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William Kaelin, Peter Ratcliffe, and Gregg Semenza receive the 2016 Albert Lasker Basic Medical Research Award

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News

The importance of adequate oxygenation has been recognized for over 200 years, but how cells and tissues are able to monitor and respond to oxygen levels remained elusive until the late twentieth century. The 2016 Albert Lasker Basic Medical Research Award honors three scientists (Figure 1), William Kaelin, Peter Ratcliffe, and Gregg Semenza, for the discovery of the molecular mechanisms by which human and animal cells sense and respond to low or inadequate oxygen levels, referred to as hypoxia. Molecular oxygen is a critical substrate for cellular metabolism and bioenergetics, and cells within each tissue require an adequate oxygen supply to meet the needs of these pathways, as either oxygen deficiency or excess can lead to rapid death of both cells and whole organisms. While organisms with just a few cells can rely on passive oxygen diffusion, large multicellular organisms require multiple complex organ systems, including respiratory, circulatory, and neuroendocrine systems, to ensure that all cells and tissues have reliable access to oxygen. In all cells and tissues, hypoxia initiates a series of physiological responses that are geared toward maintaining oxygen homeostasis over a time course of minutes to days. These events include upregulation of processes that enhance oxygen delivery, such as erythropoiesis, angiogenesis, and modulation of vascular tone, and downregulation of oxygen consumption through changes in cellular metabolism, proliferation, […]

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Early evidence of an oxygen sensor

As anyone who has traveled at high altitudes will attest, low levels of atmospheric oxygen have marked effects on physiology, ranging from relatively mild symptoms, such as breathlessness and dizziness, to severe symptoms, such as pulmonary and cerebral edema. Consequently, some of the earliest evidence for an oxygen-sensing mechanism in animal cells came from scientists visiting high-altitude locales. In 1890, François-Gilbert Viault noted that the number of erythrocytes in his blood was elevated after a stay in the Peruvian highlands (around 4,500 m above sea level). He concluded that erythropoiesis is stimulated when blood oxygen content is reduced (1). Similarly, as part of the 1910 Anglo-American expedition to Pikes Peak to investigate the effects of high altitude on breathing, Mabel Fitzgerald found that signs and symptoms of the response of humans to high altitude were set off even by small reductions in the partial pressure of oxygen (pO_2) in the arterial blood (2, 3).

Further support for an oxygen-sensing mechanism was provided by the discovery of erythropoietin (EPO), a glycoprotein hormone that stimulates erythrocyte production. In adults, EPO is produced in response to low oxygen levels in the blood by interstitial fibroblasts in the renal cortex. Human EPO was purified in 1977 (4), followed by cloning of the human *EPO* gene in 1985 (5, 6). These advances led to the development of recombinant human EPO for the treatment of anemia; however, the mechanisms underlying the regulation of EPO by oxygen levels remained enigmatic.

By the end of the 1980s, it was well established that the kidneys controlled the number of erythrocytes and thus the oxygen capacity of the blood through the release of EPO (7). Further, it was known

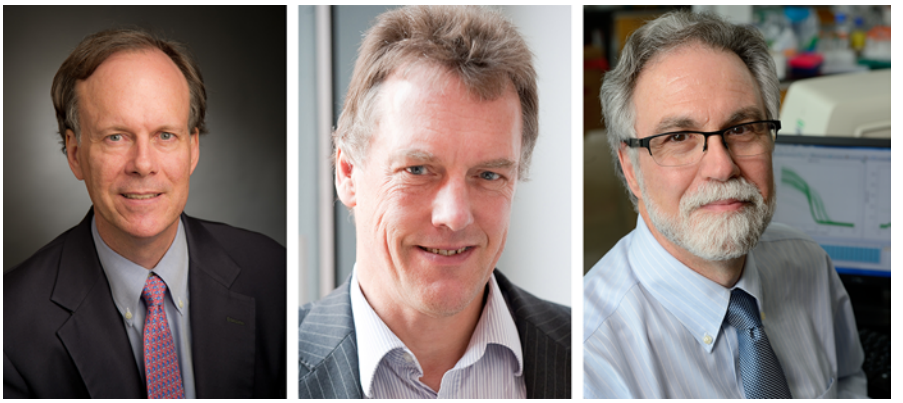

Figure 1. The recipients of the 2016 Albert Lasker Basic Medical Research Award. From left to right: William Kaelin, Peter Ratcliffe, and Gregg Semenza discovered the essential pathway by which human and animal cells sense and adapt to changes in oxygen availability. Image credits (left to right): Sam Ogden/Dana-Farber Cancer Institute, Paul Wilkinson Photography, Jay VanRensselaer/Johns Hopkins Medicine.

