OXYGEN SENSING BY HIF HYDROXYLASES

Christopher J. Schofield and Peter J. Ratcliffe‡*

The transcription factor HIF (hypoxia-inducible factor) has a central role in oxygen homeostasis in animals ranging from nematode worms to man. Recent studies have shown that this factor is regulated by an unprecedented signalling mechanism that involves post-translational hydroxylation. This hydroxylation is catalysed by a set of non-haem, Fe^{2+}-dependent enzymes that belong to the 2-oxoglutarate-dependent-oxygenase superfamily. The absolute requirement of these enzymes for molecular oxygen has provided new insights into the way cells sense oxygen.

PAS FAMILY
(period circadian protein (PER), aryl-hydrocarbon receptor (AHR), aryl-hydrocarbon-receptor nuclear translocator (ARNT) and single-minded protein (SIM)). A group of interacting and structurally related basic helix–loop–helix transcription factors.

ANGIOGENESIS
The growth and proliferation of new blood vessels from existing vasculature.

ERYTHROPOIESIS
The formation of erythrocytes (red blood cells).

**The Oxford Centre for Molecular Sciences and The Dyson Perrins Laboratory, Department of Chemistry, South Parks Road, Oxford OX1 3QY, UK.*
‡The Henry Wellcome Building of Genomic Medicine, Roosevelt Drive, Oxford OX3 7BN, UK.
Correspondence to P.J.R.
e-mail: pjr@well.ox.ac.uk
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The possibility that cells have specific interfaces with molecular oxygen that have a prime function in biological control has long interested biologists. Specific 'oxygen-sensing' mechanisms have been defined in bacteria and yeast, but, until recently, have remained elusive in higher organisms. Studies have now identified an unexpectedly direct link between the availability of oxygen and an important transcriptional cascade that regulates many responses to hypoxia in higher organisms, including humans. Molecular dissection of one of the most striking homeostatic responses to hypoxia — the induction of the haematopoietic growth factor erythropoietin — led to the discovery of the transcription factor hypoxia-inducible factor (HIF)[1]. Unexpectedly, it was found that this system operates in essentially all mammalian cells irrespective of their relevance to erythropoietin production[2], and that it directs many other responses to hypoxia. The same hypoxic responses can be induced by iron chelators or cobaltous ions, distinctive properties that led to the proposal of a ferroprotein oxygen sensor, which was originally thought to be a haem protein. Work on the HIF signalling pathway has now shown that its transcriptional activity is regulated by the post-translational hydroxylation of specific residues. Hydroxylation is catalysed by a set of oxygen-dependent enzymes that belong to the 2-oxoglutarate-dependent-oxygenase superfamily, which are, in fact, non-haem, Fe^{2+}-dependent enzymes (BOX 1). Here, we review recent findings and their implications for our understanding of cellular responses to hypoxia, and discuss the possibility of a wider role for post-translational hydroxylation in signalling.

Hypoxia-inducible factor

HIF is a heterodimeric transcription factor that is composed of two basic helix–loop–helix proteins — HIFα and HIFβ — of the PAS FAMILY (PER, AHR, ARNT and SIM family)[3]. The HIFα/β dimer binds to a core DNA motif (G/ACGTG) in hypoxia-response elements (HREs) that are associated with a broad range of transcriptional targets (for reviews, see REFS 4–8). These target genes are centrally involved in both systemic responses to hypoxia, such as ANGIOGENESIS and ERYTHROPOIESIS, and in cellular responses, such as alterations in glucose/energy metabolism (see BOX 2 and FIG. 1; for a fully referenced version of this figure, see online supplementary information S1 (figure)). The HIFβ subunit, which is identical to ARNT, is a constitutive nuclear protein that has further roles in transcription. By contrast, the levels of HIFα subunits are highly inducible by hypoxia. There are three closely related forms of HIFα, each of which is encoded by a distinct gene locus. HIF1α and HIF2α have a similar domain architecture and are regulated in a similar manner. HIF3α is less closely related and its regulation is less well understood.

HIF1α and HIF2α undergo proteolytic regulation that is dependent on the presence of two independently functioning oxygen-dependent degradation

Box 1 | 2-Oxoglutarate-dependent enzymes and related enzymes

2-Oxoglutarate-dependent oxygenases and related enzymes are involved in a wide range of metabolic and signalling processes. In plants, they catalyse steps in the biosynthesis of signalling molecules such as ethylene and the gibberellins, as well as several steps in flavonoid biosynthesis. In both bacteria and fungi, they are involved in the biosynthesis of various medicinally important antibiotics, including those of the β-lactam family such as penicillins[36], clavulanic acid[48,96] and carbapenems[97,98]. The oxidative reactions that are catalysed in the biosynthesis of these antibiotics are unusual and have little precedent in synthetic chemistry. In mammals, in addition to the hydroxylation of hypoxia-inducible factor, 2-oxoglutarate-dependent oxygenases catalyse procollagen-extracellular-matrix formation[99], as well as important metabolic reactions such as the biosynthesis of carnitine[100] and the metabolism of dietary phytanic acid (a product of chlorophyll metabolism). In phytanic-acid metabolism, genetic lesions in phytanoyl CoA hydroxylase — a 2-oxoglutarate-dependent oxygenase — lead to Refsum's disease[101]. Finally, these oxygenases are involved in DNA repair in organisms ranging from bacteria to humans[74,75,102,103].

domains (NODDD and CODDD), which are located in the central region of the molecule (FIG. 2). HIF1α and HIF2α also possess two transactivation domains — an internal activation domain (NAD) that overlaps with the CODDD and a carboxy-terminal activation domain (CAD; FIG. 2). CAD is not directly involved in proteolytic regulation, but has an enabling role in HIF-mediated transcription through its oxygen-regulated association with the CH-1 (cysteine/histidine rich) domain of the transcriptional co-activator p300. In addition, HIF1α is reported to be subject to a further control that involves its nuclear/cytoplasmic localization, which is probably mediated by its active exclusion from the nucleus in the presence of oxygen. However, the precise mechanisms and regulatory sequences that are involved are not yet known (for reviews, see REFS 5,7,9).

For HIF3α, at least one of the oxygen-regulated processes — that is, proteolytic regulation that involves a central domain that closely resembles the HIF1/2α CODDD — seems to be conserved[10]. HIF3α is also regulated by several types of alternative splicing. Most striking, is the production of a shortened form that is composed of the amino-terminal basic helix–loop–helix and PAS domains, and omits the CODDD and other carboxy-terminal sequences. This protein — known as inhibitory PAS protein (IPAS) — forms transcriptionally inactive heterodimers with HIF1α (REF. 11). Interestingly, the process of alternative splicing that forms the *IPAS* transcript is induced by hypoxia in certain tissues, although it is not yet clear whether this represents a separate interface with oxygen-sensitive pathways or a counter-regulatory response to the activation of HIF transcriptional activity.

An important aspect of the analysis of the domain architecture of HIFα subunits was the identification of specific regions that, when fused to heterologous proteins, could independently transfer responsiveness to hypoxia. So far, three regions that correspond to the NODDD, CODDD and CAD have been shown to operate in this way (FIG. 2). It was found that the responses produced by these domains were affected in a similar manner by hypoxia, cobaltous ions and iron chelators. These findings indicated that each sequence must have a discrete, but mechanistically related, interaction with the regulatory signal pathway(s), and focused analysis on these sites.

