that is buried deep within the protein interface, where it participates in multiple hydrogen-bonding interactions that are predicted to stabilize the complex (9, 12, 15). Hydroxylation of Asn-803 is predicted to disrupt these protein-protein interactions. Similarly, hydroxylation of Pro-564 has been shown to also function as a molecular switch to positively regulate the interaction of HIF-1α and VHL (21, 53). Thus hydroxylation provides a mechanism for regulating protein-protein interactions, similar to the effect of phosphorylation and other posttranslational modifications. However, what sets hydroxylation apart is that the modification occurs in an O_2-dependent manner, thus establishing a direct link between cellular oxygenation and HIF-1 activity.

One remarkable aspect of the O_2-sensing system described above is its plasticity. Although O_2 may be the limiting substrate for hydroxylation under physiological conditions, it appears that under pathophysiological conditions iron or ascorbate may also be limiting (36). Furthermore, the expression of the PHDs varies from one cell type to another as well as in response to various physiological stimuli, including hypoxia (1, 10, 13, 52). Thus the O_2 dose-response curve may be shifted to the left or right under different developmental or physiological conditions. Alternative splicing of the primary RNA transcripts for two of the PHDs provides yet another mechanism for modulating prolyl hydroxylase activity (20). Finally, the transcriptional response elicited by a hypoxic stimulus also demonstrates a remarkable degree of plasticity, because the battery of target genes that is regulated by HIF-1 is unique to each cell type (34). Thus the identification of the molecular components of the O_2-sensing system represents a milestone, rather than a finish line, on the course to defining the physiology of oxygen homeostasis.

Developmental and Physiological Consequences of HIF-1 Activity

The identification of HIF-1, VHL, FIH-1, and the PHDs over the past decade has delineated a pathway by which cells sense O_2 and respond to changes in oxygenation with changes in gene expression, a property that is fundamental to the cells of all metazoan species. Coincident with these dramatic molecular discoveries have been equally dramatic discoveries regarding the remarkable variety of biological processes in which HIF-1 plays an important role. Analyses of mice, in which expression of HIF-1α has been lost either in all cells (germline knockout) or a single cell lineage (conditional knockout), have identified multiple aspects of development and physiology that are dependent on HIF-1 (TABLE 1). Indeed, the study of HIF-1's role in development and physiology provides a basis for unifying these two central areas of biology. O_2 delivery to cells of the developing embryo becomes limited by diffusion such that establishment of a functioning circulatory system is required for embryonic survival by embryonic

TABLE 1. Developmental and physiological roles of HIF-1 as established by analysis of HIF-1α-null mice and cell lines

Role(s)	References
Development	
Cardiovascular development. embryonic survival	7, 26, 39, 60
Chondrogenesis/skeletal development	62
Adipogenesis	88
B lymphocyte development	38
Mammary gland development	64
Physiology	
Angiogenesis	5, 34, 60
Hypoxia-induced glycolysis	26, 65
Hypoxia-induced apoptosis	5
Hypoxia-induced erythropoiesis	86
Hypoxia-induced pulmonary vascular remodeling	71, 86
Carotid body sensing of arterial P_{O_2}	35
Myeloid cell-mediated inflammation	8
Hypoxia-induced myocardial preconditioning	4
Hypoxia-induced cell cycle arrest	17

HIF, hypoxia-inducible factor

day 9 (E9) in the mouse. In wild-type mouse embryos, HIF-1α expression increases dramatically between E8.5 and E9.5, whereas embryos that lack HIF-1α expression die between E9.5 and E10.5 and show cardiac malformations, vascular regression, and massive cell death (7, 26, 39, 60). Complete HIF-2α deficiency is also associated with embryonic lethality (58, 76), and because the embryos survive longer than *Hif1a*$^{-/-}$ mice, effects on multiple organ systems can be demonstrated (63).