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that EPO production had three critical regulatory properties: 1) EPO expression is tissue specific; 2) EPO exhibits developmental stage specificity, being produced prenatally in the fetal liver, then postnatally in the kidney; and 3) EPO expression is inducible, with expression increasing in response to hypoxia, anemia, or cobalt chloride exposure (8–12). These properties attracted the attention of two researchers, Gregg Semenza and Peter Ratcliffe, who would soon demonstrate that *EPO* is not the only gene regulated by hypoxia.

A genetic hypoxia response element

In 1986, Gregg Semenza was a postdoctoral fellow in medical genetics at Johns Hopkins School of Medicine, working with research teams led by Haig Kazazian and Stylianos Antonarakis, who were renowned for their ability to dissect disease-associated mutations in order to understand how these mutations affected gene expression. "At the time, I was interested in studying developmental regulation, and it was known that EPO is expressed in the fetal liver and then in the adult kidneys. The original goal was to figure out what sequences were regulating the expression in different organs during development," Semenza recently recounted to the *JCI*. At the time, the ability to create transgenic animals was relatively new, but this technique had the advantage of allowing direct manipulation of various aspects of gene expression, making it an interesting model in which to study the regulation of *EPO*. In a collaboration with John Gearhart, Semenza engineered transgenic mice carrying human *EPO*. The transgenic mice exhibited increased erythropoiesis compared with their wild-type counterparts. (13). Semenza next began expressing *EPO* constructs that contained additional 5′ or 3′ flanking regions to demonstrate that these regions conferred tissue-specific and hypoxia-inducible expression (14, 15). Narrowing down the hypoxia-responsive region of the *EPO* gene, Semenza identified a region downstream of the EPO coding sequence that was bound by multiple nuclear factors. The addition of this region to a different gene construct conferred hypoxia-inducible expression (16).

During the same time period in which Semenza was developing *EPO*-transgenic mice, Peter Ratcliffe, a physician and kidney specialist, was establishing a laboratory in Oxford University's Nuffield Department of Medicine to study the regulation of *EPO*, a venture that was to be strongly supported by Christopher Pugh, Patrick Maxwell, and other kidney specialists training at Oxford at the time, who contributed much to the laboratory's work. As a kidney specialist, Ratcliffe was initially intrigued by the unusual counter-current circulation within the kidney that results in very low oxygen tensions within specific areas of the organ. "Somewhat surprisingly, the kidney is able to distinguish changes in blood oxygen content and the numbers of red blood cells in circulation from changes in renal blood flow. Consideration of this remarkable oxygen-sensing capacity was what initially led me into the field," said Ratcliffe. By understanding how oxygen regulated EPO production, it would be possible to get at the oxygen-sensing mechanism in the kidney. One of Ratcliffe's early experiments demonstrated that *Epo* mRNA levels in isolated rat kidneys were responsive to changes in oxygen delivery, confirming that all of the components necessary for *Epo* regulation were present in the kidney (17). At the time, there was evidence that diseased kidneys and extrarenal tissues could produce EPO, but the extent and localization of EPO production were unclear. Using very sensitive assays, Ratcliffe and colleagues showed that *Epo* mRNA was detectable not only in the kidneys and livers of rats, but also that small amounts of *Epo* mRNA became detectable in the spleen, brain, and testes under hypoxic conditions, indicating that hypoxia can induce EPO expression outside of the kidney and suggesting that the oxygen-sensing process operated more widely than had previously been considered (18, 19). In a parallel line of investigation testing hypoxia-inducible expression of truncated forms of the mouse *Epo* gene, Ratcliffe identified a region downstream of the coding sequence that was required for oxygen-regulated expression (20). By coupling this region of murine *Epo* to a broadly active promoter in cell lines derived from different tissues, Ratcliffe demonstrated that oxygen sensing was possible in many different cell types, even in those that did not produce EPO, suggesting that similar oxygen-sensing mechanisms were involved in the regulation of other genes (21–23).

The identification of a genetically encoded hypoxia response element (HRE) and the demonstration that this element could be activated in a variety of tissues opened up two new avenues of research: 1) the identification of the nuclear factors that regulate hypoxia-induced gene expression and 2) the identification of other genes that respond to hypoxia through similar mechanisms.

A hypoxia-inducible transcription factor

Semenza next focused on identifying the oxygen-regulated nuclear factors that bind *EPO*. In 1992, he and his postdoc, Guang Wang, identified the nuclear factor that bound the HRE, which they termed hypoxia-inducible factor-1 (HIF-1). Moreover, HIF-1 binding of the HRE was required for transcriptional activation and was induced by hypoxia in a variety of mammalian cell lines (24). "No matter which mammalian cell line we looked at, if we exposed the cells to low oxygen for four hours, we saw the induction of HIF activity. So that was a pretty clear indicator that HIF-1 was not just regulating the expression of *EPO*, but was regulating the expression of other genes under hypoxic conditions," said Semenza. The DNA-binding activity of HIF-1 decayed rapidly when cells were exposed to increased oxygen levels, suggesting that HIF-1 activity itself was directly regulated by oxygen (25). It rapidly became clear that HIF was not restricted to mammalians cells, as the Ratcliffe group observed a similar activity in *Drosophila* cells around the same time (26).