HIF regulation by protein hydoxylation

Detailed biochemical and genetic analyses have now shown that oxygen responsiveness is indeed conveyed by separate interactions with each of the three HIFα regions that transfer responsiveness to hypoxia. And, in each case, the process is similar and involves the oxygen-dependent enzymatic hydroxylation of specific residues (FIG. 2). Although other post-translational modifications of HIFα subunits (including phosphorylation and acetylation) have been defined, their interface with oxygen-sensitive pathways remains unclear. This review therefore focuses on the newly defined hydroxylation pathways.

Prolyl hydroxylation. Hydroxylation of specific prolyl residues at conserved sites in NODDD and CODDD (FIG. 2) regulates independent interactions with the von Hippel–Lindau tumour suppressor (pVHL)[12–15]. pVHL is the recognition component of a multi-component ubiquitin ligase (pVHL–elonginB–elonginC–Cul2–Rbx) that targets HIFα subunits for proteasomal proteolysis by the UBIQUITIN–PROTEASOME PATHWAY[16,17]. Hydroxylation increases the affinity of HIFα peptides for the pVHL–elonginB–elonginC (VBC) complex by at least

Box 2 | The HIF transcriptional cascade

So far, more than 40 hypoxia-inducible-factor (HIF) target genes have been characterized by the functional delineation of HIF binding to hypoxia-response elements in transcriptional control sequences. In keeping with the complexity of oxygen homeostasis in large animals, these HIF transcriptional targets are involved in diverse aspects of cellular and integrative physiology, including, for example, energy metabolism and cell growth and migration (see FIG. 1; for a fully referenced version of this figure, see online supplementary information S1 (figure)). In addition, large-scale gene-expression arrays have been used to define global responses to hypoxia, and they indicate that, in any given cell, several hundred genes are positively or negatively regulated by hypoxia[104–106]. Furthermore, the complement of genes that is regulated by hypoxia differs greatly between cell types, so that, overall, on the order of thousands of genes might be regulated in this way. Genetic studies in HIFα-subunit- or von-Hippel–Lindau-tumour-suppressor (pVHL)-defective cells, together with studies of the effects of nonspecific hydroxylase inhibitors such as cobaltous ions and iron chelators, have indicated that most of the responses to hypoxia are dependent on a functional HIF/pVHL/hydroxylase system. However, the extent to which the responses are the direct result of HIF-target-gene transcription, as opposed to indirect effects, is unclear.

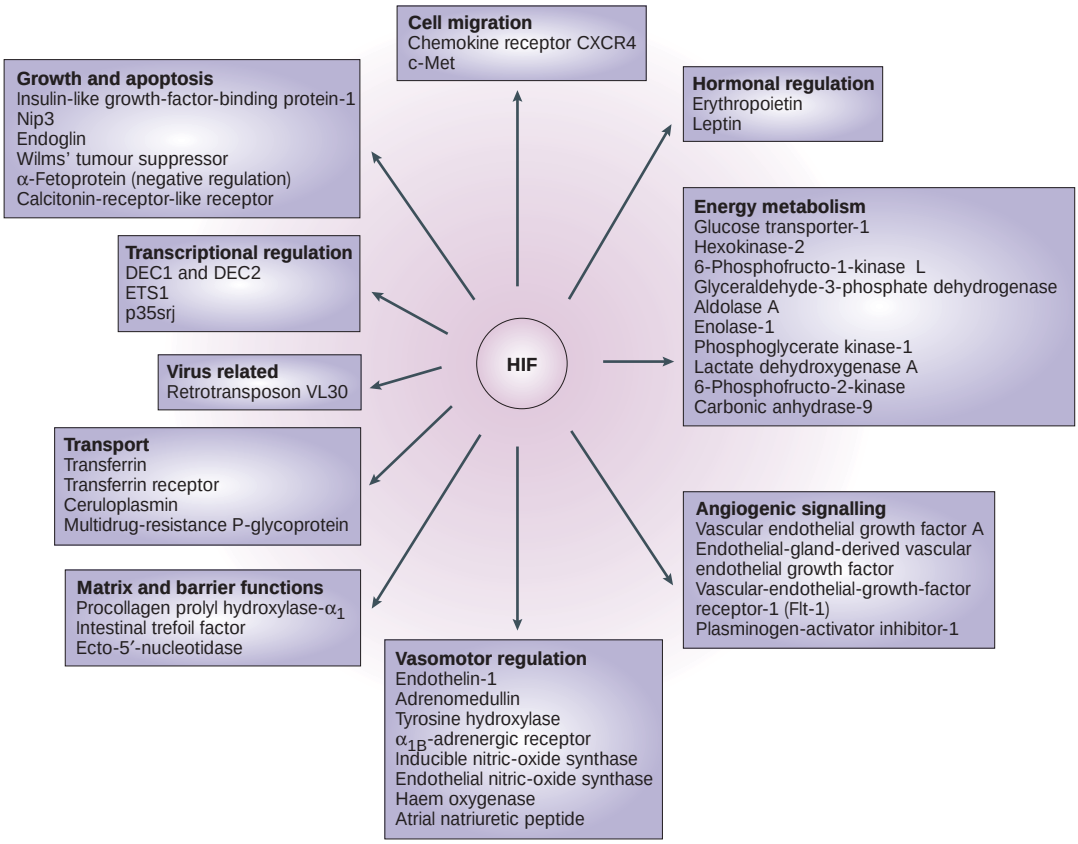

Figure 1 | **Direct transcriptional targets of HIF.** The figure shows the protein products of the genes for which there is evidence of direct transcriptional activation by hypoxia-inducible factor (HIF). For a fully referenced version of this figure, see online supplementary information S1 (figure). Differentiated embryo chrondrocyte (DEC)1 and DEC2 are transcription factors of the basic helix–loop–helix family. ETS is a DNA-binding domain that defines a family of transcription factors. p35srj is a 35-kDa protein that contains a serine/glycine-rich junction. Nip3 is a member of the Bcl2 family of cell-death factors. CXCR is a receptor for the CXC family of chemokines. c-Met is the tyrosine-kinase product of the *met* proto-oncogene.

three orders of magnitude. X-ray crystallography of a hydroxylated HIF1α CODDD peptide bound to VBC showed that the exquisite discrimination between hydroxylated and non-hydroxylated sequences is mediated by two optimized hydrogen bonds, which are formed between the alcohol of the hydroxylated proline and two residues of pVHL (Ser111 and His115)[18,19]. In hypoxia, prolyl hydroxylation is suppressed, which allows the HIFα subunit to escape pVHL-mediated destruction and therefore to accumulate to high levels. Three closely related 2-oxoglutarate-dependent oxygenases with the capacity to catalyse HIF prolyl hydroxylation — known as prolyl hydroxylase domain (PHD)1, PHD2 and PHD3 — have been identified[20,21]. They have an absolute requirement for molecular oxygen as a co-substrate and therefore provide a direct link between oxygen availability and the regulation of HIF (FIG. 3).

Asparaginyl hydroxylation. Analysis of the CADs of human HIF1α and HIF2α showed that the oxygen-dependent association of this domain with the CH-1 domain of the p300 co-activator is blocked by the hydroxylation of a specific asparaginyl residue (Asn803 in human HIF1α; Asn851 in human HIF2α)[22]. Further analysis showed that the hydroxylation occurs on the β-carbon[23]. NMR studies of a non-hydroxylated CAD polypeptide bound to CH-1 showed that Asn803 of HIF1α is part of an α-helix that is deeply buried in the molecular interface[24,25]. These findings indicate how β-hydroxylation of Asn803 prevents HIF1α binding to CH-1: it might disrupt both the hydrophobic interactions between the molecules and the α-helix that is formed by CAD in this complex. Like prolyl hydroxylation, HIF asparaginyl hydroxylation is also catalysed by a member of the 2-oxoglutarate-dependent-oxygenase superfamily, but one that is significantly different in sequence to the PHDs[26,27]. It was originally identified as a protein that binds HIF and was named FIH (factor inhibiting HIF)[28]. Asparaginyl hydroxylation therefore provides a second oxygen-regulated mechanism by which HIFα molecules that escape the prolyl-hydroxylation/degradation pathway are prevented from activating transcription through the p300 co-activator (FIG. 3).