Whereas complete HIF-1α deficiency results in developmental defects, partial HIF-1α deficiency is sufficient to result in impaired responses to physiological stimuli. A particularly dramatic example is the loss of O_2 sensing in the carotid body of *Hif1a*$^{+/-}$ mice (35). Although the carotid bodies are anatomically and histologically normal and depolarize normally in response to cyanide application, they show essentially no response to hypoxia. Thus partial HIF-1α deficiency in the carotid body results in a complete loss of the ability to sense and/or respond to changes in the arterial P_{O_2} by stimulation of the central nervous system cardiorespiratory centers. HIF-2α is also expressed in the mouse carotid body (76), which suggests that HIF-1α and HIF-2α play distinct roles in this organ. The HIF-1 target genes that are critical for O_2 sensing and/or efferent responses by the carotid body have not been identified. Remarkably, in the intact animal, other chemoreceptors are less sensitive to *Hif1a* gene dosage and compensate for the loss of carotid body activity in *Hif1a*$^{+/-}$ mice (35).

Another dramatic phenotype is the complete inability of *Hif1a*$^{-/-}$ myeloid cells (granulocytes and macrophages) to respond to inflammatory stimuli (8). Myeloid cells are dependent on glycolysis for ATP generation, perhaps reflecting the hypoxic microenvironment that is often associated with inflammation and infection. HIF-1α deficiency results in ATP deficiency, which impairs critical myeloid cell functions such as aggregation, motility, invasion, and bacterial killing. The role of HIF-1 in immunity is not restricted to myeloid cells, because HIF-1 also plays critical roles in B lymphocyte development (38) and T lymphocyte activation (47). The ability to create mice in which HIF-1α deficiency is restricted to a limited number of cell types (8, 62, 64) is likely to result in the identification of an increasing number of developmental and physiological processes that are regulated by HIF-1.

Medical Consequences of HIF-1 Activity

The preceding sections provide a brief summary of the critical role of HIF-1 in understanding oxygen sensing, development, and physiology. HIF-1 plays an equally important role in disease pathophysiology, including ischemic cardiovascular disease (3, 67) and cancer (68, 82), the most common causes of mortality in the US population. As a result, there is considerable interest in HIF-1 as a therapeutic target in these disorders (16, 68, 82). In the case of cardiovascular disease, increased HIF-1 activity induced as a result of HIF-1α gene therapy (34, 72, 78), small molecule inhibitors of prolyl hydroxylase activity (20, 24, 43), or inhibitors of HIF-1α-VHL interaction (83) may provide a means to stimulate neovascularization of ischemic tissue. In contrast, small-molecule inhibitors of HIF-1 activity may be useful as anticancer agents (84). However, because HIF-1 functions as a global regulator of oxygen homeostasis, it may not be a useful therapeutic target if the treatment results in unintended and undesirable side effects. An alternative approach may be to focus on the products of HIF-1 target genes. For example, erythropoietin administration may reduce ischemia-induced apoptosis in patients presenting with acute cerebral or myocardial infarction (4, 14, 54, 57). The translation of a rapidly growing body of basic science data into clinical applications looms as the most challenging and most important goal in this exciting field. ■

References

1. Berra E, Benizri E, Ginouves A, Volmat V, Roux D, and Pouyssegur J. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. *EMBO J* 22: 4082–4090, 2003.
2. Bruick RK and McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 294: 1337–1340, 2001.
3. Bruick RK and McKnight SL. Building better vasculature. *Genes Dev* 15: 2497–2502, 2001.
4. Cai Z, Manalo DJ, Wei G, Rodriguez ER, Fox-Talbot K, Lu H, Zweier JL, and Semenza GL. Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. *Circulation* 108: 79–85, 2003.
5. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E, and Keshet E. Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* 394: 485–490, 1998.
6. Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, Maher ER, Pugh CW, Ratcliffe PJ, and Maxwell PH. Hypoxia inducible factor-α binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J Biol Chem* 275: 25733–25741, 2000.
7. Compernolle V, Brusselmans K, Franco D, Moorman A, Dewerchin M, Collen D, and Carmeliet P. Cardia bifida, defective heart development and abnormal neural crest migration in embryos lacking hypoxia-inducible factor-1α. *Cardiovasc Res* 60: 569–579, 2003.
8. Cramer T, Yamanishi Y, Clausen BE, Forster I, Pawlinski R, Mackman N, Haase VH, Jaenisch R, Corr M, Nizet V, Firestein GS, Gerber HP, Ferrara N, and Johnson RS. HIF-1α is essential for myeloid cell-mediated inflammation. *Cell* 112: 645–657, 2003.
9. Dames SA, Martinez-Yamout M, De Guzman RN, Dyson HJ, and Wright PE. Structural basis for HIF-1α/CBP recognition in the cellular hypoxic response. *Proc Natl Acad Sci USA* 99: 5271–5276, 2002.

