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Molecular Cell
Review

OH, the Places You'll Go! Hydroxylation, Gene Expression, and Cancer

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Hydroxylation is an emerging modification generally catalyzed by a family of ~70 enzymes that are dependent on oxygen, Fe(II), ascorbate, and the Kreb's cycle intermediate 2-oxoglutarate (2OG). These "2OG oxygenases" sit at the intersection of nutrient availability and metabolism where they have the potential to regulate gene expression and growth in response to changes in co-factor abundance. Characterized 2OG oxygenases regulate fundamental cellular processes by catalyzing the hydroxylation or demethylation (via hydroxylation) of DNA, RNA, or protein. As such they have been implicated in various syndromes and diseases, but particularly cancer. In this review we discuss the emerging role of 2OG oxygenases in gene expression control, examine the regulation of these unique enzymes by nutrient availability and metabolic intermediates, and describe these properties in relation to the expanding role of these enzymes in cancer.

> Congratulations! Today is your day. You're off to Great Places! You're off and away!—Dr. Seuss (*Oh, The Places You'll Go!*)

Introduction

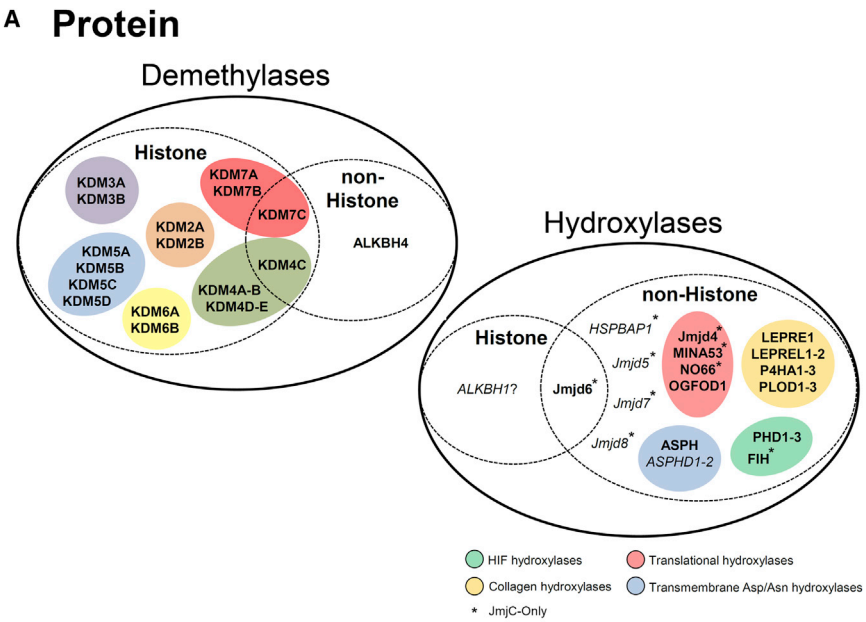
Hydroxylation was first identified as a post-translational modification following fundamental biochemical experiments performed in the mid-20th century investigating the source of hydroxy-proline and -lysine in collagen. The demonstration that these were not derived from their correspondingly modified free forms paved the way for partial purification of the first protein hydroxylases in the 1960s (Kivirikko and Prockop, 1967). Even at this embryonic stage, it was appreciated that the inhibition of hydroxylase activity due to nutrient deprivation could result in disease (i.e., scurvy). The fact that abnormal collagen hydroxylase activity was the basis of Ehlers-Danlos syndrome also highlighted that hydroxylase mutations can cause disease (Hyland et al., 1992). What was not appreciated was that the collagen hydroxylases belonged to a wider family of oxygenases and that, collectively, these enzymes are involved in a variety of fundamental biological processes and diseases, particularly cancer.

The potential importance of hydroxylation in cancer was first discussed in the 1990s following the discovery of asparaginyl and aspartyl hydroxylation in extracellular EGF repeats (Stenflo et al., 1989). Deletion of the β-aspartyl/asparaginyl hydroxylase (BAH) gene in mice promotes colorectal cancer in an APC/min model (Dinchuk et al., 2002), while an association between its overexpression in tumors and poor prognosis may relate to roles in motility, invasion, proliferation, and survival (Dong et al., 2015). The potential pro-tumorigenic properties of BAH have led researchers to consider it as a biomarker and therapeutic target in several cancers.

The new millennium heralded a surge in hydroxylation research, following the seminal discovery that oxygen-dependent modification of a hypoxia-inducible transcription factor (HIF) was the basis for oxygen sensing and hypoxic adaptation (discussed in detail below). The discovery that hydroxylation of a transcription factor could link nutrient availability to a physiological response also signaled a new paradigm in gene expression control. It has since become apparent that control of gene expression is a theme that unifies many members of this enzyme family. In this review, we describe the role of hydroxylation at each step of gene expression. We will discuss the unique position of this oxygenase family at the intersection of nutrient availability and metabolism and consider that this underlies a general role in gene expression control and cancer. We begin by providing a brief introduction to the biochemistry of this family of oxygenases (for comprehensive reviews, see Hausinger, 2004; Loenarz and Schofield, 2008; McDonough et al., 2010).

