The importance of oxygen for the survival of multicellular and aerobic organisms is well established and documented. Over the years, increased knowledge of its use for bioenergetics has placed oxygen at the centre of research on mitochondria and ATP-generating processes. Understanding the molecular mechanisms governing cellular oxygen sensing and response has allowed for the discovery of novel pathways oxygen is involved in, culminating with the award of the Nobel Prize for Medicine and Physiology in 2019 to the pioneers of this field, Greg Semenza, Peter Ratcliffe and William Kaelin. However, it is now beginning to be appreciated that oxygen can be a signalling molecule involved in a vast array of molecular processes, most of which impinge on gene expression control. This review will focus on the knowns and unknowns of oxygen as a signalling molecule, highlighting the role of 2-oxoglutarate-dependent dioxygenases as central players in the cellular response to deviations in oxygen tension.

Introduction

Molecular oxygen (O₂) is fundamental for cellular bioenergetics and metabolism and thus a critical factor in the normal function and survival of most multicellular organisms. As such, essential mechanisms have been developed in all aerobic multicellular species to sense and respond to changes in O₂ levels. This is especially pertinent in conditions of low oxygen tension (hypoxia), whereby regulating the consumption of oxygen is essential to maintain oxygen homeostasis and ensure cellular survival. Indeed, when the oxygen-sensing and response mechanisms are disrupted, it leads to defects in development as well as problems in a variety of human conditions [1]. Alterations to the oxygen-sensing pathway have been implicated in conditions...
such as diabetic wound healing, organ transplants and cancers such as renal clear cell carcinoma [1–3].

**Hypoxia-inducible factors (HIFs)**

The central pathway involved in maintaining oxygen homeostasis in response to limited O₂ availability is predominantly governed by the stabilisation of a family of α, β-heterodimeric transcription factors, known as hypoxia-inducible factors (HIFs). Identified by Semenza et al. [4], and activated in response to hypoxia, HIF-1α protein levels markedly increase when cellular O₂ concentration is decreased [5]. In humans, there are three, O₂-sensitive HIF-α isoforms, HIF-1α, HIF-2α and HIF-3α [6]. In contrast, the HIF-β subunit is constitutively expressed and, for the most part, not responsive to O₂ levels [7,8]. Furthermore, depending on tissue type, each isoform potentially functions differently (reviewed in Ref. [7]).

HIF activity is directed through oxygen-regulated signals, which are generated by a class of 2-oxoglutarate-dependent (2-OG) dioxygenases, named prolyl-hydroxylases (PHDs). This group of enzymes acts as enzymatic oxygen sensors within the cell, possessing an affinity for O₂ comparable to atmospheric concentrations [9,10]. As such, in normoxia, PHDs are fundamental in the catalysis of an O₂-dependent, post-translational modification of HIF-α, through the hydroxylation of target proline (Pro) residues. PHD-mediated hydroxylation of the proline residues results in more than 1000-fold increase in the affinity for binding to the von Hippel-Lindau ubiquitin E3 ligase complex (VHL) [11–13]. Following VHL-mediated polyubiquitination, HIF-α is targeted for rapid degradation by the proteasome. In conditions of O₂ depletion (see below), the HIF complex is functional and binds hypoxia-responsive elements (HREs) across the genome. This process promotes transcriptional regulation of numerous genes involved in processes such as angiogenesis, autophagy, metabolism and motility, culminating in increased oxygen delivery and reduced consumption (reviewed in Refs. [14,15]).

**Prolyl-Hydroxylases**

Initially identified in *Caenorhabditis elegans* [9] where the prolyl-hydroxylation reaction is catalysed by a single nonredundant prolyl-hydroxylase egl nine homolog 9 (egl9) gene, the human genome contains three homologous genes, which encode HIF PHD domains 1 (EGLN2), 2 (EGLN1) and 3 (EGLN3) [9,16]. It must be noted that whilst each of the three PHD enzymes contributes to the regulation of HIF, in *vivo*, PHD2 is the most abundant enzyme and is the main mediator of HIF-1α hydroxylation [17]. PHDs hydroxylate several proline residues in the different HIF-α isoforms: HIF-1α – Pro402 and Pro564; in HIF-2α – Pro405 and Pro531; in HIF-3α – Pro492 [18–22]. In addition, PHDs have a preference for different prolines in HIF-α subunits as well as some preference between HIF-α isoforms [23].

Under low O₂ conditions, the activity of PHDs is restricted by O₂ availability. The reported O₂ K_M for the PHDs is ~ 230 µM (Table 1) [10,24–26], which is above the approximate O₂ 220 µM concentration of atmospheric air, and far above reported O₂ levels of human tissues. This characteristic of the PHDs confers high sensitivity to changes in O₂ concentration at a cellular level, with PHD2 being reported as the most sensitive enzyme and hence are considered molecular O₂ sensors in the cell.

**Non-HIF PHD targets in Hypoxia**

Until 2011, HIF-α was the only validated target for PHDs. However, subsequent studies have identified substrates other than HIF that are hydroxylated and regulated by the PHD enzymes in response to hypoxia (reviewed in Ref. [27]). Although remaining a controversial issue [28,29], it is unlikely that organisms have evolved three separate enzymes for one single target. As such, in addition to regulating HIF levels in response to fluctuating levels of O₂, PHDs could also mediate post-translational modifications that serve to regulate other cellular pathways, which require prompt O₂-dependent regulation. A number of studies have reported novel functions for these enzymes based on cellular analysis, rather than *in vitro* readouts (Fig. 1). Tissue and PHD specificity may play important roles in facilitating significant hydroxylation beyond HIF. For example, p53, a tumour suppressor gene and regulator of numerous cellular responses, has expression levels reported to be increased [30–32] or reduced [33,34] under hypoxic conditions. These seemingly conflicting data could indicate the role of hydroxylation, as two separate studies have shown that p53 can be hydroxylated by both PHD3 at Pro359 [35] and PHD1, probably at Pro142 [36]. Here, we will explore the other published individual PHD targets affected by hypoxia in cells.