Genetic studies have clearly shown the non-redundant operation of each step in the physiological regulation of HIF. In *Caenorhabditis elegans* and *Drosophila melanogaster*, there is a single HIF prolyl hydroxylase,

UBIQUITIN–PROTEASOME PATHWAY
A system of selective, energy (ATP)-consuming protein degradation that involves the linking of ubiquitins to specific proteins and the subsequent targeting of these polyubiquitylated proteins to the 26S proteasome (a multi-catalytic protease).

and genetic inactivation of this hydroxylase results in constitutive stabilization of the HIFα orthologues and activation of the transcriptional response[20,21,29]. In mammalian cells, a small interfering RNA approach has recently been used to specifically inactivate each of the three PHD enzymes in various cell types. In a range of cell lines, these studies have shown that inactivation of PHD2 alone is sufficient to upregulate HIF1α protein levels and HIF-target-gene expression in oxygenated cells, and it has been proposed that PHD2 is the most important enzyme in regulating oxygenated levels of HIF1α under these conditions (REF. 30). However, the extent to which these findings reflect a special activity of PHD2 on HIF1α or the relative abundance of the PHD enzymes in oxygenated cell lines is not yet known. Transfection studies indicate that all three PHD enzymes have the potential to regulate HIF in cultured cells[21]. In the case of the HIF asparaginyl hydroxylase FIH, only one isoform has been identified so far, and genetic manipulation of this isoform has highlighted its non-redundant effect on HIF-dependent transcription under a range of conditions[28].

2-Oxoglutarate-dependent oxygenases

The involvement of two discrete types of 2-oxoglutarate-dependent oxygenase in directing transcriptional responses to hypoxia raises an interesting question — is this class of enzyme particularly suited to an oxygen-sensing function? Enzymes that metabolize molecular oxygen (dioxygen or O_2) are defined as: dioxygenases when both atoms of oxygen are incorporated into their products; mono-oxygenases when one atom is incorporated into a substrate and the other is reduced to water; and oxidases when both atoms are reduced to water. The HIF hydroxylases are therefore dioxygenases and belong to a large group of enzymes that use 2-oxoglutarate (a citric-acid-cycle intermediate and oxoacid) as a co-substrate (BOX 1; for reviews, see REFS 31,32). During catalysis, the splitting of molecular oxygen is coupled to both the hydroxylation of the prime substrate (for example, HIF) and the oxidative decarboxylation of 2-oxoglutarate to give succinate and CO_2. One oxygen atom of molecular oxygen is incorporated into the alcohol that is a result of oxidation of the prime substrate and the other is incorporated into succinate.

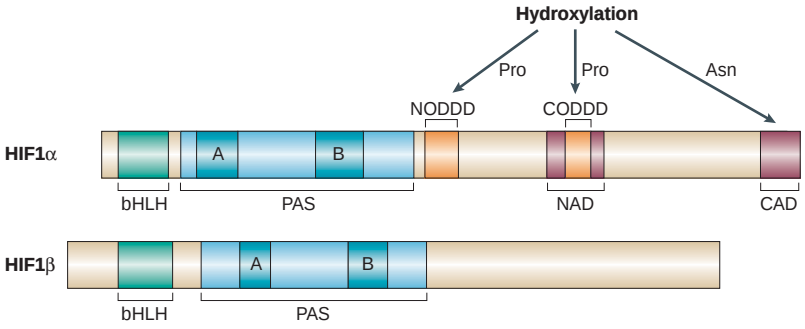

Figure 2 | **The domain structure of HIF1α and HIF1β/ARNT.** The figure highlights: basic helix–loop–helix (bHLH) domains; PAS (PER (period circadian protein), AHR (aryl-hydrocarbon receptor), ARNT (aryl-hydrocarbon-receptor nuclear translocator), SIM (single-minded protein)) domains, which consist of directly repeating A and B sequences that are contained within a region of sequence similarity; the amino-terminal oxygen-dependent degradation domain (NODDD); the carboxy-terminal oxygen-dependent degradation domain (CODDD); the amino-terminal activation domain (NAD); and the carboxy-terminal activation domain (CAD). Note the overlap of CODDD and NAD in hypoxia-inducible factor (HIF)α. The oxygen responsiveness of HIFα is conveyed by separate protein interactions with three regions of HIFα that transfer responsiveness to hypoxia (that is, NODDD, CODDD and CAD), and these interactions involve the oxygen-dependent enzymatic hydroxylation of specific residues in these regions.

The 2-oxoglutarate-dependent-oxygenase family is the largest of several families of non-haem, Fe^{2+}-dependent enzymes that use a conserved two-histidine, one-carboxylate motif to coordinate Fe^{2+} at the catalytic site. These endogenous protein ligands form a 'facial triad' that occupies three of the six possible coordination sites in an octahedral coordination geometry (FIG. 4; for a review, see REF. 32). In the enzyme–Fe^{2+} complex, the remaining three coordination sites are occupied by two to three labile water molecules that are readily displaced by substrates and co-substrates. In the enzyme–Fe^{2+}–2-oxoglutarate and enzyme–Fe^{2+}–2-oxoglutarate–O_2 complexes, 2-oxoglutarate occupies two of these coordination sites and is ligated to Fe^{2+} through its 1-carboxylate and 2-oxo groups. After the loss of a water ligand, which is induced by substrate binding, dioxygen binds to the remaining site.

The coordination chemistry of the 2-oxoglutarate-dependent oxygenases contrasts with that of haem-dependant oxygenases and oxidases, in which the porphyrin ring and the distal ligating amino acid account for five of the six coordination positions. This leaves only one vacant site for binding molecular oxygen and prohibits the binding of a further (co-)substrate to the metal. In these enzymes, the role of the 2-oxoglutarate in the two-electron reduction of molecular oxygen is typically taken by accompanying reductase/iron-sulphur proteins, which couple the reduction of O_2 to the oxidation of NAD(P)H. The flexibility in the coordination chemistry of the 2-oxoglutarate-dependent oxygenases, which is perhaps coupled to conformational changes during catalysis (see below), might facilitate the diverse oxidative reactions that are catalysed by these enzymes, including hydroxylations, oxidative ring closures and desaturations.