10. D'Angelo G, Duplan E, Boyer N, Vigne P, and Frelin C. Hypoxia up-regulates prolyl hydroxylase activity: a feedback mechanism that limits HIF-1 responses during reoxygenation. *J Biol Chem* 278: 38183–38187, 2003.

11. Dann CE III, Bruick RK, and Deisenhofer J. Structure of factor inhibiting hypoxia-inducible factor 1: an asparaginyl hydroxylase involved in the hypoxic response pathway. *Proc Natl Acad Sci USA* 99: 15351–15356, 2002.

12. Elkins JM, Hewitson KS, McNeill LA, Seibel JF, Schlemminger I, Pugh CW, Ratcliffe PJ, and Schofield CJ. Structure of factor inhibiting hypoxia-inducible factor (HIF) reveals mechanism of oxidative modification of HIF-1α. *J Biol Chem* 278: 1802–1806, 2003.

13. Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, and Ratcliffe PJ. *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107: 43–54, 2001.

14. Erbayraktar S, Grasso G, Sfacteria A, Xie QW, Coleman T, Kreilgaard M, Torup L, Sager T, Erbayraktar Z, Gokmen N, Yilmaz O, Ghezzi P, Villa P, Fratelli M, Casagrande S, Leist M, Helboe L, Gerwein J, Christensen S, Geist MA, Pedersen LO, Cerami-Hand C, Wuerth JP, Cerami A, and Brines M. Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo. *Proc Natl Acad Sci USA* 100: 6741–6746, 2003.

15. Freedman SJ, Sun ZY, Poy F, Kung AL, Livingston DM, Wagner G, and Eck MJ. Structural basis for recruitment of CBP/p300 by hypoxia-inducible factor-1α. *Proc Natl Acad Sci USA* 99: 5367–5372, 2002.

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

17. Goda N, Ryan HE, Khadivi B, McNulty W, Rickert RC, and Johnson RS. Hypoxia-inducible factor 1α is essential for cell cycle arrest during hypoxia. *Mol Cell Biol* 23: 359–369, 2003.

18. Gu YZ, Moran SM, Hogenesch JB, Wartman L, and Bradfield CA. Molecular characterization and chromosomal localization of a third α-class hypoxia inducible factor subunit, HIF-3α. *Gene Expr* 7: 205–213, 1998.

19. Hewitson KS, McNeill LA, Riordan MV, Tian YM, Bullock AN, Welford RW, Elkins JM, Oldham NJ, Bhattacharya S, Gleadle JM, Ratcliffe PJ, Pugh CW, and Schofield CJ. Hypoxia-inducible factor asparaginyl hydroxylase is identical to factor inhibiting HIF-1 (FIH-1) and is related to the cupin structural family. *J Biol Chem* 277: 26351–26355, 2002.

20. Hirsila M, Koivunen P, Gunzler V, Kivirikko KI, and Myllyharju J. Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. *J Biol Chem* 278: 30772–30780, 2003.

21. Hon WC, Wilson MI, Harlos K, Claridge TD, Schofield CJ, Pugh CW, Maxwell PH, Ratcliffe PJ, Stuart DI, and Jones EY. Structural basis for the recognition of hydroxyproline in HIF-1α by pVHL. *Nature* 417: 975–978, 2002.

22. Hu CJ, Wang LY, Chodosh LA, Keith B, and Simon MC. Differential roles of hypoxia-inducible factor 1α (HIF-1α) and HIF-2α in hypoxic gene regulation. *Mol Cell Biol* 23: 9361–9374, 2003.

23. Huang LE, Gu J, Schau M, and Bunn HF. Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci USA* 95: 7987–7992, 1998.

24. Ivan M, Haberberger T, Gervasi DC, Michelson KS, Gunzler V, Kondo K, Yang H, Sorokina I, Conaway RC, Conaway JW, and Kaelin WG Jr. Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor. *Proc Natl Acad Sci USA* 99: 13459–13464, 2002.

25. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, and Kaelin WG Jr. HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. *Science* 292: 464–468, 2001.

26. Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, and Semenza GL. Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1α. *Genes Dev* 12: 149–162, 1998.

27. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim Av Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, and Ratcliffe PJ. Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science* 292: 468–472, 2001.