**PHD1**

The process of proceeding through the cell cycle is an extremely energy-consuming event; therefore, in suboptimal conditions, such as O₂ deprivation or metabolic...
perturbation, cell cycle arrest occurs. The involvement of PHD1 in modulating levels of cellular proteins has been shown to play a role in the regulation of cell cycle progression (Fig. 1) [37–40].

The protein levels of the centrosomal protein 192 (Cep192), which is required for centrosome maturation and centriole duplication [41,42], were shown to be elevated after 2 and 4 h of 1% O2 in U2OS cells. The elevated levels were comparable to those exhibited by HIF-1α. Further investigation identified that Cep192 is hydroxylated by PHD1 at proline residue 1717 [37]. Likewise, the transcription factor forkhead box O3a (FOXO3a) has been shown to be hydroxylated by PHD1 at Pro residues 426 and 437 both in vitro and

| Type                      | Enzyme     | O₂ Kₘ (µM) | Substrate                               | Assay                                           | Reference |
|---------------------------|------------|------------|-----------------------------------------|------------------------------------------------|-----------|
| Hydroxylase               | PHD1       | 230        | HIF-1α(556-574)CODD                      | Radioactivity 2-OG turnover assay               | [10]      |
|                           | PHD2(181-426) | 229 ± 60   | HIF-1α(556-574) CODD                      | O₂ consumption assay, radioactivity 2-OG turnover assay, MALDI-TOF mass spec assay, TR-FRET | [24,10,25,26] |
|                           |            | 250        | HIF-1α(556-574) CODD                      | Radioactivity 2-OG turnover assay               | [24]      |
|                           |            | 1746 ± 574 | HIF-1α(530-698)CODD                      | O₂ consumption assay                           |           |
|                           | PHD3       | 230        | HIF-1α(556-574) CODD                      | Radioactivity 2-OG turnover assay               | [10]      |
|                           | C-P4H1     | 40         | (Pro-Pro-Gly)10                          | Radioactivity 2-OG turnover assay               | [10]      |
|                           | mPAHx      | 93 ± 43    | Isovaleryl CoA                            | O₂ consumption assay                           | [24]      |
|                           | TauD       | 76 ± 17    | Taurine                                  | O₂ consumption assay                           | [24]      |
|                           | FIH        | 150 ± 30   | HIF-1α(788-822) CAD                      | O₂ consumption assay, radioactivity 2-OG turnover assay, MALDI-TOF-MS | [24,76,77] |
|                           |            | 90 ± 20    |                                          |                                                 |           |
|                           |            | 110 ± 30   |                                          |                                                 |           |
|                           |            | 237 ± 28   | His6–HIF-1α (653–826) CAD                 | O₂ consumption assay, radioactivity 2-OG turnover assay, MALDI-TOF-MS | [24]      |
|                           |            | 110 ± 73   | HIF-2α(852–866)-CAD                      | O₂ consumption assay, radioactivity 2-OG turnover assay, MALDI-TOF-MS | [24,77]   |
|                           |            | 200 ± 10   | Non-HIF substrates (ankyrin-containing)   | MALDI-TOF-MS, radioactivity 2-OG turnover assay | [77,161]  |
| Tet1                      | 30 ± 10    | 5-mC Oligonucleotide                      | Radioactivity 2-OG turnover assay               | [122]     |
| Tet2                      | 0.31%      | Cellular 5-mC                             | Cell assay                                    | [125]     |
|                           | 30 ± 3     | 5-mC Oligonucleotide                      | Radioactivity 2-OG turnover assay               | [122]     |
| Lysine demethylase        | KDM4A(1-359)| 173 ± 23  | H3(1-15)K9me3                            | MALDI-TOF-MS                                   | [106]     |
|                           | KDM4A(1-359)| 57 ± 10   | H3(7-14)K9me3                            | O₂ consumption assay                           | [106]     |
|                           | KDM4C(1-366)| 158 ± 13  | H3(7-14)K9me3                            | O₂ consumption assay                           | [106]     |
|                           | KDM4E(1-337)| 197 ± 16  | H3(1-21)K4me3                            | Radioactivity 2-OG turnover assay               | [105]     |
|                           | KDM5A      | 90 ± 30    | H3(1-21)K4me3                            | Radioactivity 2-OG turnover assay               | [105]     |
|                           | KDM5B      | 40 ± 10    | H3(21-44)K27(me3)                        | Radioactivity 2-OG turnover assay               | [105]     |
|                           | KDM5C      | 35 ± 10    |                                          |                                                 |           |
|                           | KDM5D      | 25 ± 5     |                                          |                                                 |           |
|                           | KDM6A      | 180 ± 40   |                                          |                                                 |           |
|                           | KDM6B      | 20 ± 2     |                                          |                                                 |           |
|                           | ADO        | > 500      | RGS4(2-15)                               | UPLC-MS-TOF                                    | [134]     |
|                           |            | (55.1% ± 6.6%) |                                           |                                                 |           |
|                           | ADO        | > 500      | RGS5(2-15)                               | UPLC-MS-TOF                                    | [134]     |
|                           |            | (47.1% ± 5.01%) |                                           |                                                 |           |
in vivo [38]. Although the hydroxylation events ultimately promote the proteasomal degradation of Cep192 and FOXO3a, they do so through different mechanisms. Hydroxylation of Cep192 results in recruitment of the SKP-Cullin-F-box protein (SCF)-S-phase kinase-associated protein 2 (Skp2) E3 ligase complex and its ensuing ubiquitination, whilst hydroxylated FOXO3a blocks its deubiquitination by
destabilising its interaction with ubiquitin-specific protease 9x (USP9x). Degradation of FOXO3a, in normoxia, leads to the accumulation of cyclin D1, which is an essential cyclin in G1 of the cell cycle. These contrasting mechanisms as a result of proline hydroxylation indicate the modification can either promote or prevent protein interactions.

