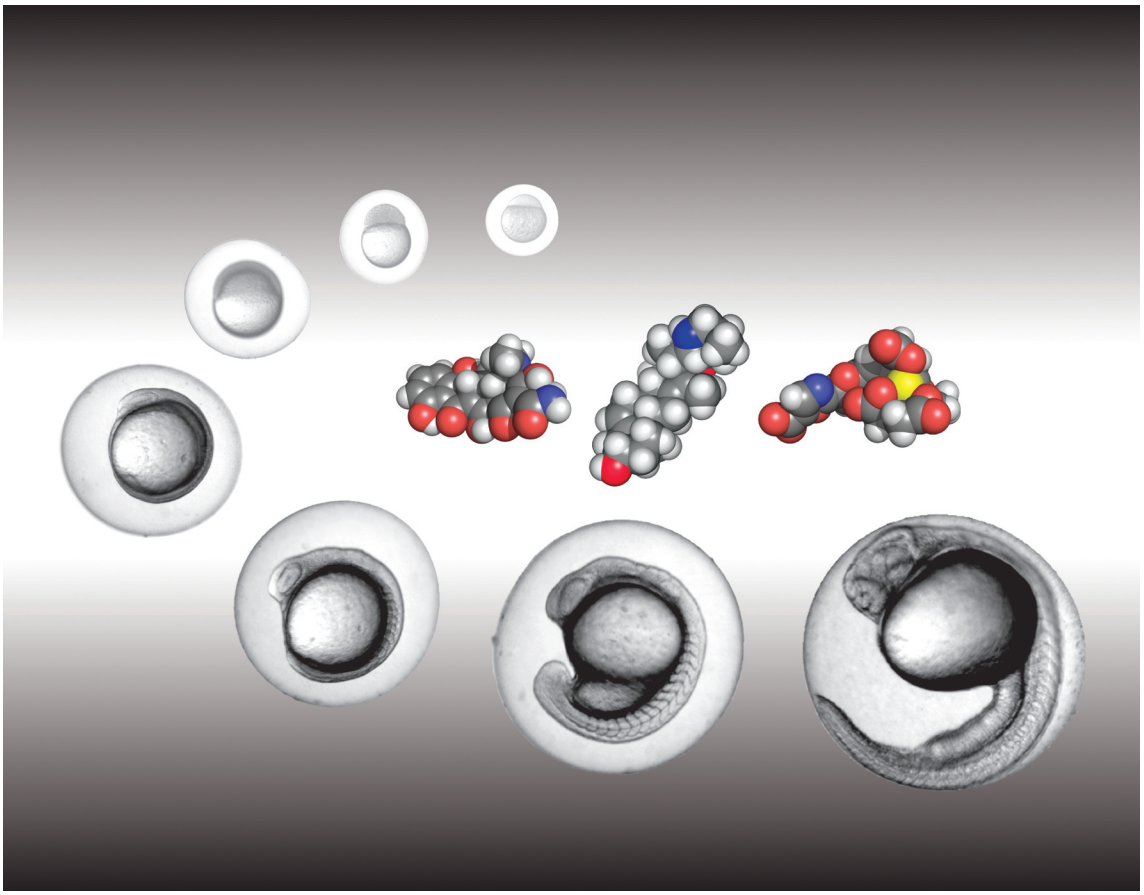
Chem Soc Rev

This article was published as part of the

2008 Chemistry–Biology Interface Issue

Reviewing research at the interface where chemistry meets biology

Please take a look at the full table of contents to access the other papers in this issue

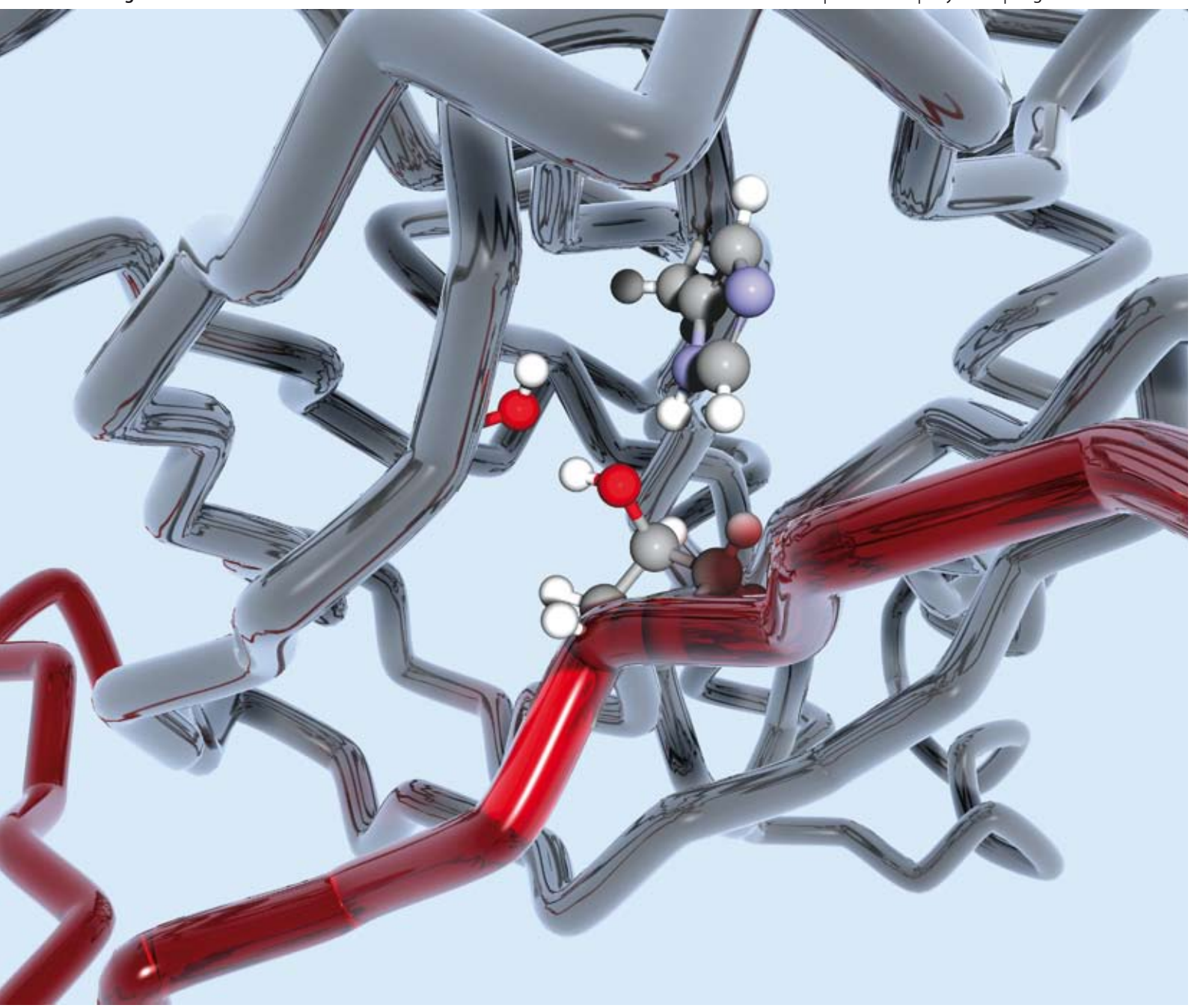


Chem Soc Rev

Chemical Society Reviews

www.rsc.org/chemsocrev

Volume 37 | Number 7 | July 2008 | Pages 1281–1452



ISSN 0306-0012

RSC Publishing

The human oxygen sensing machinery and its manipulation†

Rasheduzzaman Chowdhury, Adam Hardy and Christopher J. Schofield*

Received 1st April 2008
First published as an Advance Article on the web 27th May 2008
DOI: 10.1039/b701676j

Animals respond to the challenge of limited oxygen availability by a coordinated response that works to increase oxygen supply and minimize tissue damage. The chronic hypoxic response is mediated by the α,β-hypoxia inducible transcription factor (HIF) that enables the expression of a gene array. Because this array includes genes encoding for proteins that regulate processes including red blood cell and blood vessel formation, manipulation of the HIF system has potential for the treatment of ischemic diseases, anaemia and tumours. Hydroxylase enzymes act as oxygen sensors by regulating both the lifetime of HIF-α and its transcriptional activity. This *tutorial review* aims to provide a non-expert introduction to the HIF field by providing a background to current work, summarising molecular knowledge on the HIF system, and outlining opportunities for therapeutic intervention.

1. Physiological background

The mechanism by which animals adapt to conditions of limiting oxygen has been a long-standing physiological problem. More than a century ago, physiologists observed that limited oxygen availability, such as at high altitude, resulted in an increase in red blood cells. Evidence that the effect was mediated *via* an effector molecule, later identified as erythropoietin (EPO), came from animal work in which serum from anaemic rabbits was shown to elicit erythropoiesis after injection into normal rabbits. These fundamental observations led ultimately to the purification of EPO and cloning of the *EPO* gene. Recombinant EPO is now very widely used for the treatment of anaemia. Although EPO is normally produced continuously in order to ensure renewal of red blood cells, its rate of production is increased when oxygen becomes limiting, including at altitude, in tumours, and with CO poisoning.[1]

The Chemistry Research Laboratory and the Oxford Centre for Integrative Systems Biology, Mansfield Road, Oxford, United Kingdom OX1 3TA.
E-mail: Christopher.schofield@chemistry.oxford.ac.uk;
Fax: 44 (0) 1865 275 674; Tel: 44 (0) 1865 275 625
† Part of a thematic issue examining the interface of chemistry with biology.

2. DNA binding and transcriptional activation by HIF

The expression of eukaryotic genes, including *EPO*, is regulated by multiple transcription factors (TFs) some of which may regulate transcription in response to external stimuli. TFs are modular in structure and contain DNA binding and trans-activating domains. The trans-activating domains enable binding of the TF to other proteins involved in transcription or its regulation. Some TFs, or TF complexes, contain sensing domains that regulate the activity or level of the transcription factor, sometimes in response to binding of a small molecule. Here we discuss aspects of transcriptional regulation that are hypoxia and EPO associated.