The evolutionary origin of the oxygen-sensing function of 2-oxoglutarate-dependent oxygenases in higher animals is unclear, although the superfamily is widely represented across both prokaryotes and eukaryotes. Members of the 2-oxoglutarate-dependent-oxygenase family and related enzymes oxidize both small- and large-molecule substrates and are involved in diverse biological functions. However, none of these processes point clearly to an ancestral oxygen-sensing mechanism, and in lower organisms other types of enzyme have been implicated in this role. The striking conservation of HIF prolyl hydroxylases across nematode worms, insects and vertebrates is apparently confined to higher eukaryotes. Neither the PHDs nor the HIFα orthologues have been identified so far in bacteria, yeast or plants, although a striking exception is the presence of PHD-related proteins in *Pseudomonas aeruginosa* and *Vibrio cholera*[33]. The function of these putative 2-oxoglutarate-dependent

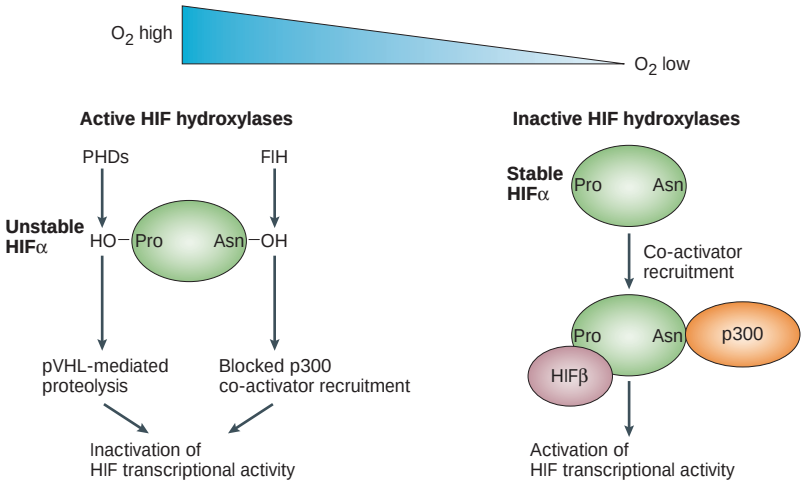

Figure 3 | **Dual regulation of HIFα subunits by prolyl and asparaginyl hydroxylation.** In the presence of oxygen, active hypoxia-inducible factor (HIF) hydroxylases — that is, prolyl hydroxylase domains (PHDs) and factor inhibiting HIF (FIH) — downregulate and inactivate HIFα subunits. PHDs hydroxylate a prolyl residue in the amino- and the carboxy-terminal oxygen-dependent degradation domains (NODDD and CODDD, respectively; see FIG. 2), which promotes von-Hippel–Lindau-tumour-suppressor (pVHL)-dependent proteolysis and results in the destruction of HIFα subunits. FIH, on the other hand, hydroxylates an asparaginyl residue in the carboxy-terminal activation domain (CAD; see FIG. 2), which blocks p300 co-activator recruitment and results in the inactivation of HIFα-subunit transcriptional activity. In hypoxia, HIF hydroxylases are inactive and these processes are suppressed, which allows the formation of a transcriptionally active complex.

oxygenases is unknown and their isolated appearance indicates that they might have arisen in these species by horizontal gene transfer.

Structures of 2-oxoglutarate-dependent oxygenases. X-ray crystallographic analyses of the 2-oxoglutarate-dependent oxygenases have highlighted a core of eight β-strands that are folded into a 'jelly-roll' motif (also known as a double-stranded β-helix)[34–36] (FIG. 5a,b). The three iron-coordinating residues of the facial triad — HXD/EX$_n$H (where X is any amino acid) — are found on or close to the second and seventh β-strands. Such an arrangement has been observed in all structural studies of 2-oxoglutarate-dependent oxygenases so far and is predicted, by sequence conservation and mutational analysis, to be present in many other members of this class[31,33,37]. Other classes of non-haem, Fe^{2+}-dependent oxygenases (for example, the cyclo-oxygenases, *para*-hydroxyphenylpyruvate dioxygenase and catechol dioxygenases)[38] use different structural platforms to present the two-histidine, one-carboxylate motif. In addition, at the start of the catalytic cycle some use Fe^{3+} rather than the Fe^{2+} that is used by 2-oxoglutarate-dependent oxygenases. Importantly, inserts might occur between the β-strands of the jelly-roll motif — in particular, between the fourth and fifth strands — that potentially confuse simple sequence analyses and structural predictions[31]. When such possibilities are allowed for, analysis of genomic data indicates that many other predicted proteins might be 2-oxoglutarate-dependent oxygenases with as-yet-unknown functions[27,33]. Indeed, the functional identification of both the PHD enzymes and the asparaginyl hydroxylase FIH as HIF hydroxylases was achieved following similar structural predictions that were coupled to biological knowledge. The jelly-roll conformation is also present in numerous other enzymes and proteins (including the CUPIN SUPERFAMILY), many of which are not 2-oxoglutarate-dependent oxygenases[39].

Mutational studies of both PHDs and FIH have confirmed the assignment and crucial importance of the predicted Fe^{2+}-coordinating residues[21,26]. For FIH, the predictions have also been verified by X-ray crystallography[40–42] (FIG. 5c). These studies showed that FIH, like other 2-oxoglutarate-dependent oxygenases, contains a single Fe^{2+} that is coordinated almost octahedrally by a two-histidine, one-carboxylate motif (that is, by the side chains of His199, Asp201 and His279; FIGS 4a,5d). The 2-oxoglutarate co-substrate binds the Fe^{2+} in the typical bidentate manner through its 1-carboxylate and 2-oxo groups. However, the FIH residues that interact with the 5-carboxylate of 2-oxoglutarate are unusual. By contrast with certain other 2-oxoglutarate-dependent oxygenases, in which an Arg residue (on or close to the eighth strand of the jelly-roll motif) and a Ser/Thr residue[34,35,43] interact with this carboxylate, in FIH the interacting residues are Lys214 (on the fourth strand of the jelly-roll motif), Thr196 and Tyr145. This and other features identify FIH as a member of a distinct subfamily of 2-oxoglutarate-dependent oxygenases.

The presence of a jelly-roll motif and sequence similarities also define FIH as one of the jumonji transcription factors, members of which have been implicated in cell growth and heart development (for a review, see REF. 44). Owing to the sequence similarities between these factors and zinc-dependent enzymes that contain a jelly-roll motif (for example, phosphomannose isomerase), some of them have been proposed to be zinc-dependent transcription factors, but might in fact be iron- and 2-oxoglutarate-dependent oxygenases. Many, but not all, of the jumonji proteins contain an HXD/EX$_n$H motif. Interestingly, several are predicted to contain a tyrosine in the place of D/E, which might indicate that the consensus sequence should be changed to HXD/E/YX$_n$H or that these proteins have alternative roles. Others, possibly including jumonji itself, do not seem to contain the conserved metal-binding ligands, which indicates that they might produce their biological effect through different, non-oxidative mechanisms.

X-ray crystallography of FIH in complex with the CAD of HIF1α or HIF2α shows that the enzyme–substrate interaction involves at least two distinct sites[41]. At the hydroxylation site, the CAD forms an extensive set of hydrogen bonds with FIH and forms a tight turn that presents the side chain of Asn803 towards the catalytic Fe^{2+} of the active site. Consistent with the importance of this structure, the identity of the adjacent residue (Val802), which is conserved throughout HIFα sequences, is also crucial for substrate activity[45]. Unusually, the active-site complex involves a hydrogen bond between the iron-coordinating carboxylate residue in FIH (Asp201) and the target asparagine in

CUPIN SUPERFAMILY
The cupins are a diverse family of plant proteins, all of which contain at least one double-stranded β-helix or jelly-roll strucural motif. This motif is also present in all structurally characterized 2-oxoglutarate-dependent oxygenases including the HIF hydroxylases, and is characteristic of the jumonji transcription factors.