PHD2

Whilst PHD2 is the foremost PHD responsible for the hydroxylation of HIF-a, it has been shown to similarly regulate dendritic spines and synaptic transmission in response to fluctuating O2 levels, by controlling filamin A (FLNA) levels (Fig. 1). The protein levels of FLNA, an actin cross-linker, are stabilised by hypoxia and dimethyloxalylglycine (DMOG) treatment in mouse hippocampal neurons, independent of Flna mRNA levels. FLNA protein levels are subsequently regulated through its interaction with VHL, though the interaction required PHD2-mediated hydroxylation of FLNA at Pro residues 2309, 2316 and potentially 2312 [43].

Likewise, PHD2 has also been shown to hydroxylate the serine/threonine kinase (AKT) (Fig. 1) at four separate Pro residues (125, 313, 318 and 423) [44]. Hydroxylation at these residues, under normoxia, induces VHL-dependent inhibition of the AKT kinase activity, but independent of its degradation. However, the authors show that in hypoxic conditions and in cells deficient of a functional VHL, nonhydroxylated AKT is activated, which promotes cell survival and tumorigenesis.

PHD3

PHD3 is the PHD enzyme, which has mostly been associated with the plethora of identified non-HIF PHD targets within the literature (Fig. 1). Firstly, using yeast 2-hybrid technology, Kéditz et al. [45] identified an O2-dependent stabilisation of the activating transcription factor-4 (ATF-4) protein, which is mediated by interaction with PHD3 within the ATF-4 zipper II domain. In addition to 1% O2, DMOG, a competitive 2-OG inhibitor, was able to induce ATF-4 reporter activity, whilst after 4 h of 1% O2 ATF-4 protein levels increased, similar to those of HIF-1a in HeLa cells. Likewise, DMOG in normoxia also increased ATF-4 protein. However, hypoxic stabilisation of ATF-4 protein decreased to normoxic levels after 24 h of hypoxia, matching the hypoxic induction of PHD3. The role of PHD3 was confirmed as PHD3 siRNA treatment led to upregulated ATF-4 protein levels. The oxygen-dependent stabilisation of ATF-4 was partially conditional on the PHD3-dependent hydroxylation of 5 proline residues, located within amino acids 154–181, and was able to confer both adaptive responses to the hypoxic environment alongside cell fate decisions [45].

Similarly, Xie et al. [46] demonstrate that G protein-coupled receptor (GPCR) stability is regulated by oxygen-dependent hydroxylation mediated by PHD3. In normoxia, PHD3 interacts directly with the b2-adrenergic receptor (b2-AR), a guanine nucleotide-binding GPCR, to hydroxylate Pro residues 382 and 395. Hydroxylation at these prolines results in interaction with and ubiquitination by the VHL-E3 ligase complex and proteasomal degradation, similar to HIF-a proteins. However, in low O2 tensions, hydroxylation and degradation of the b2-AR receptor decrease substantially, resulting in increased b2-AR stability and abundance. Since b2-AR is highly abundant in cardiac and smooth muscle in vivo, similar to PHD3 [47], the results from Xie and colleagues correlate with b2-AR function, whereby in response to ischaemic conditions it enhances alveolar fluid clearance and bronchodilation to increase O2 uptake alongside cardiac output to increase O2 delivery [48].

A study by Xie et al. [49] identified a link between the DNA damage response pathway responding to changes in ambient oxygen tensions and the hydroxylation of the DNA damage protein human biological clock protein 2 (hCLK2) by PHD3, in FLAG-PHD3 stable HeLa cells. Hydroxylation was shown to mediate its interaction with ataxia telangiectasia and Rad3-related protein (ATR), a DNA damage checkpoint kinase, which culminated in the activation of the ATR/CHK1/P53 pathway. Subsequent inhibition of PHD3, either with hypoxia or DMOG treatment, proceeded to stop activation of the pathway and decrease DNA damage-induced apoptosis. However, DMOG treatment was unable to stop HCLK2 forming a stable complex with PHD3, suggesting that the association is independent of DNA damage or hydroxylation. Not all novel targets possess HIF-independent functions, as Luo et al. [50] reported that hydroxylation of pyruvate kinase M2 (PKM2) by PHD3 promoted HIF-1a interaction and augmented transactivation of HIF target genes through enhanced HIF-1a HRE binding, and p300 recruitment. Using antihydroxyproline antibodies in addition to mass spectrometry, hydroxylation of PKM2 was identified on prolines 403 and 408 in cancer cells. Inhibition of PKM2’s co-activator function following PHD3 knockdown increases oxygen consumption, in addition to reducing glucose uptake and lactate production in cancer cells. As such,
PKM2 is involved in a positive feedback loop, which ultimately causes the promotion of gene transcription, through HIF-1α transactivation, which reprogrammes glucose metabolism in cancer cells.