Studies with transgenic mice demonstrated that there were sequences on both the 3′ and 5′ sides of the *EPO* gene that regulated its expression, some in a hypoxia dependent manner. Liver cell lines that expressed EPO in an oxygen dependent manner were then identified and used to narrow down sequences that were responsible for the hypoxic regulation of EPO. A sequence (to the 3′-side) that enhanced EPO expression under hypoxic conditions (up to 1000 fold) termed the hypoxia response element (HRE) was identified along with other sequences that are important for hypoxic regulation

Rasheduzzaman Chowdhury

Rasheduzzaman Chowdhury received baccalaureate and master degrees from the University of Dhaka (Bangladesh). After working there as a lecturer, he started doctoral research in Chemical Biology at the University of Oxford with funding from a Commonwealth Scholarship. He is currently working on structural and mechanistic studies on oxygen sensing enzymes.

Adam Hardy studied chemistry at the University of Oxford, where he obtained his M.Chem in 2003. He is currently finishing his D.Phil on substrates and enzymes involved in the hypoxic response at the University of Oxford under the supervision of Chris Schofield.

Chris Schofield works at the Chemistry Research Laboratory and Hertford College in Oxford University. Research in his group concerns attempts to contribute to the development of a chemical understanding of biology. Current fields of interest include the hypoxic response, antibiotic biosynthesis and mechanism of action, and transcriptional regulation.

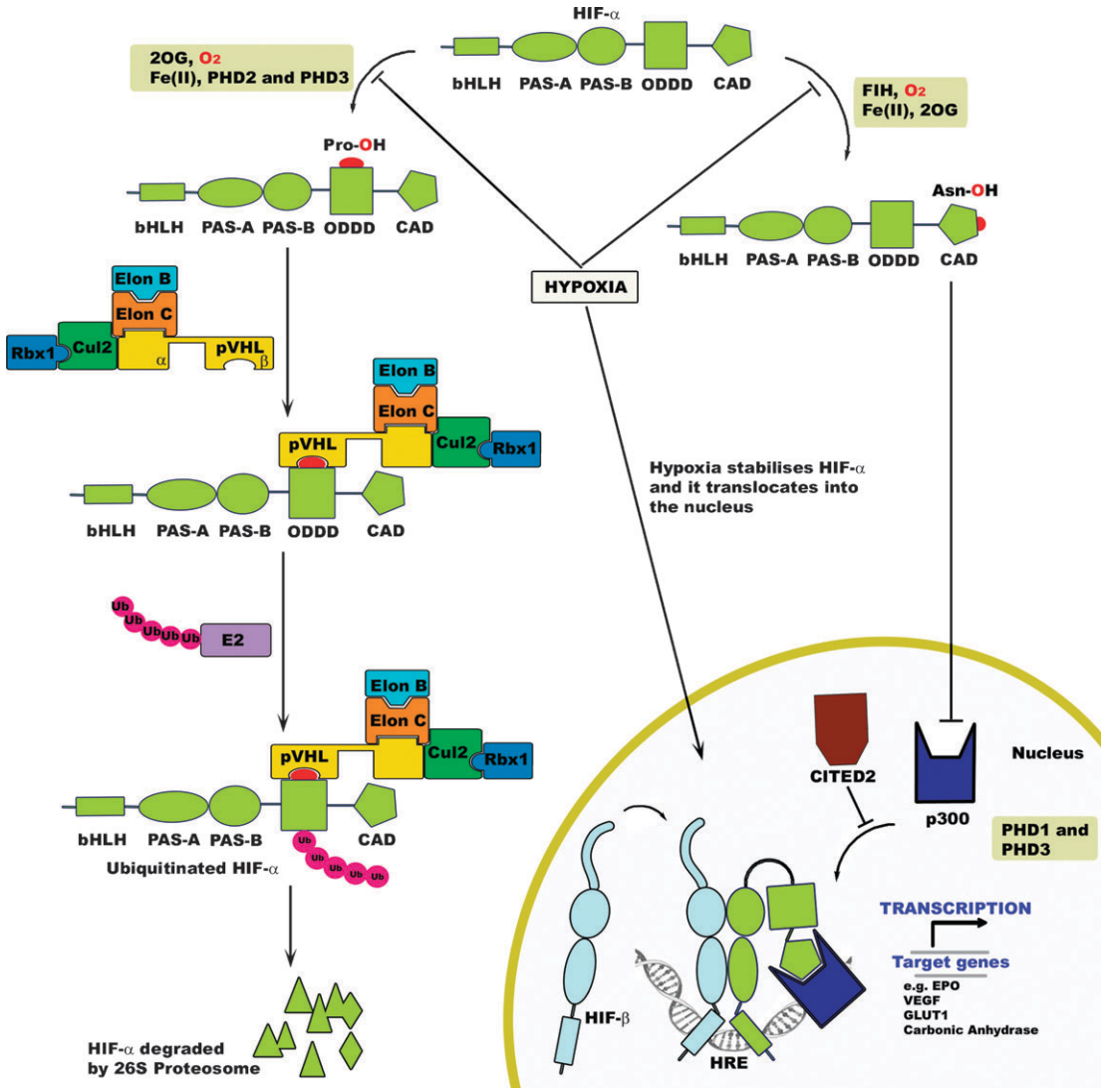


Fig. 1 Simplified overview of the HIF system including reactions of the HIF hydroxylases. Under normoxic conditions, PHDs and FIH downregulate and inactivate HIF-α subunits. In hypoxia, the activity of the HIF hydroxylases is reduced so enabling HIF-α to escape PHD and FIH mediated control. HIF-α translocates to the nucleus, dimerises with HIF-β *via* PAS domains leading to a transcriptionally active complex. The functional heterodimeric HIF then binds to hypoxia response elements (HREs) in the regulatory regions of target genes using bHLH regions of both subunits. PAS = PER ARNT SIM (see text), bHLH = basic helix1 loop helix2, ODDD = oxygen dependent degradation domain, CAD = transactivation domain, Elon B = elongin B, Elon C = elongin C, Rbx1 = ring-box 1, Cul2 = cullin 2, Ub = ubiquitin, E2 = E2 ligase, CITED2 = CBP/p300-interacting transactivator with ED-rich carboxy-terminal domain 2.

(Fig. 1).[2] The core conserved region within the HRE is a pentanucleotide sequence 3′-(A/G)CGTG-5′ that is repeated with some genes. DNA affinity chromatography led to the characterisation of hypoxia inducible factor (HIF), as a heterodimeric α,β-TF. The overall DNA binding and dimerisation domains of HIF-α and -β are not unique, being observed in other TFs. The oxygen dependency of HIF activity relies on post-translational modifications to HIF-α that occur in a central oxygen dependent degradation domain (ODD) and a C-terminal trans-activation domain (CAD). HIF-β is not known to be directly regulated by oxygen. Hypoxic regulation by the HIF system is not limited to *EPO* but involves an array of human genes revealing a complex and coordinated response to chronic hypoxia (Fig. 2) (for reviews see ref. 3–6).

Both HIF-α and -β subunits are basic helix–loop–helix (bHLH) and PAS [PER (period circadian protein), ARNT (aryl hydrocarbon receptor nuclear translocator), SIM (single-minded protein)] domain containing TF proteins (Fig. 3). The basic sequences in bHLH-PAS proteins bind to DNA and the helix–loop–helix domain enables dimerisation of the HIF-α and -β subunits (Fig. 4). PAS domains are also involved in HIF-α/β dimerisation (Fig. 5). Nuclear localisation sequences target HIF-α to the nucleus.[3–6]

The tertiary structures of sections of HIF-α have been investigated (Fig. 4 and 5). An NMR structure of the HIF-2α PAS-B domain (residues 240–350) reveals a fold comprising several α-helices flanked by a five stranded anti-parallel β-sheet (Fig. 5a).[7] Titration experiments showed that the HIF-2α PAS-B domain interacts with the HIF-β PAS domain but