28. Jewell UR, Kvietikova I, Scheid A, Bauer C, Wenger RH, and Gassmann M. Induction of HIF-1α in response to hypoxia is instantaneous. *FASEB J* 15: 1312–1314, 2001.

29. Jiang BH, Semenza GL, Bauer C, and Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O_2 tension. *Am J Physiol Cell Physiol* 271: C1172–C1180, 1996.

30. Jiang BH, Zheng JZ, Leung SW, Roe R, and Semenza GL. Transactivation and inhibitory domains of hypoxia-inducible factor 1α: modulation of transcriptional activity by oxygen tension. *J Biol Chem* 272: 19253–19260, 1997.

31. Kallio PJ, Okamoto K, O'Brien S, Carrero P, Makino Y, Tanaka H, and Poellinger L. Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1α. *EMBO J* 17: 6573–6586, 1998.

32. Kallio PJ, Wilson WJ, O'Brien S, Makino Y, and Poellinger L. Regulation of the hypoxia-inducible transcription factor 1α by the ubiquitin-proteasome pathway. *J Biol Chem* 274: 6519–6525, 1999.

33. Kamura T, Sato S, Iwai K, Czyzyk-Krzeska M, Conaway RC, and Conaway JW. Activation of HIF1α ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. *Proc Natl Acad Sci USA* 97: 10430–10435, 2000.

34. Kelly BD, Hackett SF, Hirota K, Oshima Y, Cai Z, Berg-Dixon S, Rowan A, Yan Z, Campochiaro PA, and Semenza GL. Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. *Circ Res* 93: 1074–1081, 2003.

35. Kline DD, Peng YJ, Manalo DJ, Semenza GL, and Prabhakar NR. Defective carotid body function and impaired ventilatory responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1α. *Proc Natl Acad Sci USA* 99: 821–826, 2002.

36. Knowles HJ, Raval RR, Harris AL, and Ratcliffe PJ. Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. *Cancer Res* 63: 1764–1768, 2003.

37. Koivunen P, Hirsila M, Gunzler V, Kivirikko KI, and Myllyharju J. Catalytic properties of the asparaginyl hydroxylase (FIH) in the oxygen sensing pathway are distinct from those of its prolyl-4-hydroxylases. *J Biol Chem* 279: 9899–9904, 2004.

38. Kojima H, Gu H, Nomura S, Caldwell CC, Kobata T, Carmeliet P, Semenza GL, and Sitkovsky MV. Abnormal B lymphocyte development and autoimmunity in hypoxia-inducible factor 1α-deficient chimeric mice. *Proc Natl Acad Sci USA* 99: 2170–2174, 2002.

39. Kotch LE, Iyer NV, Laughner E, and Semenza GL. Defective vascularization of HIF-1α-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. *Dev Biol* 209: 254–267, 1999.

40. Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, and Bruick RK. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev* 16: 1466–1471, 2002.

41. Lando D, Peet DJ, Whelan DA, Gorman JJ, and Whitelaw ML. Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* 295: 858–861, 2002.

42. Lee C, Kim SJ, Jeong DG, Lee SM, and Ryu SE. Structure of human FIH-1 reveals an active site pocket and interaction sites for HIF-1 and VHL. *J Biol Chem* 278: 7558–7563, 2003.

43. Linden T, Katschinski DM, Eckhardt K, Scheid A, Pagel H, and Wenger RH. The antimycotic ciclopirox olamine induces HIF-1α stability, VEGF expression, and angiogenesis. *FASEB J* 17: 761–763, 2003.

44. Mahon PC, Hirota K, and Semenza GL. FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev* 15: 2675–2686, 2001.

45. Makino Y, Cao R, Svensson K, Bertilsson G, Asman M, Tanaka H, Cao Y, Berkenstam A, and Poellinger L. Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. *Nature* 414: 550–554, 2001.

46. Makino Y, Kanopka A, Wilson WJ, Tanaka H, and Poellinger L. Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3α locus. *J Biol Chem* 277: 32405–32408, 2002.

47. Makino Y, Nakamura H, Ikeda E, Ohnuma K, Yamauchi K, Yabe Y, Poellinger L, Okada Y, Morimoto C, and Tanaka H. Hypoxia-inducible factor regulates survival of antigen receptor-driven T cells. *J Immunol* 171: 6534–6540, 2003.

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

49. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, and Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 399: 271–275, 1999.