An additional oxygen-sensitive signalling pathway was illustrated by Heir et al. [51], whereby the expression/turnover level of the cell surface erythropoietin (EPO) receptor (EPOR) is regulated by oxygen tension. The authors showed that reducing oxygen concentrations until 1% O₂ noticeably increased total EPOR protein levels in human erythroleukemia UT-7 cells, yet, EPOR mRNA did not differ at any hypoxia time point. In addition to hypoxia, molecular inhibition of PHD3 or VHL demonstrated similar EPOR turnover and downstream signalling, in the presence of EPO. An in vitro hydroxylation assay, using purified GST-PHD3 and His-CytoEPOR, followed by mass spectrometry, identified that PHD3 hydroxylates the conserved proline residues 419 and 426, within the EPOR cytoplasmic region. Hydroxylation of Pro419 was shown to be the more critical modification for high affinity binding to VHL. However, oxygen-dependent prolyl-hydroxylation on endogenous EPOR could not be specifically determined by mass spectrometry, since < 1% of total cellular endogenous EPORs are located on the cell surface [52].

The demonstration of hydroxylation for many of the non-HIF PHD substrates both in vitro and in vivo through mass spectrometry methods has up-to-now proven challenging. As such, the studies highlighted have undertaken a multitude of methods to identify the targets outlined in this review (for a more detailed review on the methods, see Strowitzki et al. [27]). As mentioned above, a recent study failed to detect hydroxylation on a vast number of the reported substrates using an in vitro method [28]. As such, in vivo analysis will be more informative, particularly when combining state-of-the-art mass spectrometry analysis with enrichment protocols of extracts derived from cells and/or tissue where PHD function has been altered.

Currently, several novel small-molecule PHD inhibitors (vadadustat, molidustat and daprodustat) are in the advanced stages of clinical trials for the treatment of anaemia associated with chronic kidney disease [53–57]. A further compound, roxadustat (FG-4592), has recently been approved for clinical use in America and China, alongside several other countries [58]. Also, pharmacological stabilisation of HIF-1α and -2α has potential therapeutic benefit for other hypoxia-related diseases [59–63], including inflammatory bowel diseases [64–66], myocardial ischaemia–reperfusion injury [67–69] and wound healing [70,71]. However, PHD inhibitors, currently in trials or approved for use, could potentially have numerous actions independent of HIF, as evidenced by the increasing numbers of potential non-HIF PHD targets, which are involved in a variety of cellular regulation.

**Factor inhibiting HIF (FIH)**

In addition to the PHD enzymes, HIF-α can be regulated by another member of the 2-OG-dependent oxygenase superfamily known as factor inhibiting HIF-1 (FIH) [72]. However, FIH differs significantly in sequence to the PHDs [73,74]. In normoxia, in mammals, FIH regulates HIF transcriptional activity through hydroxylation of the C-terminal asparagine residue Asn803 in HIF-1α and Asn851 in HIF-2α (reviewed in Ref. [75]). The subsequent HIF asparaginyl hydroxylation causes a decrease in binding of HIF to the transcriptional co-activator proteins (CREB-binding protein (CBP)/p300) [73,74]. FIH has reported O₂ K₉ of 90–237 μM [24,76,77], which could suggest that FIH is less likely to act as an O₂ sensor. Interestingly, this O₂ dependence is much less pronounced for FIH’s activity in hydroxylating ankyrin-containing substrates, suggesting that its O₂ sensitivity is peculiar to the HIF pathway.

Of note, whilst the activity of the PHD and FIH enzymes is essentially dependent on molecular O₂ as a cosubstrate, they are also 2-OG- and Fe²⁺-dependent dioxygenases. Hence, in addition to acting as cellular O₂ sensors [18], owing to the use of 2-OG as an essential cosubstrate, PHDs are also sensitive to alterations in specific Krebs cycle intermediates and hence metabolic sensors [78,79].

Further, it is interesting to note the variety of phenotypes elicited in mice in response to differential deletion of these enzymes. Whilst deletion of PHD2 results in embryonic lethality [80], highlighting its important role in developmental hypoxia, deletion of PHD1, PHD3 and FIH presents quite distinct and milder phenotypes [80–84]. PHD1 deficiency has been associated with hypoxia tolerance via reduced oxidative stress generation [82]. PHD3 deficiency resulted in altered sympathoadrenal development and systemic hypotension [83], whilst FIH deficiency has revealed altered metabolism [84]. Tissue-specific contributions for each of the PHD enzymes are therefore very likely and have been identified with the use of conditional deletions for these enzymes [85,86].

**JmjC histone demethylases**

Histone methylation is a highly dynamic and reversible modification [87]. Histones are a core part of chromatin. Eukaryotic DNA is stored in the nucleus, with
DNA–histone octamer complexes, termed nucleosomes, packaged together with linker DNA and other proteins to form chromatin. Chromatin is a fundamental regulator of gene expression, as accessibility to the DNA by the transcriptional machinery is dependent on the chromatin context [88]. Open chromatin is associated with actively transcribed or poised genes. Conversely, closed chromatin is associated with silenced genes and provides a barrier to transcription. There are various mechanisms by which chromatin structure is controlled, which together facilitate in determining the transcriptional output and cell fate decisions. Such mechanisms include histone post-translational modifications [89], covalent DNA modifications [90], incorporation of histone variants [91] and action of chromatin remodelling complexes [92]. In the context of this review, we will focus on histone and DNA methylation as mechanisms controlling chromatin with direct relevance to O2 levels.