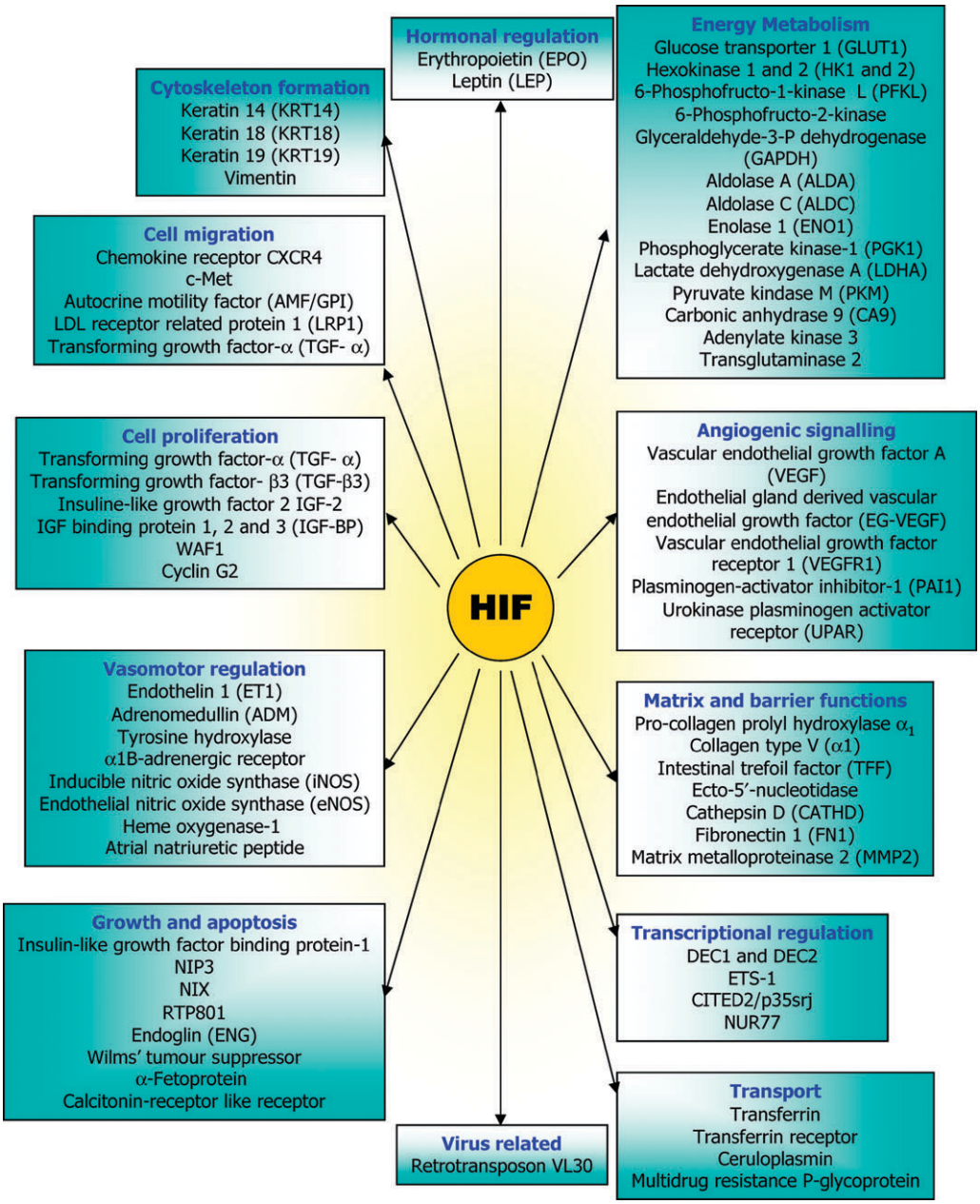


Fig. 2 HIF target genes. The complete number of genes transcriptionally activated by HIF may exceed 200 as demonstrated by microarray analyses. c-Met, tyrosine-kinase product of the *met* proto-oncogene; WAF1, protein implicated in p53 transcriptional regulation; Nip3, novel immunogenic protein 3, and NIX, Bnip3-like protein X, are members of the Bcl2 family of cell-death factors; DEC1 and DEC2, differentiated embryo chondrocyte 1 and 2; ETS is a DNA-binding domain that defines a family of transcription factors; p35srj, 35 kDa serine/glycine-rich junction; NUR77, orphan nuclear receptor; CITED2, CBP/p300-interacting transactivator with ED-rich carboxy-terminal domain 2. Figure updated from ref. 5 and 6.

not with the PAS domain from PAS kinase. These observations suggest that the HIF-2α PAS-B domain is folded in such a way as to interact specifically with the HIF-β PAS domain and not PAS domains from other proteins. HIF-1α/2α PAS-B have sequence identity of ~75% (Fig. 5); homology modelling and mutational studies imply similar folding of PAS-B in the two HIF-α isoforms.[7]

HIF-β is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) and is a ubiquitous nuclear protein that is involved in the transcription of genes other than those involved in the hypoxic response. Dimerisation of HIF-β/ARNT with the aryl hydrocarbon receptor forms a complex that senses the dioxin pollutants. Detailed information on the interaction of the HIF heterodimer and the HRE is not yet available; studies on the interactions between other bHLH proteins and response elements reveal that a conserved His-Glu-Arg triad binds to the major groove of the DNA (Fig. 4). HIF-β likely binds in a similar way though the triad appears not to be conserved in HIF-α. Other factors are involved in the regulation of

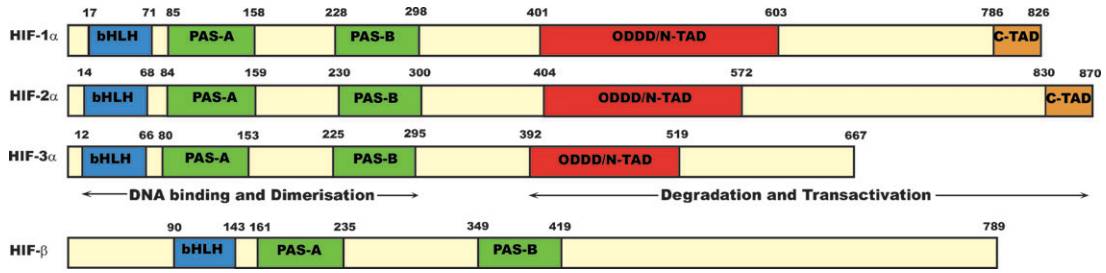


Fig. 3 Domain architectures of human HIF-1α, -2α, -3α and HIF-β. The bHLH and PAS domains are involved in DNA binding and dimerisation whilst ODDD and transactivation domains (N/C-TAD), in degradation and recruitment of transcriptional coactivators.

HIF target gene transcription. Methylation at the cytosine-5-position of the conserved 3′-(A/G)CGTG-5′ sequence blocks HIF binding providing one possible mechanism for cell-type specificity.[8] Co-transcriptional activator proteins are necessary for the expression of HIF target genes. One important interaction in the hypoxic response involves binding of the p300/CBP (CREB binding protein) transcriptional coactivators to the HIF-α CAD.

3. How HIF activity is regulated by oxygen—the HIF hydroxylases

Under conditions of normal oxygen supply (normoxia) only very low levels of HIF-α, if any, can be detected by western blotting (antibody staining). HIF-1/2α (~100 kDa proteins) contain a large central regulatory region of ~200 residues that regulates their oxygen dependent degradation. Within this oxygen dependent degradation domain (ODDD) there are two sites for oxygen dependent prolyl hydroxylation (Pro402 in the N-terminal ODDD, NODDD; Pro564 in the C-terminal ODDD of human HIF-1α, CODDD)[9,10] (for reviews see ref. 3 and 4). Both of these sites contain an LXXLAP motif that is highly conserved in other metazoans. Hydroxylation at the *trans*-4-position of either NODDD or CODDD proline residues is sufficient to target HIF-α to a ubiquitin ligase (an E3 type, a multi-protein complex with five subunits, pVHL, elongins B and C, Cul2 and Rbx1) that results in attachment of ubiquitin protein molecules.[11] The oxygen in the hydroxy-group was shown to arise from molecular oxygen providing a direct link between oxygen availability and HIF-1α stability.[12] As for other proteins labelled with ubiquitin, ubiquitinylation targets HIF-α to the proteasome (a large protease in the cytoplasm that degrades proteins labelled with ubiquitin) where it is hydrolysed into peptides. Under hypoxic conditions, the rate of prolyl hydroxylation of the NODDD and CODDD is slowed, HIF-α can avoid hydroxylation and can translocate to the nucleus where it is able to dimerise with HIF-β and enable the hypoxic response.

The targeting component of the ligase that joins ubiquitin to HIF-α is the von Hippel–Lindau protein elongin B/C complex

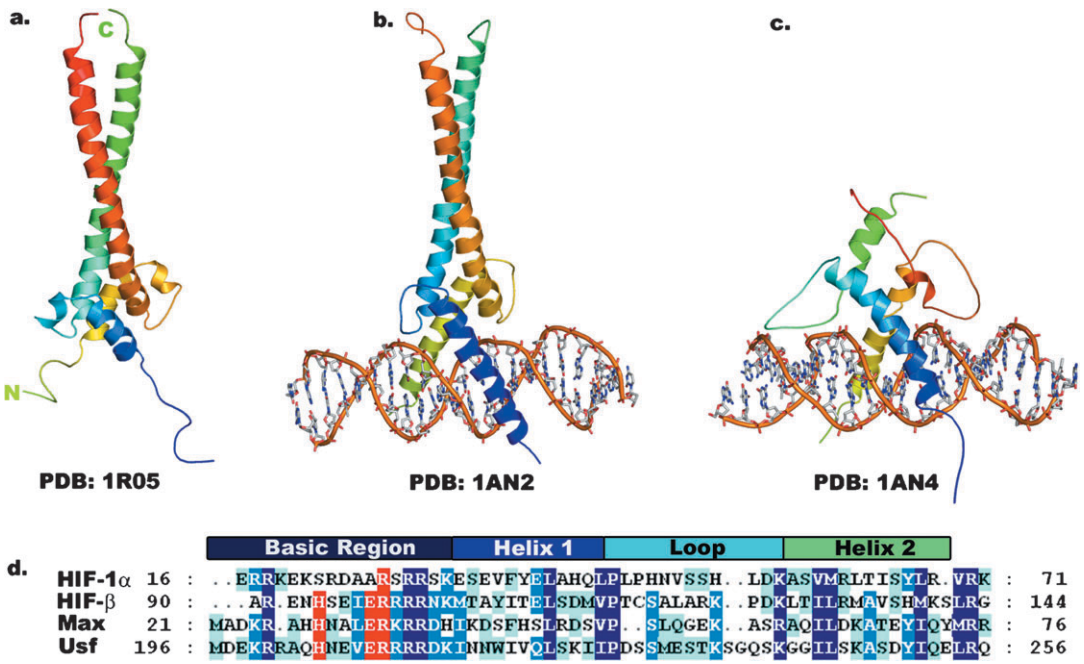


Fig. 4 Cartoon showing binding of the homodimeric Max (MYC-associated protein X) and USF (upstream stimulatory factor) transcription factors to the same recognition sequence (CACGTG) suggesting how a functional HIF complex could bind to the hypoxic response element. (a) View from an NMR structure of the bHLH of Max (PDB: 1RO5),[58] (b) view from a crystal structure of Max in complex with target gene promoter E-Box (5′-CACGTG-3′) DNA sequences (PDB: 1AN2), (c) view from a crystal structure of USF bHLH in complex with DNA (PDB: 1AN4).[59]