Purification of HIF-1 by the Semenza laboratory (27) revealed that it is a heterodimer consisting of an HIF-1α subunit and an HIF-1β subunit (also known as aryl hydrocarbon receptor nuclear translocator [ARNT] protein), both of which are basic helix-loop-helix proteins that contain a Per-Arnt-Sim (PAS) domain. While HIF-1β expression is constitutive and stable, HIF-1α protein is induced in

cells exposed to 1% oxygen and decays rapidly when the cells are returned to normal oxygen levels (21% pO_2). The genes encoding HIF-1α were assigned to murine chromosome 12 and human chromosome 14 in 1996 (28). The mRNAs encoding HIF-1α and HIF-1β were present in all human, rat, and mouse organs tested (29). A closely related gene, *HIF2A*, was identified and cloned in 1997, followed by *HIF3A* in 1998 (30–34). HIF-1α homologs were subsequently found in other organisms, including *Drosophila melanogaster* and *Caenorhabditis elegans*, indicating an evolutionary conservation of the oxygen-sensing mechanism (26, 35–37).

The identification of the HIF proteins and cloning of the genes and cDNAs allowed for an in-depth functional analysis of the HIF subunits, providing hints as to how these genes and proteins are regulated. Studies in HIF-1β–deficient cells by the Ratcliffe group demonstrated that this subunit was required for hypoxia-induced gene expression (38). Semenza's group showed that expression of a dominant-negative form of HIF-1α blocked the transcriptional response to hypoxia (39). Semenza's group also identified the stretch of amino acids required for dimerization of the HIF-1α and -1β subunits and DNA binding, as well as N-terminal and C-terminal transactivation domains in HIF-1α that interact with transcriptional coactivators such as CREB-binding protein (CBP), while both the Ratcliffe and Semenza groups identified a regulatory domain that prevents transcriptional activity in normoxic conditions (40–42). These regulatory domains would soon provide insights into the role of oxygen in the regulation of HIF activity.

Identification of hypoxia-regulated genes

Following the identification of the HRE and its operation in cell types that do not produce EPO, Ratcliffe and Semenza began hunting for other genes that exhibited hypoxia-induced expression. Both groups identified a number of genes involved in cellular metabolism, including human phosphoglycerate kinase-1 (*PGK1*), mouse lactate dehydrogenase-A (*Ldha*), mouse glucose transporter-1 (*Glut1*), human aldolase A (*ALDA*), enolase 1 (*ENO1*), and murine phosphofructokinase (*Pfkl*), which were bound and regulated by HIF-1 (43–46). Together, these studies established HIF-1 as a critical regulator of glycolysis, allowing cells to switch from aerobic to anaerobic metabolism (47).

Angiogenesis is critical for establishing a blood supply and is therefore also a means by which tissues can increase their oxygenation, making the process a likely HIF regulatory target. Ratcliffe's group showed that the expression of multiple angiogenic growth factors, including PDGFA/B, placental growth factor (PLGF), TGF-β1, and VEGF, was regulated by hypoxia in a manner similar to that seen with EPO (48). This was followed by a study from the Semenza group demonstrating that the *VEGF* gene was directly transactivated by HIF-1 (39). Further, Semenza and colleagues engineered a mouse lacking both copies of *Hif1a*, which resulted in developmental arrest and lethality at mid-gestation (E11). The HIF-1α–deficient embryos exhibited multiple malformations of the heart and blood vessels and decreased erythropoiesis (49). "Initially, the embryo is very small, and it can get all of its oxygen just from diffusion from maternal blood vessels," said Semenza. "At some point, it gets large enough that it has to have its own functioning circulatory system. When we looked at the expression of HIF-1α in the embryo, we saw that at mid-gestation, the levels of HIF-1α went up in the wild-type embryos, and that's when the mutant embryos died." These studies confirmed the role of oxygen homeostasis and HIF-1 in development. Identification of angiogenesis as a target of HIF-1 would quickly provide a link between HIF and another highly angiogenic process, tumorigenesis.

The cancer connection: von Hippel–Lindau disease

In 1993, William Kaelin, an oncologist, had just established his own laboratory after completing a postdoctoral fellowship focused on determining the functions of the retinoblastoma (RB) tumor suppressor in the laboratory of David Livingston at the Dana-Farber Cancer Institute. He was looking for a project that would be distinct from his postdoctoral work but that could potentially take advantage of some of the same experimental approaches he had used successfully to study RB. He then read a paper describing the identification of the gene that causes von Hippel–Lindau (VHL) disease (50), a familial cancer syndrome that predisposes affected individuals to hemangioblastomas in the CNS and retina, renal cell carcinoma, and pheochromocytoma, a benign tumor of the adrenal gland. From his clinical training, Kaelin knew that *VHL* mutant–associated tumors are highly vascularized (angiogenic) and are notable for their ability to secrete EPO. By the early 1990s, there was considerable interest in targeting angiogenesis to treat tumors, making VHL-associated tumors a potentially useful system for the study of angiogenesis-targeted therapeutic approaches (51). In addition, he realized that a defect in oxygen sensing could be the unifying factor underlying the induction of angiogenesis and EPO by the VHL-mutant tumors, with the tumors behaving as though they were starved of oxygen. "These tumors are constantly putting out the stress signals that would be expected under hypoxic conditions. We thought that studying the VHL protein could provide some insight into how cells respond to hypoxia," said Kaelin.