Specific histone methylation groups provide binding sites for recruitment of distinct protein complexes to genomic loci. Some histone methylations such as histone (H)3 lysine (K)4 trimethylation (me3) and H3K36me3 are associated with active gene transcription, whereas others, such as H3K27me3 and H3K9me3, are associated with gene silencing [89]. With the exception of H3K4me1/me2 and H3K9me1/me2, which can also be demethylated by the lysine-specific demethylase (LSD) family of histone demethylases, histone lysine demethylation is performed by jumonji C (JmjC) proteins, characterised by the presence of a JmjC domain [93]. There are 32 identified JmjC proteins in humans, 23 of these display histone demethylase activity in cells [87]. Depending on substrate specificity, individual JmjC proteins can function as transcriptional co-activators, repressors or both. They can be classified into 7 subfamilies (lysine demethylase (KDM)2–KDM8) based on sequence homology and substrate specificity (Table 2).

| KDM class | Gene name/other name | Histone substrate in cells |
|-----------|----------------------|-----------------------------|
| KDM2      | KDM2A/FBXL11/JHDM1A  | H3K36me1/me2                |
| KDM2      | KDM2B/FBXL10/JHDM1B  | H3K36me1/me2, H3K4me3       |
| KDM3      | KDM3A/JMJD1A/JHDM2A/TSGA | H3K9me1/me2                |
| KDM3      | KDM3B/JMJD1B/JHDM2B  | H3K9me1/me2                 |
| KDM3      | JMJD1C/KDM3C         | H3K9me1/me2                 |
| KDM4      | KDM4A/JMJD2A/JHDM3A  | H3K9me2/me3, H3K36me2, H1.4K26me2/me3 |
| KDM4      | KDM4B/JMJD2B/JHDM3B  | H3K9me2/me3, H3K36me2, H1.4K26me2/me3 |
| KDM4      | KDM4C/JMJD2C/JHDM3C/GASC1 | H3K9me2/me3, H3K36me2, H1.4K26me2/me3 |
| KDM4      | KDM4D/JMJD2D/JHDM3D  | H3K9me2/me3                 |
| KDM4      | KDM4E/JMJD2E         | H3K9me3                     |
| KDM5      | KDM5A/JARID1A/ RB2   | H3K4me2/me3                 |
| KDM5      | KDM5B/JARID1B        | H3K4me2/me3                 |
| KDM5      | KDM5C/JARID1C        | H3K4me2/me3                 |
| KDM5      | KDM5D/JARID1D        | H3K4me2/me3                 |
| KDM6      | KDM6A/UTX            | H3K27me2/me3                |
| KDM6      | KDM6B/JMJD3         | H3K27me2/me3                |
| KDM7      | KDM7A/KIAA1718/JHDM1D | H3K9me1/me2, H3K27me1/ me2 |
| KDM7      | PHF8/KDM7B/JHDM1F/KIAA1111 | H3K27me1/me2, H4K20me1 |
| KDM7      | PHF2/KDM7C/JHDM1E/CENP35 | H3K9me2/me3 |
| KDM8      | KDM8/JMJD5          | H3K36me2                     |
| NA        | HR                   | H3K9me1/me2                 |
| NA        | RIOX1/NO66           | H3K4me2/me3, H3K36me2/ me3 |
| NA        | RIOX2/MINA53         | H3K9me3                     |

In direct relevance to oxygen sensing, JmjC proteins, like the HIF hydroxylases, PHD and FIH, are 2-OG-dependent dioxygenases [87,94]. They also bear high structural similarity at the catalytic site with FIH [95,96]. Several groups have reported histone hypermethylation in hypoxia and hypoxia-associated diseases [97–105]. Importantly, in vitro studies determining O2 affinities of JmjC proteins indicate that some have the potential to function as physiological O2 sensors (Table 1) [105–108]. However, until recently, direct evidence linking histone hypermethylation to hypoxia-mediated cellular function via JmjC protein O2 sensing was lacking. Chakraborty et al. have shown that hypoxia induces H3K27me3 hypermethylation in human and mouse cell lines in a HIF-independent manner [105,109,110]. They found that the H3K27me3 targeting JmjC protein, KDM6A, is oxygen-sensitive in vitro and in cell culture. Structural and mutational analysis revealed that two residues in the 2-OG- and Fe2+–binding pocket of KDM6A (M1190 and E1335), which are nonconserved in the only other KDM6 family member, KDM6B, confer the physiological oxygen sensitivity of KDM6A. Additionally, inactivation of KDM6A in hypoxia drives H3K27me3 hypermethylation and this is needed for hypoxia-mediated inhibition of myogenic differentiation in mouse myoblasts, with increases in this repressive mark accumulating at late
myogenic genes, blocking their expression. Batie et al. reported HIF-independent increases in various histone methylation marks in human cell culture lines, which were reversed upon reoxygenation [104,109–111]. Strikingly, these changes were observed as early as 30 min of exposure to 1% O2. Increases in H3K4me3 and H3K36me3 at a subset of hypoxia-inducible gene loci were found prior to changes in their gene expression. KDM5A depletion mimicked hypoxia-induced changes in H3K4me3 and gene expression at hypoxia-inducible genes, and cell sensitivity to H3K4me3 in hypoxia was altered by changing the levels of KDM5A, suggesting it functions as an O2 sensor. Earlier work also attributed hypoxia-induced H3K4me3 hypermethylation to loss of H3K4 targeting JmjC protein demethylase activity, with KDM5A depletion increasing H3K4me3 at a subset of hypoxia-inducible genes [99]. Whilst KDM5A has the highest O2 K_M of the KDM5 family members (Table 1) [105], the oxygen sensitivity of KDM5A could not be rationalised by nonconserved active site residues, although mutational analysis found that nonconserved residues in other functional domains of the protein were regulating KDM5As demethylase activity under low oxygen in cells [104]. The authors proposed that relative levels of JmjC proteins, in addition to O2 affinities, are important in determining their ability to function as physiological oxygen sensors, and this will contribute to cell and tissue specificity of hypoxia responses and sensitivity to changes in O2 levels [104]. The aforementioned studies are supported by previous work, which also found hypoxia-mediated increases in H3K4me3 and H3K27me3 at hypoxia-regulated genes [102].