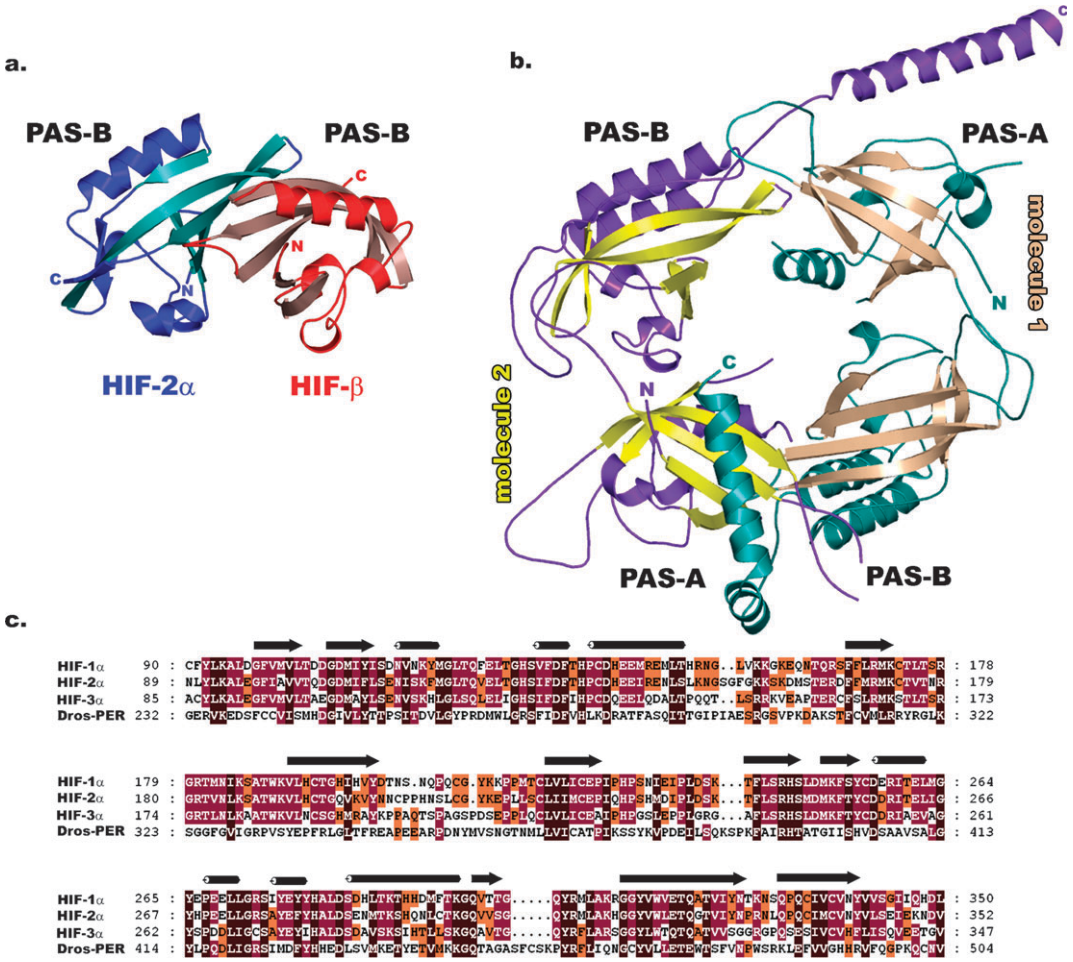


Fig. 5 PAS domains of HIF-α and HIF-β. (a) View from an NMR structure of the heterodimeric complex of HIF-2α PAS-B (residues 240–350) and HIF-β PAS-B (residues 356–470) (PDB: 2A24).[7] (b) View from a crystal structure of the PAS domain of *Drosophila* clock protein PERIOD (PDB: 1WA9).[60] (c) Sequence alignment of PAS domains of HIF-α isoforms and *Drosophila* PERIOD circadian protein. Secondary structures for PAS-A were assigned on the basis of PAS-A of the *Drosophila* PERIOD and those of PAS-B were assigned on the basis of PAS-B of *Drosophila* PERIOD/HIF-2α structures.

(VCB).[11] Prior to the observation of HIF-α prolyl hydroxylation, the oxygen dependent nature of complex formation between HIF-α and VCB had been an important step in identifying the oxygen sensing mechanism. *In vitro* studies have shown that 4-prolyl hydroxylated CODDD peptides bind ~1000 fold more tightly to the VCB complex than non-hydroxylated CODDD. X-Ray diffraction analyses have revealed that the post-translationally introduced alcohol is positioned to bind *via* hydrogen-bonds to the side chains of Ser111 and His115 of the von Hippel–Lindau protein in VCB (Fig. 6).[13] In the absence of the VCB template, NMR studies reveal this region of HIF-α as disordered. The hydroxylated CODDD prolyl residue has a C^4 *exo*-conformation in the VCB complex as observed in the collagen triple-helix fold.

Oxygen dependent post-translational hydroxylation of HIF-α also occurs within the CAD region.[14] Hydroxylation at the β-position of Asn803 (HIF-1α CAD) significantly decreases binding of HIF-α to the cysteine/histidine rich (CH-1) domain of the transcriptional co-activator complex CBP/p300 so disabling HIF mediated transcription. In the HIF-α–CBP/p300 complex,[15,16] HIF-1α Asn803 is part of an α-helix; hydroxylation is proposed to disrupt the hydrophobic binding interactions between HIF-α and CH-1 (Fig. 6).

In summary, two types of oxygen dependent post-translational hydroxylations of HIF-α have been identified. Prolyl-hydroxylation 'makes' a protein–protein interaction that signals for HIF degradation. Asparaginyl hydroxylation 'breaks' a protein–protein interaction so disabling HIF mediated transcription. Both the HIF-α prolyl and asparaginyl hydroxylations are catalysed by oxygenases that employ Fe(II) as a cofactor and 2-oxoglutarate (2OG) and oxygen as cosubstrates. The 2OG oxygenases couple the two electron oxidation of 2OG into succinate and carbon dioxide to the two electron oxidation of a substrate (Fig. 7). Substrate oxidation is carried out by an Fe(IV)=O intermediate[17] (reviewed in ref. 18). To date in humans the only type of substrate oxidation identified has been hydroxylation. In microorganisms and plants, the 2OG oxygenases catalyse a range of oxidative transformations, including desaturations and oxidative ring closures, in the secondary metabolism of, for example, antibiotics and flavonoids.

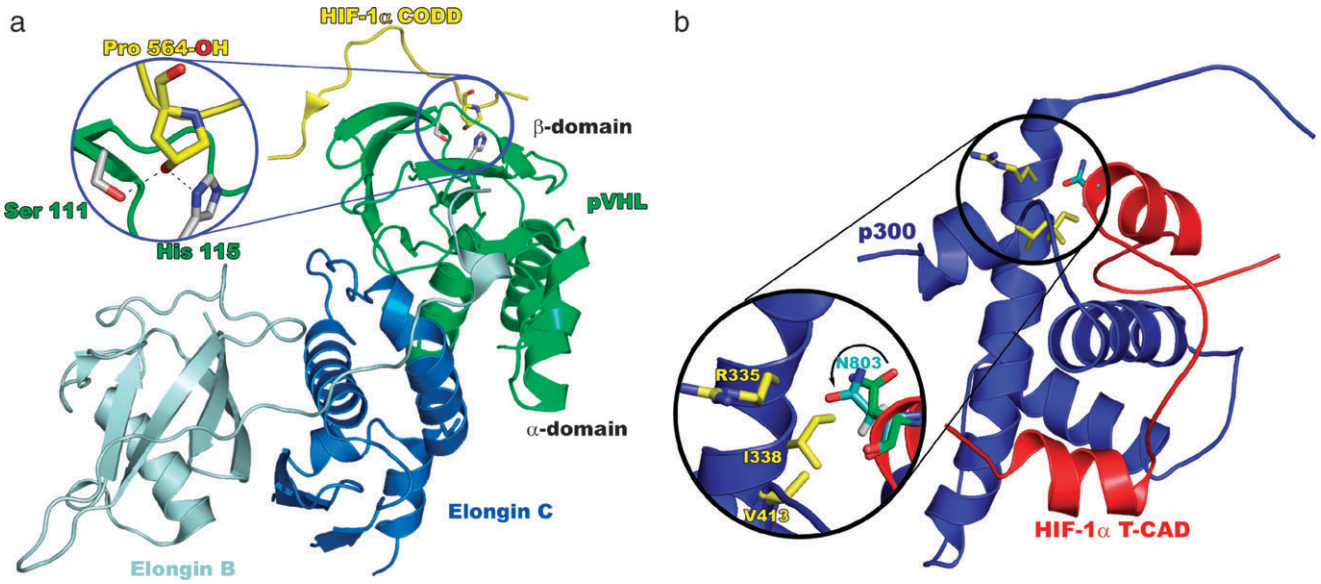


Fig. 6 Views from structures of key oxygen dependent components of the HIF system. Inset diagrams provide closer views. (a) The HIF–VCB interaction; VCB shows exquisite discrimination between hydroxylated and non-hydroxylated proline containing ODDDs. This is mediated by two hydrogen bonds formed between the alcohol of the hydroxylated proline and two residues of pVHL (Ser111 and His115). The α-domain of pVHL binds to elongin C and Cul2 whereas the β-domain interacts with hydroxylated HIF-α (PDB: 1LQB). (b) The HIF–p300 interaction; upon binding to p300, HIF-1α CAD forms two α-helices which are connected by an intervening loop (PDB: 1L3E). Hydroxylation at the pro-*S* β-carbon of Asn803 may provide a steric clash with Ile338 of p300. Note the conformational changes of Asn803 (inset) when bound to p300 (cyan sticks) and FIH (green sticks).

4. The HIF hydroxylases

There are three human prolyl hydroxylases (PHD1–3, also known as EGLN 1–3 enzymes).[19,20] All are selective for the HIF-α CODDD over the NODDD with PHD3 being the most selective, accepting almost exclusively CODDD in *in vitro* studies. PHD2 is thought to be the most important of the PHDs in catalysing HIF-α hydroxylation under normoxic conditions in healthy tissue. A recent report has demonstrated that another human prolyl hydroxylase can carry out HIF-α hydroxylation, but its relevance to the hypoxic response is not yet established.[21]

As for other 2OG oxygenases, the available evidence implies catalysis by factor inhibiting HIF (FIH, the HIF-α asparaginyl hydroxylase) and PHD2 proceeds *via* 2OG binding followed by that of the substrate then, finally, oxygen. Kinetic and biophysical analyses have revealed that PHD2 has unusually tight binding constants for Fe(II) and 2OG, compared to FIH and most other 2OG oxygenases studied (reviewed in ref. 4). The binding constants for long HIF-α fragment substrates for PHD2 and FIH are in the <0.1–10 range.[22–24] Oxygen binding to the HIF hydroxylases appears to be within the normal range for 2OG oxygenases. Overall these properties appear to make the HIF hydroxylases suitable for their role as oxygen sensors.

Crystal structures of PHD2[25] and FIH[26,27] have revealed that their overall folds follow those involved in other 2OG oxygenases (Fig. 8). Both contain the canonical double-stranded-β-helix fold that has been observed in all 2OG oxygenases identified to date. They also both contain the highly, but not fully, conserved 2His-1carboxylate HXD...H triad of iron binding residues. The structure of PHD2 reveals a narrow entrance to the active site consistent with the high affinity for Fe(II) and 2OG. Kinetic assays coupled with the structural analyses suggest that the NODDD/CODDD selectivity of the 3 PHDs is partly determined by two non-conserved regions at their C-termini or a mobile loop region that may fold to enclose the active site upon substrate binding.

FIH–HIF-1α CAD fragment complex structures have been reported.[27] They reveal that residues 795–803 of CAD bind in a groove at the FIH active site and adopt an extended conformation linked to FIH by hydrogen bonds and hydrophobic interactions (Fig. 8). CAD Asn803 and Ala804 form an inverse γ-turn, stabilized by a hydrogen bond between the backbone carbonyl of Val802 and NH of Ala804. Asn803 of the CAD is enclosed and its 3-pro-*S* Asn hydrogen that must be oxidised projects directly towards the iron.