Kaelin first demonstrated that *VHL* encodes a functional tumor suppressor (pVHL) (52), as reintroduction of wild-type, but not mutant, *VHL* in a renal cell carcinoma line prevented the cells from forming tumors in mice. Further, his work, together with findings from Richard Klausner, indicated that pVHL-mediated tumor suppression required that pVHL bind two proteins, elongins B and C, originally identified as regulators of transcriptional elongation, but which are now known to wear multiple hats (53–55). The interactions between elongins B and C were disrupted by disease-associated *VHL* mutations (53, 54, 56, 57). Importantly, Kaelin showed that various hypoxia-inducible mRNAs, such as *VEGF*, *GLUT1*, and *PDGFB*, which had recently been shown to be regulated by HIF-1 (48, 58, 59), were insensitive to oxygen in renal cancer cell lines lacking pVHL and, consequently, were over-

produced (60). In short, loss of pVHL uncoupled the accumulation of hypoxia-inducible mRNAs from actual oxygen availability.

The connection between HIF and cancer was also emerging. Using hepatoma-derived tumor xenografts, the Ratcliffe group demonstrated that *VEGF* and *GLUT1* were induced in hypoxic regions surrounding areas of tumor necrosis in an HIF-1β–dependent manner. Notably, HIF-1β–deficient xenografts exhibited slower growth and reduced vascularization compared with HIF-1β–expressing xenografts (61). Around the same time, the Semenza group demonstrated that the level of HIF-1 expression was correlated with tumor growth in murine xenografts (62). Further, Semenza's group found that HIF-1α was overexpressed in multiple human cancers under both normoxic and hypoxic conditions (63), while Ratcliffe and his collaborators found that HIF-1α and HIF-2α were expressed in a number of human cancers and tumor-associated macrophages (64). Together, these studies suggested a broader role for HIFs in oncogenesis, as well as a potential connection to pVHL.

Linking VHL and HIF

Differences in HIFα regulation at the protein and mRNA levels pointed to potential regulatory mechanisms. Lorenz Poellinger and colleagues found that *HIF1A* and *HIF1B* mRNAs are constitutively expressed under both normoxic and hypoxic conditions in transformed cell lines; in contrast, HIFα protein levels are highly sensitive to changes in oxygen levels, while HIF-1β protein levels are stable (65). These findings suggested that some form of posttranslational modification was responsible for regulating HIF-1α levels. The Ratcliffe laboratory identified different domains of HIF-1α that conferred oxygen-regulated activity, distinguishing domains that altered protein levels from others that did not. They also showed that oxygen-regulated activity persisted in one domain, even when all phospho-acceptor amino acids were mutated, directing attention away from protein phosphorylation as the signal transduction mechanism (41). A study by Jaime Caro demonstrated that HIF-1α undergoes rapid ubiquitination and subsequent proteasomal degradation under normoxic conditions (66). Shortly thereafter, Frank Bunn and colleagues showed that HIF-1α contains an oxygen-dependent degradation domain (ODD) consisting of approximately 200 amino acid residues that makes the protein unstable in the presence of oxygen and allows it to be degraded via the ubiquitin/proteasome pathway (67). The Semenza group then engineered missense mutations and deletions within HIF-1α that blocked ubiquitination, resulting in constitutive expression and transcriptional activity in normoxic conditions (68).

Structural analyses of the pVHL complex provided key support for a role in HIF-1α degradation. Kaelin's group found that pVHL-mediated regulation of hypoxia-inducible mRNAs required binding of pVHL to protein complexes containing elongins B and C and cullin 2 (CUL2) (69), a protein that was suspected to be involved in targeting proteins for ubiquitin-dependent proteolysis (70). Structural studies of the pVHL-elongin C-elongin B complex by Kaelin's collaborator Nikola Pavletich revealed that it is structurally similar to SKP1-CUL1-F-box protein (SCF) E3 ubiquitin ligases (57). This structure also showed that pVHL has two hotspots for VHL disease–associated mutations: the α-domain, which Kaelin had shown is required for binding to the elongins and CUL2, and the β-domain, which was predicted to be a substrate docking site (57). Two additional observations increased the suspicion that the pVHL complex was an E3 ubiquitin ligase. First, Kaelin's collaborator Joan Conaway showed that the pVHL complex associates with ring-box 1 (RBX1), a known ubiquitin-conjugating enzyme (71). Second, two groups of researchers led by Richard Klausner and Wilhelm Krek showed that pVHL immunoprecipitated from cells displays E3 ubiquitin ligase activity (72, 73).