Several other studies have investigated JmjC protein demethylase activity in response to low oxygen. In HeLa cells, KDM3A maintained its activity in 0.2% O2, but KDM4B showed reduced activity [112]. KDM4A activity in U2OS cells was reduced in a step-wise fashion at 5%, 1% and 0.1% O2 [108]. Determination of the KDM4 O2 K_M revealed a lower functional level of O2 affinity compared to PHDs for KDM4C (158 µM) and KDM4E (197 µM), but not KDM4A [106]. The ability of KDM4A to function as an O2 sensor was supported by in vitro work with a reported K_M for oxygen of 173 µM (Table 1). Interestingly, work from the Hammond laboratory found that depletion or inhibition of KDM4A in normoxia or 2% O2 in RKO cells increased H3K9me3 at the HIF-1α gene and reduced HIF-1α mRNA and protein, whereas no effect was observed when depleting or inhibiting KDM5A at > 0.1% O2 [103]. In this context, KDM4A activity under less severe oxygen deprivation helps maintain hypoxia-induced HIF, possibly counteracting residual PHD activity, and this function is likely lost under more severe O2 deprivation, where PHDs and KDM5A are strongly inhibited. KDM4C has also been shown to maintain its histone demethylase activity at low oxygen concentrations and both KDM4C and KDM3A can function as HIF co-activators via demethylation of HIF target gene promoters in hypoxia [113,114]. KDM6B has also been identified as a potential HIF co-activator [115].

Many JmjC proteins are hypoxia-inducible, some of which have been validated as HIF target genes [87,116]. This was initially thought to be a compensatory mechanism to account for reduced activity in response to low oxygen, similar to the upregulation of PHDs. However, it is now clear that interplay between hypoxia, JmjC proteins and histone methylation is much more complex and comprises more than a feedback loop. Given that specific histone methylation marks are associated with open and closed chromatin and can recruit protein complexes that alter chromatin accessibility [117,118], JmjC histone demethylase inhibition may affect chromatin accessibility in hypoxia. Work from the Ratcliffe and Mole laboratories, using Capture C, complemented by chromatin immunoprecipitation sequencing (ChIP-seq), found that distal contacts at a subset of HIF target gene promoters are already established and primed in normoxia [119]. However, to date, no studies have mapped chromatin accessibility and chromatin loops on an unbiased genome-wide scale.

Ten-eleven translocations (TETs)

DNA methylation represents a well-established mechanism of transcriptional repression, which blocks the recruitment of transcriptional activators to genomic loci or leads to the recruitment of transcriptional repressor complexes [90]. Whilst DNA methyltransferases (DNMTs) add a methyl group to 5-cytosine on DNA, ten-eleven translocation (TET) enzymes mediate removal of the methyl group. In mammals, active DNA methylation is achieved through TET-mediated oxidation of 5-methylcytosine, 5-hydroxymethylcytosine and potential additional oxidation reactions to 5-formylcytosine, and 5-carboxylcytosine [120]. The oxidised forms of 5-methylcytosine are then diluted in a replication-dependent manner, or 5-formylcytosine and 5-carboxylcytosines are removed by thymine DNA glycosylase-mediated excision coupled with base excision repair [120]. TETs are also 2-OG-dependent dioxygenases. HIF-1-dependent TET1 upregulation and TET1-mediated hydroxymethylation of DNA drive hypoxic gene induction in neuroblast cells [121].
Here, TET1 was elucidated to be active at 1% O₂. In vitro, O₂ KM for TET1 and TET2 are roughly 30 µM (Table 1) [122], which suggests TET enzymes do not function as physiological O₂ sensors and retain their activity in hypoxic conditions. This supports the role of TETs in the bone marrow and during development where O₂ tensions are low [123,124]. However, there is also evidence that TET activity is impaired in more severe hypoxic environments, namely tumour hypoxia and 0.5% O₂ in some human and murine cell lines [125]. Using cell models and patient samples, tumour hypoxia and 0.5% oxygen have been shown to induce DNA hypermethylation, with loss of 5-hydroxymethylcytosine, and increase in DNA methylation at gene promoters driven by reduced TET activity [125]. In this study, TET1 and TET2 activity was hypoxia inhibited in a cell-based assay with 50% activity measured at 0.3% and 0.5% O₂, respectively. Thus, TETs may be characterised as tumour oxygen sensors; however, more work is needed to establish the physiological contexts in which TETs can sense changes in oxygen availability.