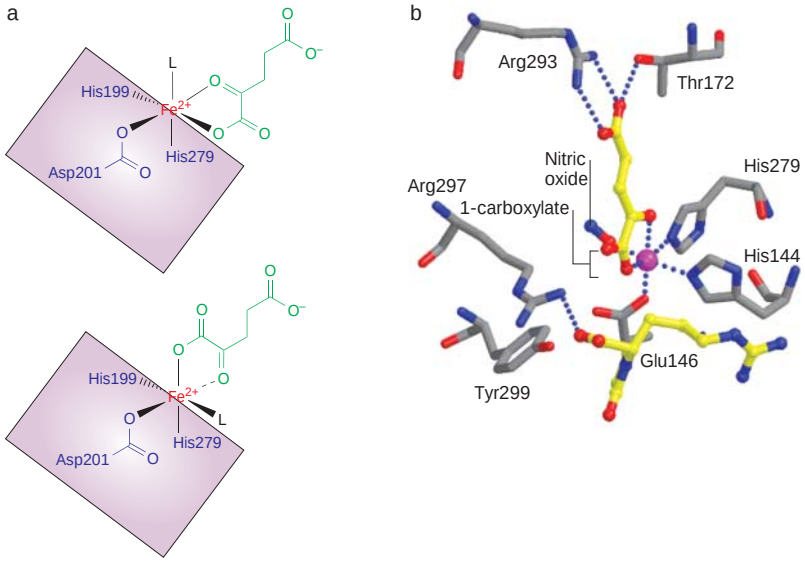

Figure 4 | **The coordination chemistry of 2-oxoglutarate-dependent oxygenases.** **a** | The facial triad of metal-ion-binding residues (highlighted by blue text and the pink shaded region) in the octahderal coordination of Fe^{2+} at the active site of 2-oxoglutarate-dependent oxygenases[32]. The factor inhibiting hypoxia-inducible factor (FIH) numbering system is used. Structural evidence shows that the 1-carboxylate of 2-oxoglutarate (green) can adopt both of the coordination positions that are shown. L represents ligand, which can be either water or dioxygen[48,96]. **b** | The crystal structure of the active site of the 2-oxoglutarate-dependent-oxygenase clavaminate synthase. The enzyme is complexed with Fe^{2+} (purple sphere), 2-oxoglutarate (upper yellow structure), the substrate *N*-acetyl arginine (lower yellow structure), and nitric oxide, which was used as a substitute for molecular oxygen to produce a stable complex[48].

HIF1α (Asn803; FIGS 5d,6). This might require disruption before oxygen can bind at the active site and might therefore affect the oxygen sensitivity of the hydroxylation reaction. Distal to this site, a portion of CAD that is necessary for efficient hydroxylation adopts an α-helical conformation and interacts with a hydrophobic region on the surface of FIH. Comparisons of FIH–CAD crystal structures with NMR structures of CAD bound to the CH-1 domain of p300 show that the conformation of the CAD region surrounding Asn803 changes from an α-helical conformation when bound to p300 (REFS 24,25) to an extended loop when bound to FIH[41]. The CAD that is present in HIFα subunits has been classified as an 'intrinsically unstructured protein', such that in the absence of binding partners the CAD region is disordered. The apparently disordered state for the isolated CAD might reflect the requirement to adopt different conformational states when bound to alternative partners.

Although structural data for PHD–HIFα complexes is not yet available, mutational and deletional studies of HIFα-subunit NODDD and CODDD prolyl hydroxylation sites indicate that recognition by PHD enzymes is probably also complex[15,46,47] and involves further interactions that are discrete from the hydroxylation site.

Enzyme mechanism. Although there are exceptions, spectroscopic, kinetic and structural studies on several different 2-oxoglutarate-dependent oxygenases have indicated a common mechanism in which an enzyme–Fe^{2+} complex first binds 2-oxoglutarate, then its prime substrate and, finally, molecular oxygen (FIG. 6). The binding of 2-oxoglutarate and the prime substrate is reversible. The binding of the prime substrate displaces a water molecule that is coordinated to the Fe^{2+}, and this allows Fe^{2+} to react with TRIPLET-STATE MOLECULAR OXYGEN. Structures of the 2-oxoglutarate-dependent-oxygenase clavaminate synthase bound to Fe^{2+}, 2-oxoglutarate and substrate were compared in the presence and absence of nitric oxide (NO; which was used as a substitute for molecular oxygen to produce a stable complex; FIG. 4b). Various other Fe^{2+} and 2-oxoglutarate complexes of these enzymes were also studied and the results indicate that the binding of molecular oxygen might involve the rearrangement of the 1-carboxylate of 2-oxoglutarate, and the approach of molecular oxygen through the jelly-roll motif[48] (FIGS 5c,6). Interestingly, if such a process occurs in HIF hydroxylases, it could provide a mechanism for oxygen sensing by regulating the access of oxygen to the active site.

Binding of oxygen is followed by the oxidative decarboxylation of 2-oxoglutarate to give succinate, CO_2 and a ferryl species (Fe^{IV}=O) at the iron centre (FIG. 6). This highly reactive intermediate can then oxidize an unactivated C–H bond in the prime substrate. Evidence for intermediates comes from substrate-analogue studies[49], model compounds[50] and spectroscopic analyses[51–53]. The prime-substrate product is released before succinate. The point at which CO_2 is released is uncertain. The sequential binding of co-substrate and prime substrate, which is necessary to trigger oxygen binding, is probably important to limit the generation of reactive oxidizing species in the absence of prime substrate. The generation of such species in a prime-substrate-uncoupled manner can inactivate 2-oxoglutarate and the related oxygenases through self-oxidation, which sometimes leads to fragmentation[54,55]. Typically, the uncoupled turnover of 2-oxoglutarate occurs at ~5% of the rate of its coupled turnover in the presence of saturating concentrations of prime substrate.

Several 2-oxoglutarate-dependent oxygenases, including procollagen prolyl hydroxylase and anthocyanidin synthase, also have a requirement for ascorbate for full catalytic activity[56,57]. Although ascorbate might stimulate activity by reducing Fe^{3+} to Fe^{2+} (either free in solution or at the active site), the stimulation of oxygenase activity by ascorbate might occur by other mechanisms, for instance, by promoting completion of uncoupled cycles. For uncoupled reaction cycles that are catalysed by procollagen prolyl hydroxylase in the absence of prime substrate, the oxidation of 2-oxoglutarate to succinate has been shown to be stoichiometrically coupled to ascorbate[58]. It is believed that one role of ascorbate is to function as a surrogate reducing substrate to 'rescue' the enzyme in the event of the uncoupled production of a ferryl (Fe^{IV}=O) intermediate. Ongoing structural studies on anthocyanidin synthase indicate that ascorbate might also have a role in the binding of prime substrate to the enzyme[57].

TRIPLET-STATE MOLECULAR OXYGEN
Most 'natural' molecules exist in the singlet state — that is, they contain paired electrons. However, the most stable form of molecular oxygen (O_2) is the triplet state, in which there are two unpaired electrons.