Soon thereafter, the Ratcliffe group demonstrated that cells lacking pVHL cannot target HIF-1α for oxygen-dependent proteolysis in vivo (74). HIFα subunits were constitutively and stably expressed in VHL-defective cells, but reexpression of wild-type pVHL restored oxygen-dependent degradation of HIFα. They also found that HIFα subunits and pVHL formed stable complexes in either normoxia or hypoxia, but that these complexes were disrupted by treatment with Co^{2+} or the iron chelator desferrioxamine. This observation initially led them to believe (as it turns out, erroneously — see below) that an iron-requiring protein was essential for complex formation and could potentially be involved in oxygen sensing through a subsequent action. Subsequently, Kaelin's group showed that the pVHL-elongin B/C-CUL2 complex binds directly to the HIF-1 ODD domain and polyubiquitinates HIF-1α (75). Similar conclusions were reached in parallel by the Ratcliffe, Poellinger, and Conaway laboratories and were published soon thereafter (76–78).

An oxygen-dependent signaling mechanism

The studies described above established that pVHL targets HIFα for ubiquitination and subsequent proteasomal degradation, but it was still unclear exactly how oxygen regulated this process. The Semenza group had shown that HIF was activated by Co^{2+} and iron chelators, as well as by hypoxia; these same factors induced the transactivation domain function of HIF (42, 79). These findings suggested the existence of an oxygen- and iron-dependent modification governing the interaction of the HIFα ODD with pVHL. In April 2001, the Kaelin and Ratcliffe groups published independent back-to-back studies identifying proline hydroxylation as the crucial oxygen- and iron-dependent posttranslational modification of HIFα that was required for recognition by the pVHL complex (80, 81); the same mechanism was identified by Frank Lee's group a few months later (82). Ratcliffe had initially been surprised when, as described above, they could isolate the HIFα-pVHL complex from hypoxic cells, and postulated that reoxygenation of cell lysates during the experimental procedure might promote this interaction. "We made a slight mistake at the time, which is an interesting one," said Ratcliffe. "We initially thought that the association between VHL and HIF was regulated by cobalt and iron chelators, which matched the properties of the system, but not by oxygen. We were extremely puzzled by that, as normally those properties were all