RNA demethylases

RNAs carry a diverse range of chemical modifications; one of the most abundant in eukaryotes is N6 methyladenosine (m6A), which impinges on all aspects of RNA biology [126]. Modulation of this highly dynamic and reversible modification on internal regions of mRNA and other types of nuclear RNA is orchestrated by m6A writers and erasers, with readers of the modification important in conferring its functional output [126]. The 2-OG-dependent dioxygenases, FTO alpha-ketoglutarate-dependent dioxygenase (FTO) [127,128] and AlkB homolog 5, RNA demethylase (ALKBH5) [129] are, thus far, the only two known mammalian m6A RNA demethylases. Work from the Mansfield laboratory has shown that m6A levels increase in response to hypoxia in oncogene-transformed human mammary epithelial cells [130] and increased m6A at a subset of key hypoxia-inducible gene transcripts increases their stability and translation in hypoxic cancer cells [131]. There is evidence that ALKBH5 can remain active in low O₂, with hypoxia-induced m6A removal at Nanog homeobox (NANOG) mRNA, mediated by ALKBH5, driving a breast cancer cell phenotype [132]. In vitro oxygen affinities for FTO and ALKBH5, coupled with cell-based activity assays in response to changes in O₂ levels, are needed to determine their ability, or lack thereof, to function as O₂ sensors.

**Cysteamine (2-aminoethanethiol) dioxygenase (ADO)**

Plant O₂ sensing is known to proceed from a cysteine N-degron system, and although there was evidence that O₂- and nitric oxide(NO)-dependent cysteine N-degron-mediated protein degradation occurred in humans (for the substrates regulator of G protein signalling (RGS)4 and RGS5) [133], only recently the mechanism has been reported. The human enzyme cysteamine (2-aminoethanethiol) dioxygenase (ADO) was demonstrated to catalyse oxidation of N-terminal cysteines resulting in arginylation and degradation. The ADO O₂ KM was reported as > 500 µM, which implies even greater O₂ sensitivity than the PHDs, suggesting an important role as an O₂ sensor [134]. ADO targets several N-cysteine proteins including RGS4, RGS5 and interleukin (IL)-32. Stabilisation of these proteins in hypoxia via ADO inhibition represents an additional layer of crosstalk between O₂ levels, gene expression and cellular responses.

**O₂ KM**

The large variability of determined in vitro O₂ KM indicates the difficulty of relating a reported oxygen affinity to cellular oxygen sensitivity. One limitation is that enzyme activity has been assayed by a variety of techniques, and despite other conditions being comparable the resulting O₂ KM can be very variable (e.g. with PHD2, there is a 2~7-fold difference in reported O₂ KM) (Table 1). A further complication occurs with the choice of substrates. Substrates used in most assays have been short peptide sequences, which may not reflect well the physiological substrates. One study that examined the effect of substrate size on enzymatic output found that longer polypeptide substrates of HIF-1α were progressively less O₂-sensitive in their assay. Therefore, the most physiological substrate (HIF-1α 502–697) had a substantially lower O₂ KM of 67 µM, which is far below atmospheric O₂ concentration, and even below some physiological tissues. However, an opposite effect was observed with FIH, with a longer polypeptide yielding an O₂ KM of 237 µM. This variability in O₂ KM obtained in vitro provides ground for argument that other O₂-sensitive dioxygenases do indeed function as true cellular O₂ sensors. Dioxygenases with low O₂ KM do not preclude them from functional roles as O₂ sensors, as proposed for the TET enzymes, which can have significantly altered activity in acute hypoxia, which may occur in either physiological or disease niches. Indeed, what defines hypoxia would be dependent on the tissue as well as in
intracellular concentration of O₂ in a given cell type. Technological developments in the measurement of O₂ in tissues, cells and subcellular compartments are therefore required to answer what truly represents a hypoxic event. The PHD-HIF pathway undoubtedly coordinates the largest effect of cellular reprogramming in response to hypoxia, but it has become clear that there exist many other important mechanisms of regulation and adaptation.

**Nuclear factor-κB (NF-κB)**

Although hypoxia leads to the activation of HIFs, other transcription factors are also regulated by reducing O₂ levels [7,135]. Perhaps, the one with the greatest degree of regulation is the nuclear factor kappa B (NF-κB) family of transcription factors and components of its activation pathway (Fig. 2). NF-κB is the key regulator of immune and inflammatory responses [136]. The NF-κB family constitutes five distinct members, including RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) that associate with each other to form active homo- or heterodimeric complexes. Activation of the NF-κB dimers can be through the stimulation of canonical, noncanonical and atypical pathways, which are all initiated by different stimuli [137]. The canonical pathway activation is triggered by cytokines, bacterial lipopolysaccharides and viral infections. This leads to the oligomerisation and recruitment of a number of adaptor molecules, which subsequently activate NF-κB through ubiquitin-dependent activation of transforming growth factor β-activated kinase 1 (TAK1) and inhibitor of κB kinase (IKK) complex [138]. The IKK complex is composed of two catalytic proteins, IKKα and IKKβ, and a regulatory protein, IKKγ (NEMO). Activated IKK complex mediates the phosphorylation of IκBα, which holds the NF-κB dimers inactive in the cytoplasm. Phosphorylated IκBα is then lysine-48-linked polyubiquitinated and eliminated through the proteasomal degradation system. This releases NF-κB dimers from the cytoplasm to translocate into the nucleus and initiate their regulatory role in gene transcription (Fig. 2).