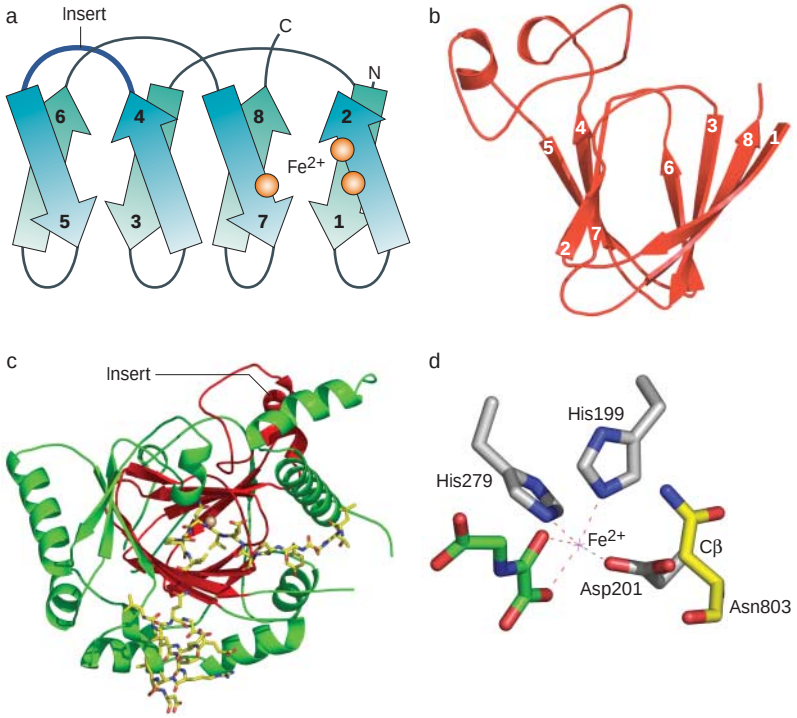

Figure 5 | **Structural insights into 2-oxoglutarate-dependent oxygenases. a** | A schematic representation of the jelly-roll (double-stranded β-helix) core motif that is found in 2-oxoglutarate-dependent oxygenases. Inserts might occur between the β-strands of the jelly-roll motif — in particular, between the fourth and fifth strands (see label). The three iron-coordinating residues of the facial triad are found on or close to the second and seventh β-strands (orange circles)[31,36]. **b** | The jelly-roll core motif of factor inhibiting hypoxia-inducible factor (FIH). **c** | The X-ray crystal structure of FIH showing the Fe^{2+} (orange sphere) and the substrate (yellow). The jelly-roll core motif of FIH is shown in red, whereas other strands and helices are shown in green. Note the apparent 'tunnel' through the double-stranded β-helix[40–42]. The insert between the fourth and fifth β-strands of the jelly-roll core motif is highlighted. **d** | Part of the active site of FIH showing the FIH residues that bind Fe^{2+} (grey), the ligating N-oxalylglycine (a 2-oxoglutarate analogue; green), and the side chain of Asn803 of hypoxia-inducible factor-1α (yellow), the methylene (Cβ) of which becomes hydroxylated[41].

Studies with several enzymes have shown that certain substrate analogues and mutants can also stimulate uncoupled 2-oxoglutarate turnover. This leads to the idea that uncoupled turnover might reflect a proof-reading process, in which incorrect substrates or incorrectly bound substrates can be rejected following the activation of molecular oxygen[35].

Control of HIF-hydroxylase activity

At present, it is not yet clear whether the HIF hydroxylases have evolved unique catalytic features or are relatively 'ordinary' 2-oxoglutarate-dependent oxygenases that simply use their absolute requirement for molecular oxygen in a signalling role. It is also unclear how such a simple and direct interface with oxygen could be used in an intact organism to direct diverse responses in cells that operate at substantially different oxygen tensions (BOX 3). Nevertheless, emerging data indicate that the enzymes are sensitive to moderate hypoxia[20,46], and that their activity might be controlled at several other levels, which potentially provide flexibility for directing physiological responses to hypoxia.

Oxygen. Assays of HIF hydroxylation by cell extracts and recombinant PHD enzymes have been conducted in graded-hypoxia conditions using a controlled-atmosphere work station. A reduction in hydroxylation activity was detected when the oxygen concentration was reduced from 20% to 10%, and progressive inhibition was observed at lower oxygen tensions[20]. This indicates that the effective K_m of PHD enzymes for oxygen might be high in relation to the physiological range of oxygen tensions in tissues (lower K_m values can reflect tighter substrate binding), which supports a sensing function. Although results that are obtained under *in vitro* conditions using non-physiological substrate concentrations are difficult to interpret, this result is consistent with recent studies of 2-oxoglutarate turnover by recombinant PHD enzymes, in which the pO_2 (partial pressure of oxygen) was measured directly in the reaction mix. Under these conditions, the apparent K_m for oxygen for each of the PHD enzymes was in the range of 230–250 μM — a result that contrasts with measurements on procollagen prolyl hydroxylase, which had a K_m of 40 μM (REF. 46). The data for the PHD enzymes are broadly in line with studies of the induction of both HIF1α protein expression and HIF DNA-binding activity by graded hypoxia in tissue-culture cells[59], and indicate that PHD enzymes do manifest the appropriate sensitivity to hypoxia.

For the asparaginyl hydroxylase FIH, similar studies indicate that its apparent K_m for oxygen is lower (being ~90 μM) than that for the PHD enzymes and that its catalytic properties towards other co-substrates are also distinct (the K_m for 2-oxoglutarate is 25 μM for FIH and 55–60 μM for the PHDs, and the K_m for ascorbate is 260 μM for FIH and 140–170 μM for the PHDs)[60]. However, how these parameters translate into kinetic behaviour *in vivo* is still unclear. Transfection experiments have indicated that, at least when overexpressed, FIH can reduce HIF transcriptional activity even at low oxygen tensions[26,28]. This result apparently contrasts with data for transfected PHD3. In this case, suppression of HIF transcriptional activity was observed in oxygenated, but not hypoxic, cells[21]. However, the studies (by different groups) are difficult to compare, particularly as the cells were not pre-equilibrated in hypoxic conditions prior to transfection. Furthermore, as FIH could, in theory, simply compete with CH-1 to bind CAD, it is not yet clear whether all of the suppressive function of FIH on HIF transcriptional activity is enzymatic.

Nitric oxide. NO is an *in vitro* inhibitor of the 2-oxoglutarate-dependent oxygenases. Several studies of the HIF/hydroxylase pathway have shown both apparent inhibition of hydroxylation and activation of HIF transcriptional activity by different NO donors[61–63]. A mechanism for this can be readily envisaged, because NO is known to compete with O_2 for binding to the iron at the active site of 2-oxoglutarate-dependent oxygenases[48]. Nevertheless, other studies have shown that NO donors might reduce HIF transcriptional activity in hypoxia[64,65], and in one study the promotion of

K_m
The Michaelis constant. A kinetic parameter for a specific substrate in an enzyme-catalysed reaction. Providing certain conditions are met, the K_m for a substrate can equate to its binding constant, and the lower the value of K_m, the tighter the substrate binds.

pO_2
The partial pressure that is exerted by molecular oxygen in a mixture of gases. It is also used to define the concentration of molecular oxygen in a solution or biological tissue that is at equilibrium with such a gas mixture.