5. HIF target genes and the hypoxic response

The transcriptional control over the hypoxic genes that is elicited by HIF is probably the most well-defined molecular mechanism for O_2 homeostasis in multicellular organisms. HIF has been termed a master regulator for the hypoxic response.[6] More than 70 HIF regulated genes have been directly identified by demonstration that HIF binds to an associated HRE (reviewed in ref. 4–6). Array analyses imply that the number will be substantially larger, possibly up to 200 or more genes that are up or down regulated by hypoxia or hypoxia mimics.[28] It is uncertain how many of these genes have functional HREs. The large number of genes that are HIF regulated is consistent with the complex nature of the hypoxic response.

Functions associated with maximising oxygen delivery, preventing damage or optimizing metabolism for hypoxia can be assigned to some of the HIF target genes.[6] Significantly

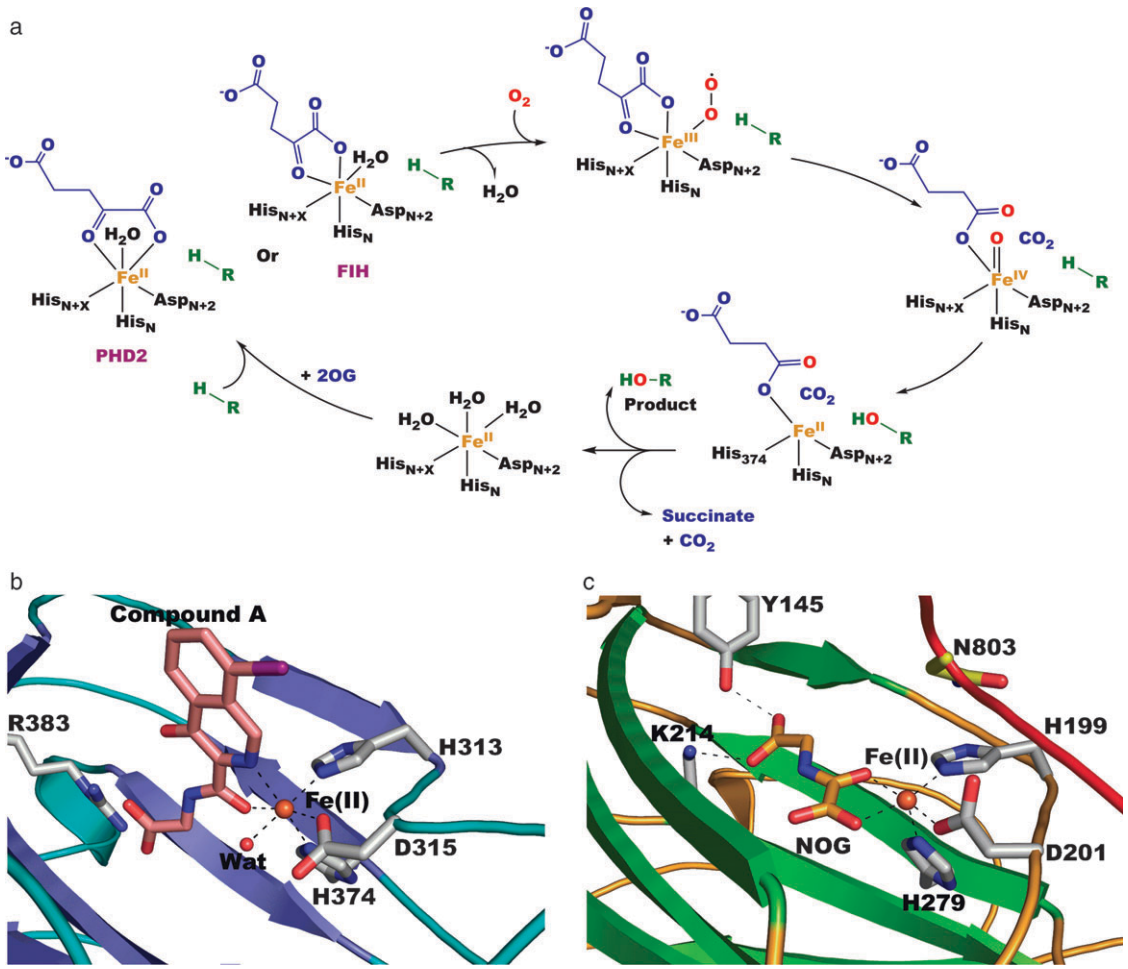


Fig. 7 Outline mechanism and active sites of the HIF hydroxylases. (a) Outline mechanism for the HIF hydroxylases. Fe(II) is octahedrally coordinated at the enzyme active site by the conserved HXD...H triad and the remaining coordination sites are occupied by water molecules. Crystal structures of FIH and other 2OG-dependent oxygenases in the presence/absence of the substrate molecule suggest that the cofactor 2OG and then the substrate (HIF-α) bind sequentially to the active site. This is followed by binding of molecular oxygen which is proposed to replace the remaining water molecule from the iron centre, leading to decarboxylation of 2OG and a highly reactive Fe(IV)=O intermediate. The latter is responsible for hydroxylating the peptide substrate. In the case of FIH, binding of the substrate displaces the water molecule from Fe(II). Views from (b) the PHD and (c) the FIH active sites.

expression of genes encoding for glycolysis enzymes are HIF regulated as well as those increasing red blood cell production (EPO) and angiogenesis (blood vessel formation from a pre-existing bed of vessels). There is also evidence that TCA cycle activity is downregulated in hypoxic conditions (reviewed in ref. 6). Given that the HIF hydroxylases are Fe(II) dependent it is notable that a number of HIF related genes encode for Fe(II) related proteins including transferrin, transferrin receptor (both involved in iron transport), haem oxygenase, inducible NO synthase, tyrosine hydroxylase, and procollagen prolyl-hydroxylase (CPH) α-subunit (like the HIF hydroxylases, CPH is a 2OG oxygenase).

6. Factors affecting HIF levels and activity

Cells become adapted to hypoxia after extended periods and lose the ability to stabilise HIF-α, only inducing HIF-α when made severely hypoxic. Two of the PHDs (PHD2 and 3) are themselves hypoxically regulated providing a feedback mechanism that may enable for the more rapid degradation of HIF-α upon reoxygenation of hypoxic cells. Regulation of PHD levels, and as a consequence HIF-α levels, provides a possible mechanism for adjusting the set-point of the HIF system, an important consideration if the hypoxic response is to operate in the very different cellular environments encountered in humans.

Various other feedback and modulating processes may regulate HIF levels *via* regulation of the activity of the HIF hydroxylases (discussed in ref. 29). Factors to be considered for which there is already evidence, include: (i) the intrinsic catalytic activity of the HIF hydroxylases under different conditions, (ii) the forms of HIF and the HIF hydroxylases present in a given cell type; modifications may occur *via* the production of different transcripts or post-translational modifications such as phosphorylation, (iii) the levels of, and the rates of production and degradation of HIF, the HIF hydroxylases and associated proteins involved in transcription and transport, (iv) the availability of the Fe(II) cofactor for the

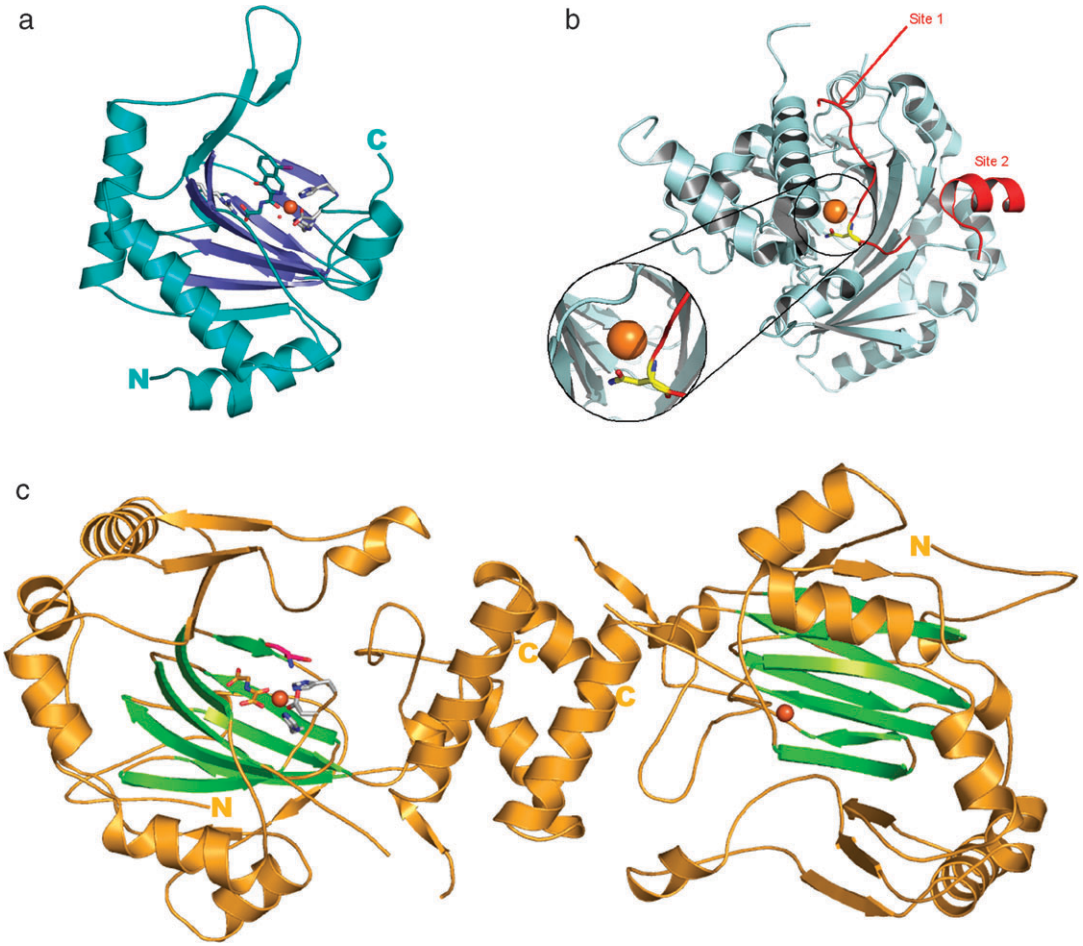


Fig. 8 Ribbon representation of the crystal structures of HIF hydroxylases. Overall structures of (a) monomeric PHD2 (cyan, PDB: 2G19); (b) FIH (pale cyan) bound to HIF-1α CAD (PDB: 1H2K) and (c) dimeric FIH (bright orange, PDB: 1H2N) showing the conserved double-stranded β-helix fold in dark blue (PHD2) and lemon green (FIH), the iron and 2OG binding sites (residues highlighted in white sticks) and orange spheres for iron centre.