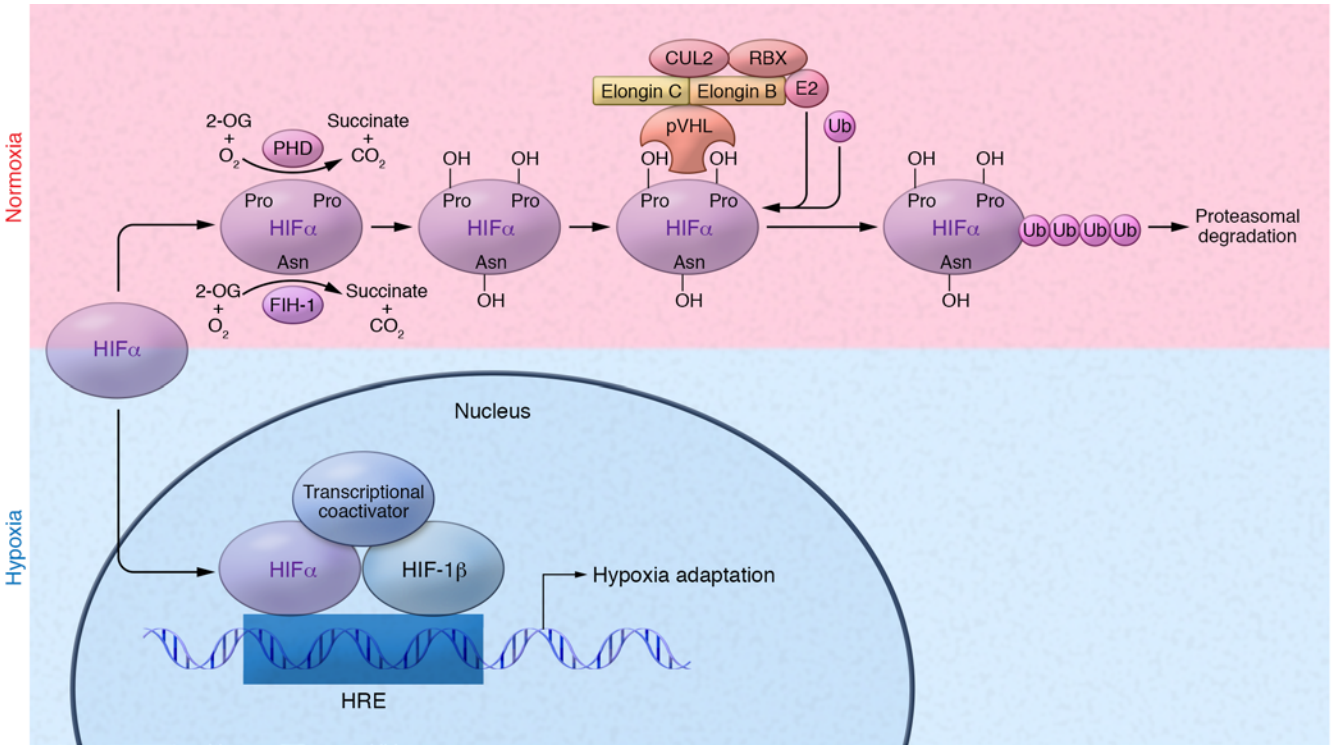

Figure 2. Regulation of hypoxia-inducible factors. Under normoxic conditions, two proline residues on the HIFα subunit are hydroxylated (OH) by PHD enzymes (PHD1, -2, and -3), in the presence of O_2, Fe^{2+}, 2-OG, and ascorbate (not shown). Hydroxylated HIFα is recognized by the pVHL E3 ubiquitin ligase complex, which tags HIFα with polyubiquitin, allowing for proteasomal recognition and subsequent degradation. Additionally, the 2-OG dioxygenase FIH-1 hydroxylates an asparagine residue in the C-terminal transactivation domain of HIFα, preventing its interaction with transcriptional coactivators. Under hypoxic conditions, HIFα prolyl hydroxylation is inhibited, preventing recognition of HIFα by pVHL. HIFα can then accumulate and translocate to the nucleus, where it dimerizes with HIF-1β. The HIF dimer binds to HREs within the promoters of target genes and recruits transcriptional coactivators such as CBP to induce transcription. Asn, asparagine; E2, ubiquitin-conjugating enzyme; Pro, proline; Ub, ubiquitin.

concordant. On reflection, we realized that oxygen would be in the buffers and all the reagents that we used to do the work on the bench. So it was possible that although the cells were made hypoxic, by the time we'd gotten the extracts, oxygen had gotten in, which turned out to be correct."

By repeating these experiments in a hypoxia workstation with deoxygenated buffers, two highly skilled postdocs in the Ratcliffe laboratory, Panu Jaakkola and David Mole, showed that the association between HIFα subunits and pVHL was governed by both oxygen and iron availability. Kaelin had reached the same conclusion by multiple means, including experiments with mouse cells expressing a temperature-sensitive mutant of the E1 ubiquitin–activating enzyme that, when grown at nonpermissive temperatures, accumulated HIFα under both normoxic and hypoxic conditions. Using so-called "far Western blots," his group showed that pVHL could bind directly to HIFα unless the HIFα was derived from cells exposed to hypoxia or iron chelators. Additionally, both groups demonstrated that a cellular factor was required for the posttranslational modification of HIFα, as pVHL only recognized recombinant HIF after incubation with vertebrate cell lysates. A systematic mutation analysis of the minimal pVHL-binding domain of HIFα zeroed in on a proline residue, Pro^{564}, which was conserved in human, *Xenopus*, *Drosophila*, and *C. elegans* HIFα proteins. Mass spectrometric analysis confirmed that Pro^{564} was hydroxylated. Importantly, a hydroxyproline substitution at Pro^{564} promoted the interaction of HIF-1α with pVHL. Follow-up studies by the Kaelin and Ratcliffe groups showed that the hydroxyproline inserts into a gap within the pVHL hydrophobic core within a site that is a hotspot for tumorigenic mutations (83, 84). A second prolyl hydroxylation site, Pro^{402}, which mediates the interaction of HIFα with pVHL, was identified by the Ratcliffe group a few months later (85). These data revealed that prolyl-4-hydroxylase activity was necessary to promote the interaction between HIFα and pVHL.

The well-studied prolyl hydroxylases at that time, which modify collagen, required Fe^{2+}, ascorbate, molecular oxygen, and 2-oxoglutarate (2-OG, also known as α-ketoglutarate) (86). These enzymes split molecular oxygen, using one atom to add a hydroxyl group to a target protein and the other to react with 2-OG, resulting in the generation of succinate and CO_2. Ratcliffe and colleagues determined that the prolyl hydroxylase activity mediating HIFα hydroxylation also required these cofactors and that HIF was induced by the 2-OG analog dimethyloxalylglycine (DMOG). Through a combination of structurally informed prediction and candidate test-

ing, Ratcliffe and his collaborator Christopher Schofield identified the dioxygenase EGL-9 as the enzyme responsible for hydroxylating the HIFα ortholog in *C. elegans*. Further, they identified a set of mammalian HIF prolyl hydroxylases (PHD1, -2, and -3; also known as EglN2, EglN1, and EglN3, respectively) that mediate hydroxylation of human HIFα. Moreover, the activity of these enzymes was modulated by graded hypoxia, iron chelation, and Co^2, as well as the 2-OG analog DMOG, mirroring the in vivo characteristics of HIF regulation (87). Rick Bruick and Steve McKnight identified these same three enzymes, after similar studies that began with the *Drosophila* ortholog (88).