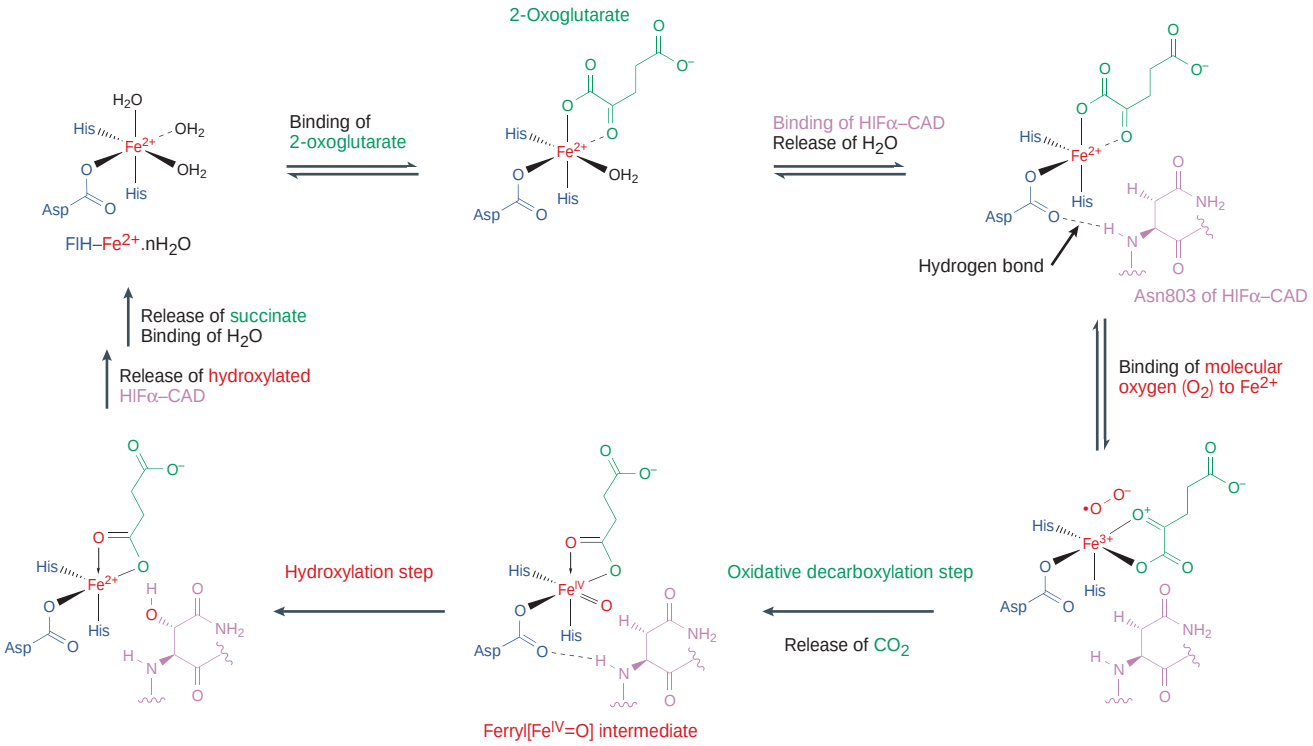

Figure 6 | **Mechanism of the 2-oxoglutarate-dependent-oxygenase FIH.** The figure shows an outline of the mechanism of the 2-oxoglutarate-dependent-oxygenase FIH (factor inhibiting HIF (hypoxia-inducible factor)), which is a HIF asparaginyl hydroxylase. Fe^{2+} is bound to the enzyme through the 'facial triad' of two histidinyl residues and one aspartyl residue[40–42], and the remaining three coordination sites are occupied by two to three labile water molecules. Studies with FIH and other 2-oxoglutarate-dependent oxygenases indicate that, in most cases, the sequential binding of 2-oxoglutarate and then the protein substrate (HIFα subunit) to the active site occurs[31,32,41,52,53]. Binding of the latter displaces a water molecule from Fe^{2+}, which leaves a vacant coordination site. Together, these processes allow the productive binding of triplet-state molecular oxygen. Subsequent oxidative decarboxylation of 2-oxoglutarate generates carbon dioxide, succinate and a ferryl (Fe^{IV}=O) species. The latter is responsible for hydroxylating the β-position of Asn803 in the CAD (carboxy-terminal activation domain) of HIFα subunits. Release of the hydroxylated product probably precedes that of succinate. The point at which carbon dioxide leaves the active site is unclear. Coloured text is used in the figure to highlight the changes that occur at each step.

hydroxylase activity by NO donors has been reported[66]. Overall, this indicates that either NO — or another component of the chemical probes that were used in these studies — has more than one action on the HIF system. Interestingly, a recent study has indicated that NO might reduce the activation of HIF in hypoxia, because it might effectively relieve cellular hypoxia by acting as an endogenous inhibitor of cytochrome-*c* oxidase (complex IV of the mitochrondrial electron-transport chain)[67].

Iron/ascorbate. Iron binding by the two-histidine, one-carboxylate motif is relatively labile, particularly for Fe^{2+} enzymes such as the 2-oxoglutarate-dependent oxygenases. In keeping with this, many 2-oxoglutarate-dependent oxygenases are readily inhibited by iron chelators: inhibition of both the PHDs and FIH by this mechanism readily explains the stabilization of HIFα and the activation of HIF transcriptional activity that is observed when cells are exposed to the iron-chelator desferrioxamine. Active-site Fe^{2+} might also be substituted by other metals that are unable to support the catalytic cycle. Indeed, spectroscopic and structural studies have shown that the active-site Fe^{2+} can be substituted by Co^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+}. So, the classic hypoxia-mimetic function of cobaltous ions can probably be explained by their direct inhibition of the HIF hydroxylases, although the direct binding of cobalt to the HIFα CODDD has been reported, which might contribute to HIFα stability[68].

From the biological perspective, these findings raise an important question: to what extent is HIF-hydroxylase activity regulated by physiological or pathological changes in cellular Fe^{2+} availability? Experiments in tissue-culture cells indicate that such effects could be important, particularly with respect to cancer. The activation of oncogenic pathways is often associated with the upregulation of HIFα protein levels even in oxygenated cells (for reviews, see REFS 69,70). Using hydroxylation-specific antibodies, it has been shown that the HIF1α CODDD is not always fully hydroxylated in such cells[71]. As oxygen availability should not be limiting in these cultures, this indicates that other mechanisms are limiting hydroxylase activity. In a separate line of investigation, it has been shown that supplementation with iron or ascorbate dramatically reduces HIF1α levels in a manner that is dependent on a functional hydroxylase system[72].

SYSTEMIC HYPOXIA
A reduction in the partial pressure of oxygen (pO_2) throughout the organism.

TISSUE ISCHAEMIA
Inadequate blood supply to a tissue, which causes other metabolic abnormalities in addition to hypoxia (for example, the defective delivery of substrates and removal of waste products).

It therefore seems probable that under normal conditions of tissue culture, HIF-hydroxylase activity can be limited by the availability of iron and/or ascorbate.

The mode of action of ascorbate in this situation is unclear. It is possible that ascorbate functions, as discussed above, to reduce the catalytic iron centre in the event of uncoupled activation. However, as similar effects are observed with iron and ascorbate, it is possible that ascorbate functions in a more general way to promote the availability of Fe^{2+}. Several lines of evidence indicate that rapidly growing cancers are associated with a cellular iron deficiency in the tumour mass[73]. Understanding the extent to which this contributes to HIF activation, and potentially to tumour angiogenesis, should be of interest in future studies. The precise definition of the *in vivo* roles of ascorbate is of increasing interest, not only because of its potential involvement in the hypoxic response, but also because of its possible role in DNA repair through stimulation of the 2-oxoglutarate-dependent-oxygenase AlkB[74,75] and related enzymes.

2-oxoglutarate and succinate. Use of the citric-acid-cycle intermediates 2-oxoglutarate, as a co-substrate, and succinate, as a product, provides a potential interface between the HIF hydroxylases and energy metabolism. Although the possibility of dual control by oxygen and energy metabolism is appealing, it is not clear whether cellular 2-oxoglutarate levels are ever limiting. Intriguingly, however, genetic defects in several citric-acid-cycle enzymes have been associated with an increased susceptibility to particular types of tumour that have indirect connections with the hypoxia response. For example, defects in the genes that encode the succinate dehydrogenase subunits B, C and D[76,77] have been associated with familial paraglioma, a type of tumour that most commonly affects the oxygen-sensitive carotid body and the incidence of which is greatly increased in populations living at a high altitude[77]. In this case, impaired hydroxylase activity might be caused by both reduced 2-oxoglutarate concentrations and elevated succinate concentrations, because the latter is both a product and a weak inhibitor of 2-oxoglutarate-dependent oxygenases. Defective fumarate hydratase has been associated with familial susceptibility to papillary renal carcinoma, leiomyomata of the skin, and uterine fibroids[78]. Uterine fibroids are a type of tumour that is (albeit rarely) associated with excessive erythropoeitin production. However, the dysregulation of HIF has not yet been clearly demonstrated in these tumours and it is possible that different mechanisms underlie the tumour predisposition.