In the noncanonical NF-κB pathway, NF-κB-induced

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**Fig. 2.** The intersection of cellular oxygen sensor 2-OG-dependent dioxygenases and the NF-κB pathway. Schematic diagram of the two main NF-κB activation pathways and the crosstalk with the 2-OG-dependent dioxygenases. T-bars indicate an inhibitory effect; dashed lines indicate a regulatory effect with incomplete mechanism. Hydroxylation of NF-κB signalling components has been described in both canonical and noncanonical pathways. These include p100 and p106 NF-κB proteins; inhibitor of κB-a (IκB-α); inhibitor of κB kinase (IKK); otubain 1 (OTUB1); OTU domain-containing protein 7B (OTUB7B); ubiquitin-conjugating enzyme variant 1A (Uev1a); Ubc13-ubiquitin-conjugating enzyme 13 (Ubc13); receptor interaction serine/threonine kinase 4 (RIPK4). KDM2A and PHD2 have been shown to bind RelA at NF-κB promoters. Finally, HIF-1α has been shown to restrict NF-κB signalling at the level of transforming growth factor β-activated kinase 1 (TAK1) by an unknown mechanism.
kinase (NIK) acts as a central signalling mediator, which cooperates the IKKα homodimer phosphorylation and subsequently NF-κB activation without the involvement of IκB proteins (Fig. 2).

The concept of hypoxia-induced inflammation has been widely recognised in many studies [139,140]. The link between the two pathways was first reported in people suffering from mountain sickness, as they had...
elevated levels of proinflammatory cytokines and vascular leakage causing pulmonary or cerebral oedema [141,142]. In addition, elevated serum levels of IL-6, IL-6 receptor and C-reactive protein, which are the known markers of inflammation, were detected in healthy volunteers who stayed at an altitude higher than 3400 metres for 3 nights [143].

Relevant to this review, a number of the aforementioned 2-OG dioxygenases, including PHDs and FIH, can regulate NF-κB activity. PHDs are shown to control NF-κB activation through the IKK complex. PHD1 can negatively regulate IKKβ; therefore, it is hypothesised that hypoxia leads to NF-κB activation [144]. PHD3 was shown to inhibit both IKKβ and IKKγ independent of its hydroxylation activity [145]. On the other hand, PHD2 has been shown to interact with the NF-κB member, p65/RelA, and acts as its co-activator [146]. FIH was shown to hydroxylate the NF-κB p105 and 1κBα [147]. However, the functional significance of this hydroxylation is not yet determined. Likewise, receptor-interacting serine/threonine protein kinase (RIPK) 4, which is known to be involved in the NF-κB activation in an IKK-dependent manner, is hydroxylated by FIH [148]. These findings demonstrate that the activity of oxygen-sensitive hydroxylases is not restricted to HIF and suggests their involvement in the inflammatory pathway.

Furthermore, the two components of an E2 ubiquitin-conjugating complex, ubiquitin-conjugating enzyme 13 (Ubc13) and ubiquitin-conjugating enzyme variant 1A (Uev1a), which are known to be important in the hypoxia-induced NF-κB pathway activation [149,150], were identified to be hydroxylated by PHD1 [151]. The deubiquitinating (DUB) enzyme, otubain 1 (OTUB1), that negatively regulates Ubc13 and Uev1a has also been associated with FIH [151]. Taken together, these findings highlight a role for O2-dependent hydroxylases in the ubiquitination mechanisms involved in the NF-κB pathway activation, independently of HIF (Fig. 2).

More recently, the DUB OTU domain-containing protein 7B (OTUD7B or Cezanne) has been shown to be hydroxylated by FIH [152]. Cezanne is known to be involved in the NF-κB pathway by controlling HIF protein levels [153,154]. Also, Cezanne negatively regulates the NF-κB pathway by targeting RIP1 signalling [155]. It can also lead to the inhibition of TNF receptor-associated factor 6 (TRAF6) [156], another important regulator in the pathway (Fig. 2). However, a clear role for FIH via Cezanne in the NF-κB pathway is currently unknown.

JmjC proteins have also been shown to impinge on NF-κB pathway activation (Fig. 2). KDM2A and KDM2B are hypoxia-inducible in a HIF-1-dependent manner [116]. KDM2A associates with p65 in human colon cancer cell lines and directly controls its transcriptional activity [157]. KDM2B was identified in a RNAi screen for IL-1β required cofactors of p65 [158]. KDM6B has been shown to be necessary for full NF-κB activation of its targets in different cell types [159,160]. However, whether other JmjC proteins control NF-κB signalling remains under investigation.

Conclusions

The identification of 2-OG-dependent dioxygenases and their roles outside the HIF transcription factors has opened new avenues for research, as well as therapeutic interventions in areas where low O2 is prevalent. Additional exciting new knowledge is therefore coming in the near future in these areas. Given our current understanding of these enzymes, one can speculate that low O2 is involved in almost all aspects of gene expression regulation (Fig. 3), from chromatin to RNA fate, as well as protein stability.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

JWW researched the literature and wrote the section on PHDs. DS researched the literature and wrote the section on NF-κB crosstalk. MB researched the literature and wrote the section on epigenetic regulation. MF researched the literature and wrote the section of dioxygenase Km. SR researched the literature, wrote the abstract, introduction and conclusion and compiled the final manuscript.

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