Prolyl hydroxylation has a profound effect on HIF stability and activity, making the PHDs potential targets for pharmacological mimicking of the effects of hypoxia. The Kaelin group had also been working to identify HIFα PHDs and, using biochemical approaches, identified PHD2, which has emerged as the workhorse member of the family (89). In collaboration with Joan and Ron Conaway, Kaelin's group purified PHD2 and demonstrated that some small molecules designed to inhibit the related collagen prolyl hydroxylases, including selected iron chelators and 2-OG antagonists, also inhibited the activity of PHD2 (89). Moreover, treatment of cultured cells with these inhibitors stabilized HIF-1α, increased expression of VEGF (89), and induced EPO in mice, including mice made anemic by partial nephrectomy (90). In collaboration with Christopher Schofield, the Ratcliffe group also developed and tested different analogs of the PHD cofactor 2-OG, which stabilized HIFα (91). These studies established PHDs as bona fide pharmacological targets for HIF regulation. Given the growing role of HIF signaling in human disease, many such inhibitors have been developed in the past decade (92). Notably, Josef Prchal, Frank Lee, and others have linked genetic variants of PHD2, HIF2α, and VHL to familial polycythemia and high-altitude adaptation. Therefore, there is genetic validation in humans as well as in multiple model organisms that ties prolyl hydroxylation to oxygen sensing (93–95).

A second oxygen-mediated HIF regulatory pathway

By 2001, it was clear that HIF signaling can be induced in any cell type under hypoxic conditions and that it plays a critical role in the response to hypoxia through transcriptional activation of genes encoding proteins that either increase oxygen availability or mediate adaptive responses to intracellular oxygen deprivation. Upon the return of normal oxygen levels, oxygen-dependent binding of pVHL mediates the destruction of the HIFα subunit, terminating the response to hypoxia. Additionally, the C-terminal domains of HIFα subunits were known to contain regulatory domains (41, 42), which bind transcriptional coactivators, including CBP, p300, steroid receptor coactivator-1 (SRC1), and transcriptional intermediary factor-1 (TIF1) (96–98). Ratcliffe, Semenza, and others had shown that the C-terminal transactivation domains were regulated by oxygen, but this regulation influenced transcriptional activity independently of protein stability (41, 42).

In order to understand the regulation of the transactivation domains, the Semenza group conducted a yeast two-hybrid screen to identify proteins that interact with HIF-1 to modulate its biological activity. They identified and characterized a protein that they named factor inhibiting HIF-1 (FIH-1), which negatively regulates the function of the HIF C-terminal transactivation domain (99). They also found that FIH-1 binds to pVHL and that pVHL can function as a transcriptional corepressor that inhibits its HIF-1α transactivation function by recruiting histone deacetylases, thereby closing down the chromatin.

In 2002, Murray Whitelaw and colleagues showed that an asparagine residue in the C-terminal transactivation domain of HIF was hydroxylated under normoxia, but that the modification was not present under hypoxia. Asparagine hydroxylation was also prevented by iron chelators or inhibitors of 2-OG–dependent dioxygenases (100). Shortly thereafter, both the collaborating Schofield and Ratcliffe laboratories and the Whitelaw group showed that FIH-1 is an iron- and 2-OG–dependent dioxygenase that hydroxylates HIF-1α on an asparagine residue, thereby inactivating one of its two transactivation domains (101–103).

The identification of the prolyl and asparginyl hydroxylation events revealed a dual regulatory system of HIF activity (Figure 2) involving two oxygen-dependent hydroxylases: a PHD (most commonly PHD2) and FIH-1 (104). Both HIFα and HIFβ are constitutively produced, but HIFα is only stable and active under hypoxic conditions (around 1% pO_2). As oxygen levels rise, FIH-1, which has activity at lower oxygen levels than does PHD2 (105, 106), becomes active and hydroxylates an asparagine residue in the C-terminal transactivation domain of HIFα, preventing interaction with transcriptional coactivators and thereby partially abrogating HIFα transcriptional activity (as FIH-1 does not inhibit the N-terminal transactivation domain). As oxygen levels continue to increase, PHD2 becomes active and hydroxylates one (or both) of two prolines on HIFα. Hydroxylation of either proline promotes the interaction of HIFα with the pVHL E3 ubiquitin ligase complex (107), resulting in ubiquitination and subsequent proteasomal degradation. Thus, these hydroxylation events allow cells to tightly control responses to alterations in oxygen levels, only allowing for HIF accumulation and transcriptional activity under the appropriate environmental conditions (108).