50. Maynard MA, Qi H, Chung J, Lee EH, Kondo Y, Hara S, Conaway RC, Conaway JW, and Ohh M. Multiple splice variants of the human HIF-3α locus are targets of the von Hippel-Lindau E3 ubiquitin ligase complex. *J Biol Chem* 278: 11032–11040, 2003.

51. McNeill LA, Hewitson KA, Claridge TD, Seibel JF, Horsfall LE, and Schofield CJ. Hypoxia-inducible factor asparaginyl hydroxylase (FIH-1) catalyzes hydroxylation at the beta-carbon of asparagine-803. *Biochem J* 367: 571–575, 2002.

52. Metzen E, Berchner-Pfannschmidt U, Stengel P, Marxsen JH, Stolze I, Klinger M, Huang WQ, Wotzlaw C, Hellwig-Burgel T, Jelkmann W, Acker H, and Fandrey J. Intracellular localisation of human HIF-1α hydroxylases: implications for oxygen sensing. *J Cell Sci* 116: 1319–1326, 2003.

53. Min JH, Yang H, Ivan M, Gertler F, Kaelin WG Jr, and Pavletich NP. Structure of an HIF-1α-pVHL complex: hydroxyproline recognition in signaling. *Science* 296: 1886–1889, 2002.

54. Moon C, Krawczyk M, Ahn D, Ahmet I, Paik D, Lakatta EG, and Talan MI. Erythropoietin reduces myocardial infarction and left ventricular functional decline after coronary artery ligation in rats. *Proc Natl Acad Sci USA* 100: 11612–11617, 2003.

55. Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, Huang LE, Pavletich N, Chau V, and Kaelin WG. Ubiquitination of hypoxia-inducible factor requires direct binding to the β-domain of the von Hippel-Lindau protein. *Nat Cell Biol* 2: 423–427, 2000.

56. Park SK, Dadak AM, Haase VH, Fontana L, Giaccia AJ, and Johnson RS. Hypoxia-induced gene expression occurs solely through the action of hypoxia-inducible factor 1α (HIF-1α): role of cytoplasmic trapping of HIF-2α. *Mol Cell Biol* 23: 4959–4971, 2003.

57. Parsa CJ, Matsumoto A, Kim J, Riel RU, Pascal LS, Walton GB, Thompson RB, Petrofski JA, Annex BH, Stamler JS, and Koch WJ. A novel protective effect of erythropoietin in the infarcted heart. *J Clin Invest* 112: 999–1007, 2003.

58. Peng J, Zhang L, Drysdale L, and Fong GH. The transcription factor EPAS-1/hypoxia-inducible factor 2α plays an important role in vascular remodeling. *Proc Natl Acad Sci USA* 97: 8386–8391, 2000.

59. Pugh CW, O'Rourke JF, Nagao M, Gleadle JM, and Ratcliffe PJ. Activation of hypoxia-inducible factor-1; definition of regulatory domains within the α subunit. *J Biol Chem* 272: 11205–11214, 1997.

60. Ryan HE, Lo J, and Johnson RS. HIF-1α is required for solid tumor formation and embryonic vascularization. *EMBO J* 17: 3005–3015, 1998.

61. Salceda S and Caro J. Hypoxia-inducible factor 1α (HIF-1α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem* 272: 22642–22647, 1997.

62. Schipani E, Ryan HE, Didrickson S, Kobayashi T, Knight M, and Johnson RS. Hypoxia in cartilage: HIF-1α is essential for chondrocyte growth arrest and survival. *Genes Dev* 15: 2865–2876, 2001.

63. Scortegagna M, Ding K, Oktay Y, Gaur A, Thurmond F, Yan LJ, Marck BT, Matsumoto AM, Shelton JM, Richardson JA, Bennett MJ, and Garcia JA. Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1$^{-/-}$ mice. *Nat Genet* 35: 331–340, 2003.

64. Seagroves TN, Hadsell D, McManaman J, Palmer C, Liao D, McNulty W, Welm B, Wagner KU, Neville M, and Johnson RS. HIF-1α is a critical regulator of secretory differentiation and activation, but not vascular expansion, in the mouse mammary gland. *Development* 130: 1713–1724, 2003.