HIF hydroxylases, (v) the redox state of the cell; in addition to exogenous iron supply, Fe(II) availability is regulated by the redox state of the cell which affects the Fe(II) : Fe(III) ratio; as for CPH activity, PHD and (with some substrates) FIH activity is stimulated by ascorbate presumably *via* stabilising the Fe(II) state, (vi) the number and availability of HRE sequences for HIF binding; availability of HREs may be regulated by changes to chromatin structure (*e.g.* cytosine methylation) or protein (*e.g.* histone modification), and (vii) small molecules (*e.g.* succinate, fumarate, NO) (Fig. 9).

From a medicinal perspective, the possibility of endogenous and exogenous regulation of HIF activity by small molecules is attractive. Succinate dehydrogenase and fumarate hydratase mutants causing increases in fumarate and succinate levels are characteristic of some tumour cells.[30] Because fumarate and, to a lesser extent, succinate are PHD inhibitors these mutations may contribute to activation of the hypoxic response *via* HIF hydroxylase inhibition. The interfaces between NO and the HIF system appear complex. Under normoxic conditions, NO upregulates HIF (possibly *via* hydroxylase inhibition involving direct interaction) whereas under hypoxic conditions it downregulates HIF.

The links between the HIF system and reactive oxygen species (ROS) are a subject of ongoing discussion.[31] Mitochondrial electron-transport chain inhibitors inhibit hypoxically mediated HIF stabilisation. It has been proposed that mitochondria produce a HIF hydroxylase inhibitor;[32] alternatively it could be that the set-point for oxygen dependency of the HIF hydroxylases is changed, for example, by altered local oxygen availability or by one of the potential regulatory factors described above. Mitochondrial respiration accounts for >90% of oxygen consumption in humans. It is proposed that under hypoxia NO can reduce mitochondrial O_2 consumption *via* inhibition of cytochrome C oxidase (redox centre IV in the mitochondrial electron-transport chain) making more O_2 available for oxygenases such as the PHDs.[33] The transcription factor JunD enables upregulation of antioxidants and deletion of JunD leads to HIF stabilisation in processes proposed to be mediated directly or indirectly *via* increased ROS.[34] One possibility is that the ROS inhibit the HIF hydroxylases by irreversible active site oxidation.

Recent studies reveal that HIF-1α can be degraded in a PHD/pVHL-independent mechanism *via* interaction of the receptor of activated protein kinase C (RACK1) with the

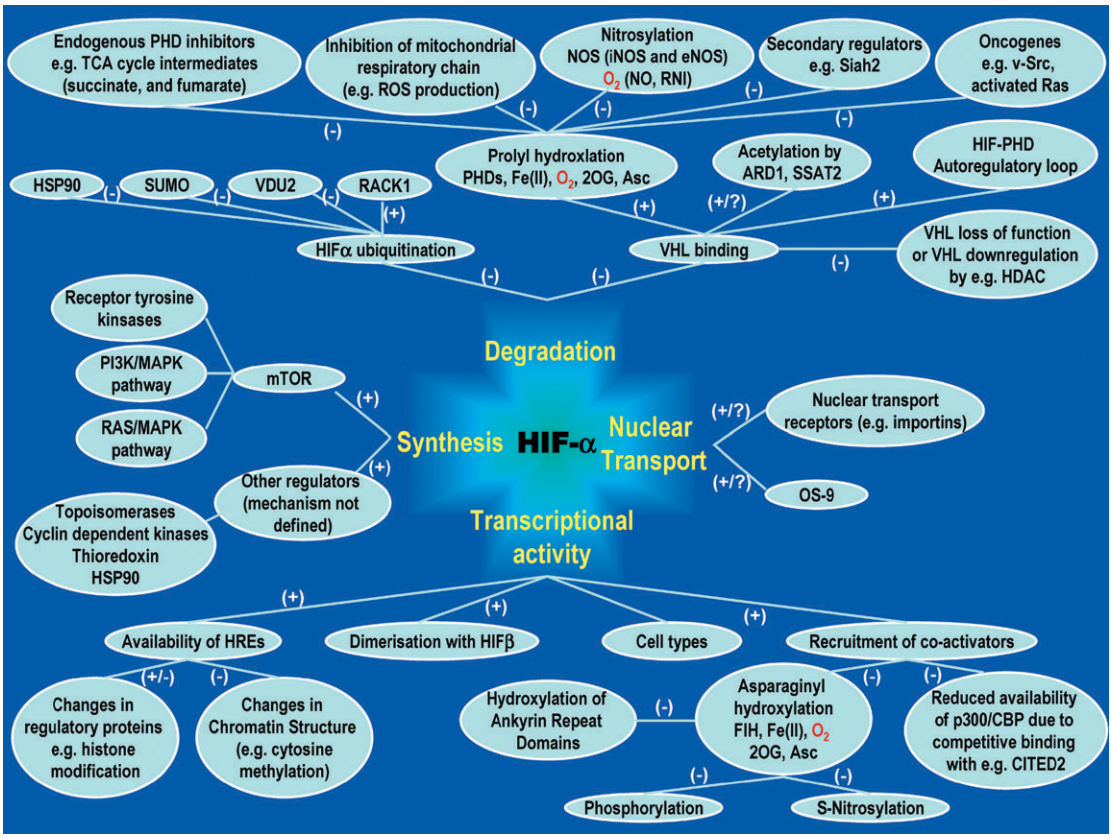


Fig. 9 Major factors that (may) affect the HIF system. ROS, reactive oxygen species; iNOS and eNOS, inducible and endothelial nitric oxide synthase; RNI, reactive nitrogen intermediates; Siah2, seven in absentia homolog 2; ARD1, arrest-defective protein 1; SSAT2, spermidine/spermine N1-acetyltransferase 2; HSP90, heat shock protein 90; RACK1, receptor of activated protein kinase C 1; pVHL, von Hippel–Lindau tumour suppressor protein; VDU2, pVHL-interacting deubiquitinating enzyme 2; HDAC, histone deacetylases; PI3K, phosphoinositide-3-kinase; MAPK, mitogen-activated protein kinase; CITED2, CBP/p300-interacting transactivator with ED-rich carboxy-terminal domain 2; SUMO, small ubiquitin-related modifier.

PAS-A domain of HIF-1α.[35] It is proposed that RACK1 forms a ubiquitin ligase complex similar to that formed by pVHL by recruiting elongin C plus other subunits and trigger for the proteasomal degradation of HIF-1α in a parallel yet O_2-independent pathway. However, the question of how rapidly and efficiently HIF-1α is destroyed in this pathway relative to the HIF/PHD pathway as a function of oxygen availability remains to be investigated.

Ongoing studies are identifying other factors that impact on the HIF system. HIF could be stabilised by promoting degradation of PHD3 *via* the ubiquitin ligase seven in absentia homologue 2 (Siah2).[36] G-protein-coupled receptor agonists, including angiotensin II and thrombin, have been shown to induce HIF-1 in vascular smooth muscle cells.[37] Inhibition of PHDs by the NADPH oxidase mediated ROS production was proposed to be a regulatory mechanism of HIF-1 induction under angiotensin II treatment. Other studies have shown that angiotensin II might suppress SM-20 (a rat homologue of PHD3) mRNA expression in a rat pheochromocytoma cell line and thereby promote HIF stabilisation.

It is important to appreciate that although the available evidence suggests that regulation of HIF levels is an important, and under some conditions probably dominant mechanism in the adaptation of cells to hypoxia, there are other transcriptional regulation systems that are regulated by oxygen including the NFκB TF (NF = nuclear factor) inflammatory response system. One connection between the HIF and NFκB systems is provided by the observation that PHD1 interacts with IκB kinases that regulate NFκB activity by regulating levels of the IκBα protein that in turn inhibits NFκB activity.[38]

7. Other substrates for the HIF hydroxylases and related human 2OG oxygenases

One important question has been whether the HIF hydroxylases have alternative substrates. Some of the PHDs have been reported to interact with other proteins including OS-9 (a protein commonly expressed in the endoplasmic reticulum with an unassigned function),[39] ING4 (a tumour suppressor protein),[40] RNA polymerase II[41] and IκB kinase-β,[38] (an enzyme involved in regulating the inflammatory response). As yet prolyl-hydroxylation of these potential targets has not been directly demonstrated by, *e.g.* mass spectrometry.

Protein interaction screens led to the identification of ankyrin repeat domain (ARD) proteins as potential FIH substrates.[42] The ARD is a 33-residue loop–helix–turn–helix motif that is amongst the most common of structural motifs

in human cells. To date, FIH has been reported to catalyze the hydroxylation of ARD proteins from the NFκB and Notch ARD protein families (intracellular notch domain proteins and some NFκB proteins are TFs, involved in the inflammatory response and development).[43] Recently, the ARD of a SOCS (suppressor of cytokine signalling) box protein (ASB4) has also been reported as an FIH substrate.[44] The link with Notch proteins is notable because HIF-1α is recruited to notch responsive promoters under hypoxic conditions (independently of HIF-β). Collectively these results suggest that ARD hydroxylation by FIH may be common; they imply that hydroxylation of cytoplasmic proteins may be much more ubiquitous than previously believed.

The significance of ARD hydroxylation in terms of effects on non-HIF pathways is unclear. Like some post-translational modifications, such as phosphorylation, it may have different consequences in different contexts. Crystallographic analyses on hydroxylated mouse notch 1 have revealed that asparaginyl hydroxylation enables an additional hydrogen bond.[43] It may be that as procollagen prolyl-hydroxylation works to stabilise the collagen triple helix, asparaginyl hydroxylation may stabilise the ARD fold. ARD proteins compete with HIF-α for FIH in the cytoplasm, and hydroxylated ARDs bind less tightly than non-hydroxylated. It has been proposed that the hydroxylation status of all ARDs accessible to FIH regulates the quantity of FIH free to hydroxylate HIF-α. Regulation by a motif common to a pool of proteins has not been widely considered in terms of signalling and may apply in other pathways.