An expanding role for HIF in physiology and disease

The discovery of the HIF pathway not only unveiled a new signaling mechanism mediated by oxygen, but also demonstrated that every cell in the body is capable of sensing and responding to oxygen levels. Kaelin, Ratcliffe, and Semenza have all worked to identify new roles for the pathway and to delineate the mechanisms by which HIF signaling is regulated in a given context. At the cellular level, HIF signaling has a profound effect on metabolism, allowing cells to switch from the oxygen-consuming TCA cycle to glycolysis (109). Additionally, HIF signaling contributes to cell fate decisions, including differentiation, senescence, and apoptosis (110–115). At the tissue level, HIF signaling is involved in the devel-

opment and maintenance of numerous organs and tissues, including those in the cardiovascular, skeletal, and immune systems (116–118). Further, HIF has been shown to play a critical role in mucosal barrier functions and inflammation (119, 120). "It really has become a situation where I assume that HIF is involved in a given process until proven otherwise, because in so many contexts this pathway is important," said Semenza.

HIF-signaling pathways have been implicated in a variety of diseases states, with HIF playing a beneficial or a detrimental role, depending on the context. HIF signaling has been shown to mediate protective responses in diseases characterized by impaired tissue oxygenation and inflammation, such as coronary artery disease (CAD) (121–124), peripheral arterial disease (PAD) (125–127), wound healing (128–130), organ transplant rejection (131, 132), and colitis (133–135). In contrast, HIF signaling might be maladaptive in other disease states, including hereditary erythrocytosis (136), pulmonary arterial hypertension (137–139), chronic ischemic cardiomyopathy (140, 141), and obstructive sleep apnea (142, 143).

HIF signaling plays complex role in cancer (144–146). Hypoxia and expression of HIF in tumors are associated with poor prognosis and have been shown to promote tumor angiogenesis, epithelial-to-mesenchymal transition, stem cell maintenance, invasion and metastasis, therapy resistance, and induction of metabolic alterations (147–149). The role of HIF in cancer has best been illustrated in the context of pVHL-defective kidney cancers. In this setting, HIF-2, rather than its better-studied cousin HIF-1, appears to the be the main culprit (150–157). The identification of HIF-2 as a driving force in kidney cancer helped to motivate and accelerate the successful development of drugs that inhibit the HIF-responsive growth factor VEGF for the treatment of this disease (145). Delineation of the pathways and factors that interact with HIF in a specific disease context has and will continue to help identify therapeutic strategies centered on HIF signaling.

Given its role in so many different disease states, a number of therapies targeting the HIF-signaling pathway are under development, with several therapeutic modalities advancing to late-stage clinical trials. Since PHD inhibitors stabilize HIFα, they cause increased HIF signaling. Such drugs could potentially be used in disease states such as anemia, in which HIF signaling is beneficial, or to protect against ischemic injury in CAD and PAD (158). Conversely, in diseases in which HIF signaling is detrimental, HIFα inhibitors may be beneficial. For example, a direct HIF inhibitor is currently in phase II clinical trials for kidney cancer (159), while drugs that indirectly downregulate HIF-1α, including the cardiac glycoside digoxin (160), are currently in clinical trials for various forms of cancer.

Coda

Each of these researchers approached this essential biological question in a slightly different way, based on their area of specialty: Kaelin from oncology, Ratcliffe from nephrology, and Semenza from medical genetics. Since their initial discoveries, they have all continued to examine the mechanisms by which oxygen sensing impacts human physiology and disease. William Kaelin is currently a professor in the Department of Medicine and associate director of Basic Science at the Dana-Farber Institute, Harvard Medical School, where he and his team study the function of specific tumor-suppressor genes, including *VHL*. Peter Ratcliffe is the clinical research director at the Francis Crick Institute, director of the Target Discovery Institute and the Oxford Hypoxia Biology Laboratory at the University of Oxford, and a member of the Ludwig Institute for Cancer Research. His current research explores mechanisms of oxygen sensing mediated by 2-OG oxygenases, as well as the oncogenic mechanisms mediated by HIF signaling. Gregg Semenza is the C. Michael Armstrong Professor of Genetic Medicine and director of the vascular program at the Institute for Cell Engineering at the Johns Hopkins University School of Medicine, where his research group investigates oxygen homeostasis and HIF signaling in ischemic cardiovascular disease and cancer.

As Kaelin told the *JCI*, "in order to make big advances in science, you need to ask big questions. When you say the question out loud, it should sound a little audacious." These three physician-scientists became interested in pursuing one of the most audacious of questions: what are the mechanisms that underlie oxygen sensing in animals? The answer to that question has expanded our understanding of many different aspects of biology, ranging from metazoan evolution to cancer biology, and underscores the importance of research focused on the most basic of scientific questions.

Jillian H. Hurst

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