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

66. Semenza GL. Perspectives on oxygen sensing. *Cell* 98: 281–284, 1999.

67. Semenza GL. Surviving ischemia: adaptive responses mediated by hypoxia-inducible factor 1. *J Clin Invest* 106: 809–812, 2000.

68. Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 3: 721–732, 2003.

69. Semenza GL. *Transcription Factors and Human Disease*. New York: Oxford University Press, 2000.

70. Semenza GL and Wang GL. 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, 1992.

71. Shimoda LA, Manalo DJ, Sham JS, Semenza GL, and Sylvester JT. Partial HIF-1α deficiency impairs pulmonary arterial myocyte electrophysiological responses to hypoxia. *Am J Physiol Lung Cell Mol Physiol* 281: L202–L208, 2001.

72. Shyu KG, Wang MT, Wang BW, Chang CC, Leu JG, Kuan P, and Chang H. Intramyocardial injection of naked DNA encoding HIF-1α/VP16 hybrid to enhance angiogenesis in an acute myocardial infarction model in the rat. *Cardiovasc Res* 54: 576–583, 2002.

73. Sowter HM, Raval R, Moore J, Ratcliffe PJ, and Harris AL. Predominant role of hypoxia-inducible transcription factor (Hif)-1α versus Hif-2α in regulation of the transcriptional response to hypoxia. *Cancer Res* 63: 6130–6134, 2003.

74. Sutter CH, Laughner E, and Semenza GL. Hypoxia-inducible factor 1α protein expression is controlled by oxygen-regulated ubiquitination that is disrupted by deletions and missense mutations. *Proc Natl Acad Sci USA* 97: 4748–4753, 2000.

75. Tanimoto K, Makino Y, Pereira T, and Poellinger L. Mechanism of regulation of the hypoxia-inducible factor-1α by the von Hippel-Lindau tumor suppressor protein. *EMBO J* 19: 4298–4309, 2000.

76. Tian H, Hammer RE, Matsumoto AM, Russell DW, and McKnight SL. The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. *Genes Dev* 12: 3320–3324, 1998.

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

78. Vincent KA, Shyu KG, Luo Y, Magner M, Tio RA, Jiang C, Goldberg MA, Akita GY, Gregory RJ, and Isner JM. Angiogenesis is induced in a rabbit model of hindlimb ischemia by naked DNA encoding an HIF-1α/VP16 hybrid transcription factor. *Circulation* 102: 2255–2261, 2000.

79. Wang GL, Jiang BH, Rue EA, and Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. *Proc Natl Acad Sci USA* 92: 5510–5514, 1995.

80. Wang GL and Semenza GL. Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. *Blood* 82: 3610–3615, 1993.

81. Wang GL and Semenza GL. Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem* 270: 1230–1237, 1995.

82. Welsh SJ and Powis G. Hypoxia inducible factor as a cancer drug target. *Curr Cancer Drug Targets* 3: 391–405, 2003.

83. Willam C, Masson N, Tian YM, Mahmood SA, Wilson MI, Bicknell R, Eckardt KU, Maxwell PH, Ratcliffe PJ, and Pugh CW. Peptide blockade of HIFα degradation modulates cellular metabolism and angiogenesis. *Proc Natl Acad Sci USA* 99: 10423–10428, 2002.

84. Yeo EJ, Chun YS, Cho YS, Kim J, Lee JC, Kim MS, and Park JW. YC-1: a potential anticancer drug targeting hypoxia-inducible factor 1. *J Natl Cancer Inst* 95: 516–525, 2003.

85. Yu AY, Frid MG, Shimoda LA, Wiener CM, Stenmark K, and Semenza GL. Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. *Am J Physiol Lung Cell Mol Physiol* 275: L818–L826, 1998.

86. Yu AY, Shimoda LA, Iyer NV, Huso DL, Sun X, McWilliams R, Beaty T, Sham JS, Wiener CM, Sylvester JT, and Semenza GL. Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1α. *J Clin Invest* 103: 691–696, 1999.

87. Yu F, White SB, Zhao Q, and Lee FS. HIF-1α binding to VHL is regulated by stimulus-sensitive proline hydroxylation. *Proc Natl Acad Sci USA* 98: 9630–9635, 2001.

88. Yun Z, Maecker HL, Johnson RS, and Giaccia AJ. Inhibition of PPAR γ2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for regulation of adipogenesis by hypoxia. *Dev Cell* 2: 331–341, 2002.