The PHDs appear to belong to a relatively small sub-family of human 2OG oxygenases.[20,25] There is some evidence that other prolyl hydroxylases may accept HIF-α as a substrate but the relevance of these observations to oxygen sensing in the hypoxic response has not yet been demonstrated. In contrast FIH is one of a significantly larger sub-family of homogenous genes/proteins.[45] Prior to the assignment of FIH as a HIF hydroxylase, these proteins had been proposed to form a group of zinc dependent TFs, termed the JmjC TFs. Subsequent to the functional assignment of FIH and the solution of its crystal structure, it was clear that these proteins were much more likely to be Fe(II) dependent 2OG oxygenases. An important advance was made when one of these proteins (JHDM1A) was shown to be a 2OG dependent Nε-methyllysine demethylase.[46] Subsequent work has shown that the Jmj 2OG oxygenases are a ubiquitous family of histone demethylases with likely >25 human members. The Jmj and related demethylases are only the second family of histone demethylases to be identified after the FAD dependent lysyl demethylases. Different lysyl demethylases display different selectivity both for lysyl methylation status (mono-, di-, or trimethyl) and histone sequence. Their mechanisms likely operate *via* hydroxylation on the methyl group followed by fragmentation. The discovery of 2OG dependent histone demethylases has opened up a new sub-field in chromatin biochemistry that is beyond the scope of this review. However, it is worth noting that whilst the oxygen dependence of the Jmj demethylases offers the potential for them to act as sensors, they are not necessarily sensors since other factors may compensate for variations in oxygen availability. The human AlkB homologue (ABH) enzymes are related to the *E. coli* enzyme AlkB that catalyses demethylation of N-methylated DNA and RNA bases and also have the potential to regulate transcription, and translation, in an oxygen dependent manner.[47] Recently the fat mass and obesity protein (FTO) has been shown to be homologous to the ABHs and to catalyse nucleic acid N-demethylation, raising the possibility of links between oxygen availability and obesity.[48]

8. Diseases associated with HIF

HIF regulated genes such as *EPO* (for the treatment of anaemia) and *VEGF* (targeted for tumour treatment) are already the subject of therapies. Because of the pivotal role of HIF itself in oxygen homeostasis it is attracting interest from a therapeutic perspective. Ischemic diseases, *e.g.* coronary heart disease, result from a restricted blood supply leading to a lack of oxygen. For ischemic disease, upregulation of HIF activity is desirable. In contrast, tumours are normally hypoxic leading to upregulation of HIF; thus for tumour therapy it is desirable to starve the tumours of oxygen/other nutrients by down-regulating HIF activity. There is evidence that renal cell carcinomas (RCCs) selectively overexpress the gene encoding for HIF-2α rather than that for HIF-1α; inhibition of HIF-2α was shown to be sufficient to suppress tumour growth.[49] Mice in which HIF-2α was ablated were reported to display anaemia implying HIF-2α as the predominant HIF isoform regulating EPO in adults. Other examples of HIF-α isoform selective gene regulation are reported, for example, HIF-1α selective regulation of carbonic anhydrase IX.[50] However, further work is required to achieve a molecular understanding of how selectivity is achieved. Beyond the scope of this introductory review, there are also links between the HIF system and other signalling systems, such as the inflammatory response, that may influence selectivity.

Two inherited mutations to PHD2 have been linked with familial erythrocytosis (an abnormally high red blood count).[51] One of these, Pro317, is located two residues away from one of the Fe(II) binding residues (Asp315) and is located close to the apparently unusually narrow entrance to the PHD2 active site. The other, Arg371, is also close to another of the triad of metal-binding residues, His374. Both mutations have been shown to result in a decrease in catalytic activity, but they may also effect the *in vivo* stability of PHD2.

von Hippel–Lindau (VHL) disease is an autosomal dominant inherited cancer syndrome that is characterized by RCCs and tumours. VHL syndrome occurs at a frequency of ~ 1 in 35 000 humans and is caused by mutations in the *VHL* gene including point mutations resulting in VHL variants. The lack of wildtype VHL results in HIF overproduction and the subsequent overexpression of HIF target genes. Abnormal increases in red blood cell production, or polycythaemia, endemic in the Chuvash population (in Russia) also arise from *VHL* mutations. However, these *VHL* mutations do not result in tumours but cause venous abnormalities and a tendency towards thrombosis. Why different *VHL* mutations lead to different phenotypes is currently unknown.

9. Possibilities for therapeutic intervention

Basic research on oxygen sensing and the HIF system has uncovered opportunities for therapeutic intervention that can be divided into those that upregulate or those that downregulate HIF target genes/proteins. Knowledge of the HRE sequences can be used to selectively target the expression of exogenous genes to hypoxic tissues, either for imaging or to deliver therapeutic genes. However, the effects of HIF-silencing are not straightforward, and do not necessarily correspond with a decrease in tumour size or vasculature. The potential for use of elements of the HIF system for employing either the HRE or ODDD components has been elegantly demonstrated by work on mice.[52] Use of polyamides that bind to specific regulatory sequences is a possible route to achieving regulation of a subset of HIF target genes.[53]

Screens to identify small molecules that downregulate HIF-α have been carried out (for a review see ref. 54). These have led to molecules that target HIF associated molecules, though how selective these targets are to HIF and the hypoxia response is not clear. Inhibitors of the mammalian target of rapamycin (mTOR) result in lowered HIF, and are in clinical development as anti-cancer agents. Curcumin, which is present in some spices, is reported to promote HIF-α degradation. Inhibitors of heat shock protein 90 (HSP90) that stabilises HIF-1α *via* a PHD/pVHL independent manner, also reduce HIF-1α levels. Promoting the activity of the HIF hydroxylases in cancer cells may reduce the availability of HIF; treatment with 2OG has shown decreased VEGF production in Hep3B cells,[55] though it is unclear if 2OG will be limiting *in vivo* and there is a possibility that in some tumours succinate and fumarate compete for 2OG binding to the PHDs.[30] Ensuring PHD activity is not limited by ascorbate, or the actual *in vivo* reducing agent—if there is one, may also help to suppress HIF levels.

Other potential small molecule targets to block HIF function include blocking HIF-α/β dimerisation and preventing HIF from binding to its cotranscriptional activators, specifically p300. The natural product chetomin, a disulfide containing diketopiperazine (epidithiodiketopiperazine), is a potent blocker of binding of HIF-α to p300 both with isolated proteins and in cells.[56]

In the case of upregulating HIF for the treatment of ischemic disease or anaemia (by upregulating EPO), blocking either the HIF–VHL interaction or the activity of the HIF hydroxylases are two clear possibilities. Studies with peptides and the 2OG analogue, *N*-oxalylglycine (first used as a procollagen prolyl hydroxylase inhibitor) verified these approaches.[20] Further evidence that targeting the HIF hydroxylases will be a therapeutically viable approach comes from the use of Co(II) as a hypoxia mimic—at least in part, the ability of Co(II) to act in this way results from inhibition of the HIF hydroxylases. Indeed, Co(II) has been used as a clinical treatment for anaemia prior to the advent of EPO, but has toxic effects that are probably unrelated to its HIF-based mechanism of action. Recent efforts have focused on identifying other organic inhibitors of the HIF hydroxylases.[52,57] Most, if not all, of those reported act as 2OG competitors and chelate to the active site Fe(II) in a bidentate manner. It will clearly be desirable to develop inhibitors that are selective for the HIF hydroxylases over other 2OG oxygenases. To what extent selectivity in terms of HIF target genes is desirable is uncertain and may depend on the therapeutic goal. Indeed, for some applications induction of the hypoxic response in a manner as close as possible to the natural response may be desirable; if so the points of oxygen sensing, including the HIF hydroxylases, may be a preferred target.

Acknowledgements

We thank all our co-workers and collaborators in Chemistry and Clinical Medicine (Peter Ratcliffe, Chris Pugh and co-workers) for their efforts and contribution to work on hypoxia sensing. We thank the Commonwealth Scholarship Commission (to R. C.), the BBSRC (to A. H.) and the Wellcome Trust for funding. We apologize for the lack of complete citations in this tutorial review.

References

1. W. Jelkmann, *Eur. J. Haematol.*, 2007, **78**, 183.
2. B. H. Jiang, E. Rue, G. L. Wang, R. Roe and G. L. Semenza, *J. Biol. Chem.*, 1996, **271**, 17771.
3. W. G. Kaelin, *Annu. Rev. Biochem.*, 2005, **74**, 115.
4. A. Ozer and R. K. Bruick, *Nat. Chem. Biol.*, 2007, **3**, 144.
5. C. J. Schofield and P. J. Ratcliffe, *Nat. Rev. Mol. Cell Biol.*, 2004, **5**, 343.
6. G. L. Semenza, *Biochem. J.*, 2007, **405**, 1.
7. P. B. Card, P. J. Erbel and K. H. Gardner, *J. Mol. Biol.*, 2005, **353**, 664.
8. R. H. Wenger, I. Kvietikova, A. Rolfs, G. Camenisch and M. Gassmann, *Eur. J. Biochem.*, 1998, **253**, 771.
9. M. Ivan, K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J. M. Asara, W. S. Lane and W. G. Kaelin, Jr, *Science*, 2001, **292**, 464.
10. P. Jaakkola, D. R. Mole, Y. M. Tian, M. I. Wilson, J. Gielbert, S. J. Gaskell, A. Kriegsheim, H. F. Hebestreit, M. Mukherji, C. J. Schofield, P. H. Maxwell, C. W. Pugh and P. J. Ratcliffe, *Science*, 2001, **292**, 468.
11. P. H. Maxwell, M. S. Wiesener, G. W. Chang, S. C. Clifford, E. C. Vaux, M. E. Cockman, C. C. Wykoff, C. W. Pugh, E. R. Maher and P. J. Ratcliffe, *Nature*, 1999, **399**, 271.
12. L. A. McNeill, K. S. Hewitson, J. M. Gleadle, L. E. Horsfall, N. J. Oldham, P. H. Maxwell, C. W. Pugh, P. J. Ratcliffe and C. J. Schofield, *Bioorg. Med. Chem. Lett.*, 2002, **12**, 1547.
13. J. H. Min, H. Yang, M. Ivan, F. Gertler, W. G. Kaelin, Jr and N. P. Pavletich, *Science*, 2002, **296**, 1886.
14. D. Lando, D. J. Peet, D. A. Whelan, J. J. Gorman and M. L. Whitelaw, *Science*, 2002, **295**, 858.
15. S. A. Dames, M. Martinez-Yamout, R. N. De Guzman, H. J. Dyson and P. E. Wright, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 5271.
16. S. J. Freedman, Z. Y. Sun, F. Poy, A. L. Kung, D. M. Livingston, G. Wagner and M. J. Eck, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 5367.
17. L. M. Hoffart, E. W. Barr, R. B. Guyer, J. M. Bollinger, Jr and C. Krebs, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 14738.
18. I. J. Clifton, M. A. McDonough, D. Ehrismann, N. J. Kershaw, N. Granatino and C. J. Schofield, *J. Inorg. Biochem.*, 2006, **100**, 644.
19. R. K. Bruick and S. L. McKnight, *Science*, 2001, **294**, 1337.
20. A. C. Epstein, J. M. Gleadle, L. A. McNeill, K. S. Hewitson, J. O'Rourke, D. R. Mole, M. Mukherji, E. Metzen, M. I. Wilson, A. Dhanda, Y. M. Tian, N. Masson, D. L. Hamilton, P. Jaakkola, R. Barstead, J. Hodgkin, P. H. Maxwell, C. W. Pugh, C. J. Schofield and P. J. Ratcliffe, *Cell*, 2001, **107**, 43.
21. P. Koivunen, P. Tiainen, J. Hyvarinen, K. E. Williams, R. Sormunen, S. J. Klaus, K. I. Kivirikko and J. Myllyharju, *J. Biol. Chem.*, 2007, **282**, 30544.