Enzyme synthesis/abundance. As the 2-oxoglutarate-dependent oxygenases are not equilibrium enzymes — that is, they do not catalyse the reversal of hydroxylation — the abundance of the HIF hydroxylases will dictate the rate of HIF hydroxylation at any given concentration of oxygen. Studies of their tissue distribution show large differences in expression, at least at the messenger RNA level. For example, PHD1 is particularly abundant in the testis, whereas PHD3 is strongly expressed in the heart[79]. However, whether these differences affect the oxygen dependence of HIF induction *in vivo* remains to be determined. Interestingly, the PHD enzymes can be induced to a high level in response to various stimuli. So far, this has been most completely studied in the context of the response to hypoxia. Both PHD2 and PHD3 (but not PHD1) are strongly induced by hypoxia[20,30]. In keeping with this, the prior incubation of cells in hypoxic conditions results in an enhanced total HIF-hydroxylase activity[20] and a more rapid destruction of HIF1α when cell cultures are re-oxygenated[80]. Induction of the PHDs by hypoxia is at least partly dependent on the transcriptional activity of HIF, so this mechanism effectively provides a feedback control on HIF signalling[30,81].

The expression of certain PHD enzymes is also highly inducible by other stimuli. PHD3 has previously been identified in different cell types as a gene product that is induced by p53, by stimuli that induce smooth-muscle differentiation and by nerve-growth-factor withdrawal[82–84]. PHD1 mRNA has been reported to be an oestrogen-inducible transcript in breast cancer cell lines[85]. However, it is unclear at present how these characteristics impinge on the regulation of the HIF system. Interestingly, both PHD1 (REF. 86) and PHD3 (REF. 83) have been reported to have growth-suppressive effects, although, again, it is not known whether these arise from effects on the HIF system or effects on other pathways.

Other controls on the rate of HIF hydroxylation could be generated by: the production of alternative hydroxylase transcripts[46] that encode less-active or

Box 3 | The oxygen dependence of HIF activation

Studies of cultured cells showed an exponential induction of hypoxia-inducible factor (HIF)1α protein and the DNA-binding activity of HIF as oxygen concentration is reduced over the range 10.0–0.6% (REF. 59). This pattern correlates well with erythropoietin production (one of the most striking homeostatic responses to hypoxia) by the hepatoma cell line Hep3b (REF. 107). Although there are only a few such detailed studies of graded hypoxia, analyses of HIF-target-gene expression in a range of tissue-culture cells, which are derived from widely differing sources, have indicated surprisingly similar characteristics, with maximal induction being observed at ~1% oxygen. This response range overlaps that of oxygen tensions *in vivo* in normal tissues, although a tissue pO_2 (partial pressure of oxygen) equivalent to 1% oxygen would rarely be found in non-pathological states in the intact organism. In keeping with this, immunohistochemical staining of human and rodent tissues for induced HIFα proteins showed that HIFα-subunit levels are generally low in the basal state, but are strikingly increased by SYSTEMIC HYPOXIA or TISSUE ISCHAEMIA.

Interestingly, by contrast with the tissue-culture observations, marked cell-type-specific differences in oxygen thresholds for HIF activation seem to exist *in vivo*. This is highlighted by rodent kidneys, in which a marked heterogeneity in pO_2 is generated by the countercurrent exchange of oxygen between blood vessels that loop from the cortex into the medulla and papilla. Cells near the papillary tip are at a pO_2 of a few mmHg physiologically (equivalent to <1% oxygen), but do not express HIF1α. Nevertheless, papillary HIF1α is strongly induced by systemic hypoxia[108]. In the renal cortex, the pO_2 is much higher. Here, HIF1α is strongly induced in proximal nephron segments by systemic hypoxia, but not in the adjacent distal nephron segments that presumably experience a similar pO_2 (REF. 108). Nevertheless, HIF1α can be induced in the distal nephron by cobaltous ions, which indicates that the regulatory hydroxylase pathway is functioning. The HIF system therefore seems to be set differently in different cells, in a manner that is appropriate for the physiological control of oxygen homeostasis.

interfering products; the post-translational (allosteric) modification of the enzymes; or the post-translational modification of HIFα-subunit target regions, which would affect enzyme–substrate interactions. For example, evidence has been provided for the functionally important phosphorylation of a conserved Thr residue in CAD (Thr796 in the human HIF1α CAD)[87] that is close to the asparaginyl hydroxylation site and might therefore affect the action of FIH.

Protein hydroxylation in non-HIF signalling?

Overall, accruing data on the HIF hydroxylases indicates the existence of numerous controls that could provide the flexibility that is required to govern the physiological activation of HIF by hypoxia. Furthermore, the discovery of protein hydroxylation as a new regulatory pathway that controls HIF raises the possibility that similar processes could be involved in signalling other biological responses to hypoxia. Recently, it has been proposed that the large subunit of RNA polymerase II is also targeted for pVHL-dependent ubiquitylation by prolyl hydroxylation[88]. Interestingly, on the basis of the responses of iron regulatory protein-2 to the inhibitory 2-oxoglutarate analogue dimethyl-oxalylglycine, it has been proposed that 2-oxoglutarate-dependent oxygenases are also involved in cellular iron sensing[89]. Similar arguments have indicated a role for 2-oxoglutarate-dependent dioxygenases in the HIF-independent, but oxygen-sensitive, generation of the signalling molecule phosphatidic acid[90]. A 2-oxoglutarate-dependent oxygenase has also been described that hydroxylates the Asp and Asn residues in human epidermal-growth-factor domains[91]. And, although the functional significance of this hydroxylation is unknown, it seems reasonable to propose that it is involved in signalling. The discovery that a 2-oxoglutarate-dependent-oxygenase AlkB can repair DNA that is methylated at 1-methyladenine and 3-methylcytosine by oxidation of the methyl group[74,75] raises the possibility that signalling and regulation involving oxygen might also occur through the direct modification of (methylated) nucleic acids. This might extend to 5-methylcytosine, which is a common site of methylation in higher eukaryotes that is important in the regulation of gene expression. Together with database predictions of additional 2-oxoglutarate-dependent enzymes that are related to the HIF hydroxylases[41], including the jumonji proteins, these observations indicate that 2-oxoglutarate-dependent oxygenases might be widely involved in cell signalling. Indeed, it might be that many of the jumonji proteins would be more accurately described as 2-oxoglutarate-dependent oxygenases that are involved in transcriptional regulation.

In considering potential targets, it is important to recognize that, as far as we know, the reactions catalysed by these enzymes are irreversible. In the case of HIFα subunits, their rapid re-synthesis enables the inactivation to be reversed. We would therefore predict that other protein substrates that are involved in irreversible signalling pathways should also be rapidly turned over, or be targeted to other enzymes that could reduce, or otherwise modify, the hydroxylation site to reverse the direction of signalling (for example, by the reduction or phosphorylation of hydroxyl groups).

Overall, the new insights into the regulation of HIF by protein hydroxylation offer many exciting possibilities for future research. As well as furthering our understanding of the physiological response to hypoxia, there is the possibility — which is attracting widespread interest — that pharmacological manipulation of these signalling pathways by hydroxylase inhibitors[60,92–95] might offer a new route to the therapy of ischaemic/hypoxic disease.

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Competing interests statement

The authors declare competing financial interests: see Web version for details.

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