22. D. Ehrismann, E. Flashman, D. N. Genn, N. Mathioudakis, K. S. Hewitson, P. J. Ratcliffe and C. J. Schofield, *Biochem. J.*, 2007, **401**, 227.
23. E. Flashman, E. A. Bagg, R. Chowdhury, J. Mecinovic, C. Loenarz, M. A. McDonough, K. S. Hewitson and C. J. Schofield, *J. Biol. Chem.*, 2008, **283**, 3808.
24. P. Koivunen, M. Hirsila, K. I. Kivirikko and J. Myllyharju, *J. Biol. Chem.*, 2006, **281**, 28712.
25. M. A. McDonough, V. Li, E. Flashman, R. Chowdhury, C. Mohr, B. M. Lienard, J. Zondlo, N. J. Oldham, I. J. Clifton, J. Lewis, L. A. McNeill, R. J. Kurzeja, K. S. Hewitson, E. Yang, S. Jordan, R. S. Syed and C. J. Schofield, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 9814.
26. C. E. Dann, 3rd, R. K. Bruick and J. Deisenhofer, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 15351.
27. J. M. Elkins, K. S. Hewitson, L. A. McNeill, J. F. Seibel, I. Schlemminger, C. W. Pugh, P. J. Ratcliffe and C. J. Schofield, *J. Biol. Chem.*, 2003, **278**, 1802.
28. G. P. Elvidge, L. Glenny, R. J. Appelhoff, P. J. Ratcliffe, J. Ragoussis and J. M. Gleadle, *J. Biol. Chem.*, 2006, **281**, 15215.
29. C. J. Schofield and P. J. Ratcliffe, *Biochem. Biophys. Res. Commun.*, 2005, **338**, 617.
30. M. A. Selak, S. M. Armour, E. D. MacKenzie, H. Boulahbel, D. G. Watson, K. D. Mansfield, Y. Pan, M. C. Simon, C. B. Thompson and E. Gottlieb, *Cancer Cell*, 2005, **7**, 77.
31. T. Klimova and N. S. Chandel, *Cell Death Differ.*, 2008, **15**, 660.
32. B. Brune and J. Zhou, *Cardiovasc. Res.*, 2007, **75**, 275.
33. T. Hagen, C. T. Taylor, F. Lam and S. Moncada, *Science*, 2003, **302**, 1975.
34. D. Gerald, E. Berra, Y. M. Frapart, D. A. Chan, A. J. Giaccia, D. Mansuy, J. Pouyssegur, M. Yaniv and F. Mechta-Grigoriou, *Cell*, 2004, **118**, 781.
35. Y. V. Liu, J. H. Baek, H. Zhang, R. Diez, R. N. Cole and G. L. Semenza, *Mol. Cell*, 2007, **25**, 207.
36. K. Nakayama, I. J. Frew, M. Hagensen, M. Skals, H. Habelhah, A. Bhoumik, T. Kadoya, H. Erdjument-Bromage, P. Tempst, P. B. Frappell, D. D. Bowtell and Z. Ronai, *Cell*, 2004, **117**, 941.
37. G. Wolf, *Antioxid. Redox Signaling*, 2005, **7**, 1337.
38. E. P. Cummins, E. Berra, K. M. Comerford, A. Ginouves, K. T. Fitzgerald, F. Seeballuck, C. Godson, J. E. Nielsen, P. Moynagh, J. Pouyssegur and C. T. Taylor, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 18154.
39. J. H. Baek, P. C. Mahon, J. Oh, B. Kelly, B. Krishnamachary, M. Pearson, D. A. Chan, A. J. Giaccia and G. L. Semenza, *Mol. Cell*, 2005, **17**, 503.
40. A. Ozer, L. C. Wu and R. K. Bruick, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 7481.
41. A. V. Kuznetsova, J. Meller, P. O. Schnell, J. A. Nash, M. L. Ignacak, Y. Sanchez, J. W. Conaway, R. C. Conaway and M. F. Czyzyk-Krzeska, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 2706.
42. M. E. Cockman, D. E. Lancaster, I. P. Stolze, K. S. Hewitson, M. A. McDonough, M. L. Coleman, C. H. Coles, X. Yu, R. T. Hay, S. C. Ley, C. W. Pugh, N. J. Oldham, N. Masson, C. J. Schofield and P. J. Ratcliffe, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 14767.
43. M. L. Coleman, M. A. McDonough, K. S. Hewitson, C. Coles, J. Mecinovic, M. Edelmann, K. M. Cook, M. E. Cockman, D. E. Lancaster, B. M. Kessler, N. J. Oldham, P. J. Ratcliffe and C. J. Schofield, *J. Biol. Chem.*, 2007, **282**, 24027.
44. J. E. Ferguson, 3rd, Y. Wu, K. Smith, P. Charles, K. Powers, H. Wang and C. Patterson, *Mol. Cell. Biol.*, 2007, **27**, 6407.
45. K. S. Hewitson, L. A. McNeill, M. V. Riordan, Y. M. Tian, A. N. Bullock, R. W. Welford, J. M. Elkins, N. J. Oldham, S. Bhattacharya, J. M. Gleadle, P. J. Ratcliffe, C. W. Pugh and C. J. Schofield, *J. Biol. Chem.*, 2002, **277**, 26351.
46. Y. Tsukada, J. Fang, H. Erdjument-Bromage, M. E. Warren, C. H. Borchers, P. Tempst and Y. Zhang, *Nature*, 2006, **439**, 811.
47. B. Sedgwick, P. A. Bates, J. Paik, S. C. Jacobs and T. Lindahl, *DNA Repair*, 2007, **6**, 429.
48. T. Gerken, C. A. Girard, Y. C. Tung, C. J. Webby, V. Saudek, K. S. Hewitson, G. S. Yeo, M. A. McDonough, S. Cunliffe, L. A. McNeill, J. Galvanovskis, P. Rorsman, P. Robins, X. Prieur, A. P. Coll, M. Ma, Z. Jovanovic, I. S. Farooqi, B. Sedgwick, I. Barroso, T. Lindahl, C. P. Ponting, F. M. Ashcroft, S. O'Rahilly and C. J. Schofield, *Science*, 2007, **318**, 1469.
49. R. R. Raval, K. W. Lau, M. G. Tran, H. M. Sowter, S. J. Mandriota, J. L. Li, C. W. Pugh, P. H. Maxwell, A. L. Harris and P. J. Ratcliffe, *Mol. Cell. Biol.*, 2005, **25**, 5675.
50. C. C. Wykoff, N. J. Beasley, P. H. Watson, K. J. Turner, J. Pastorek, A. Sibtain, G. D. Wilson, H. Turley, K. L. Talks, P. H. Maxwell, C. W. Pugh, P. J. Ratcliffe and A. L. Harris, *Cancer Res.*, 2000, **60**, 7075.
51. M. J. Percy, P. W. Furlow, P. A. Beer, T. R. Lappin, M. F. McMullin and F. S. Lee, *Blood*, 2007, **110**, 2193.
52. M. Safran, W. Y. Kim, F. O'Connell, L. Flippin, V. Gunzler, J. W. Horner, R. A. Depinho and W. G. Kaelin, Jr, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 105.
53. B. Z. Olenyuk, G. J. Zhang, J. M. Klco, N. G. Nickols, W. G. Kaelin, Jr and P. B. Dervan, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 16768.
54. G. L. Semenza, *Drug Discovery Today*, 2007, **12**, 853.
55. K. Matsumoto, S. Imagawa, N. Obara, N. Suzuki, S. Takahashi, T. Nagasawa and M. Yamamoto, *J. Cell. Physiol.*, 2006, **209**, 333.
56. A. L. Kung, S. D. Zabludoff, D. S. France, S. J. Freedman, E. A. Tanner, A. Vieira, S. Cornell-Kennon, J. Lee, B. Wang, J. Wang, K. Memmert, H. U. Naegeli, F. Petersen, M. J. Eck, K. W. Bair, A. W. Wood and D. M. Livingston, *Cancer Cell*, 2004, **6**, 33.
57. M. A. McDonough, L. A. McNeill, M. Tilliet, C. A. Papamicael, Q. Y. Chen, B. Banerji, K. S. Hewitson and C. J. Schofield, *J. Am. Chem. Soc.*, 2005, **127**, 7680.
58. S. Sauve, L. Tremblay and P. Lavigne, *J. Mol. Biol.*, 2004, **342**, 813.
59. A. R. Ferre-D'Amare, P. Pognonec, R. G. Roeder and S. K. Burley, *EMBO J.*, 1994, **13**, 180.
60. O. Yildiz, M. Doi, I. Yujnovsky, L. Cardone, A. Berndt, S. Hennig, S. Schulze, C. Urbanke, P. Sassone-Corsi and E. Wolf, *Mol. Cell*, 2005, **17**, 69.