2-Oxoglutarate Oxygenases

Hydroxylases generally belong to a family of ~70 oxygenase enzymes in mammals whose activities depend on key nutrients, including oxygen, Fe(II), the Kreb's cycle intermediate 2-oxoglutarate (2OG), and in some cases a reducing agent such as ascorbate (Loenarz and Schofield, 2011). The family can be subdivided into groups based on sequence and structural homology and/or function (Figure 1). 2OG oxygenases are ancient enzymes present in the earliest forms of life, where they catalyze a variety of different oxidative modifications (Hausinger, 2004). Although the only reaction described in higher organisms thus far is hydroxylation, a large subset of 2OG oxygenases utilize this activity to catalyze demethylation (Figure 2A) (Klose et al., 2006). Despite this more restricted biochemistry, biological diversity is driven by their ability to target all the major biological macromolecules, including DNA, RNA, protein, and lipid (Loenarz and Schofield, 2008). In the context of protein, those eukaryotic 2OG oxygenases characterized thus far catalyze the hydroxylation of prolyl, lysyl, asparaginyl, aspartyl, and histidyl residues (see below). Additional activities in prokaryotes (e.g.,



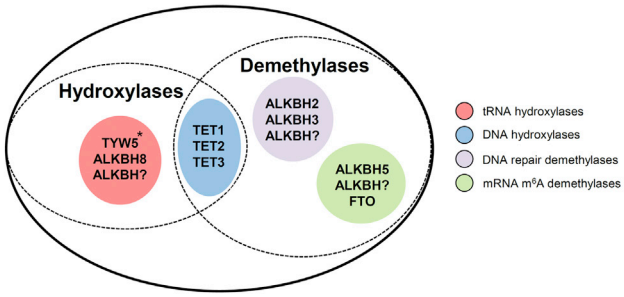


Figure 1. Functional Clustering of 2-Oxoglutarate Oxygenases

(A) 2OG oxygenases that target proteins. 2OG oxygenases catalyze hydroxylation (lower right) and demethylation (upper left) (via a hydroxylation reaction). Upper left: The JmjC histone demethylases (KDMs) are one of the largest groups of 2OG oxygenases. These can be divided into subgroups (colored circles/ovals) that share sequence homology and common histone targets (also see Figure 3A). Note that some KDMs also have non-histone substrates (e.g., KDM7C/4C). ALKBH4 is an actin lysyl demethylase. Lower right: 2OG oxygenases catalyzing stable protein hydroxylation are a major functional family. These can also be divided into sub-groups (colored circles/ovals, see key), where clustering represents a common biological target (e.g., collagen, yellow circle) or physiological process (e.g., translation, red oval). Enzyme names are written in light italic font if the corresponding substrate is uncertain or unknown. (B) 2OG oxygenases that target nucleic acids. TYW5 and ALKBH8 catalyze hydroxylation of specific tRNAs (red circle). TET enzymes (blue oval) hydroxylate methylated DNA at 5-mC. ALKBH5 and FTO demethylate m^6A in mRNA. ALKBH2/ALKBH3 demethylate DNA damaged by alkylation (purple circle). Also see Figure 3B. JmjC-only 2OG oxygenases are marked with an asterix. Not all members of the 2OG oxygenase family are represented here (lipid oxygenases have been omitted, for example).

argininyl hydroxylation) (Ge et al., 2012), together with the relaxed substrate specificities of some 2OG oxygenases (Yang et al., 2013a), suggest that hydroxylation of other amino acids may yet be described in eukaryotes. Despite the biochemical diversity of the 2OG oxygenase family, individual enzymes generally catalyze highly context-dependent modifications, with specificity driven by the primary sequence of the substrate and structural motifs within the enzyme (McDonough et al., 2010).

The 2OG oxygenase catalytic domain is characterized by a double-stranded β helix (DSBH) fold comprised of eight antiparallel β strands that fold into a barrel-like structure with an opening at one end (Figure 2B). This arrangement brings together key amino acid side chains involved in binding the co-factors (Fe(II) and O_2), co-substrate (2OG), and the prime substrate (McDonough et al., 2010). Whereas Fe(II) is coordinated by a conserved "2-His/1-carboxylate" motif (HxD/E … H) (Figure 2C), residues involved in 2OG binding are more variable, but consistent within sub-families. The catalytic cycle is initiated by 2OG binding, which facilitates substrate and subsequent oxygen binding. Oxidative decarboxylation of 2OG generates a highly reactive intermediate that mediates oxidation of the prime substrate, with the production of CO_2 and succinate as byproducts (Figure 2D).

Gene Expression Control: Modify DNA, RNA, and Protein

Pathways that signal to gene expression and growth control are tightly regulated by mitogens, nutrient availability, and metabolism and are frequently the target of genetic and epigenetic alterations in cancer (Hanahan and Weinberg, 2011). Accumulating evidence indicates that 2OG oxygenases often act in these pathways and are commonly altered in tumors. Before discussing how this emerging theme may relate to their position at the interface of nutrient availability and metabolism, we will first describe 2OG oxygenases that target each step in gene expression (from DNA to RNA to protein) and summarize evidence supporting a role for these enzymes in cancer.

DNA Hydroxylation and Demethylation

Ten-Eleven Translocation

DNA methylation is a critical regulator of gene expression that is commonly altered in cancer. Methyltransferases generally target the carbon-5 position of cytosine in CpG dinucleotides to create 5-methylcytosine (5-mC), a dynamic epigenetic modification that can be actively and passively reversed. Active reversal is initiated by the "Ten-Eleven Translocation" (TET) sub-family of 2OG oxygenases (TET1–TET3) (Figures 2A and 3B), so named because the TET1 gene on chromosome 10 is the target of a translocation with the mixed-lineage leukemia (MLL) gene (chromosome 11) in myeloid and lymphoid malignancies (Huang and Rao, 2014). TETs hydroxylate the methyl group of 5-mC to form 5-hydroxymethylcytosine (5-hmC), followed by additional subsequent oxidations.

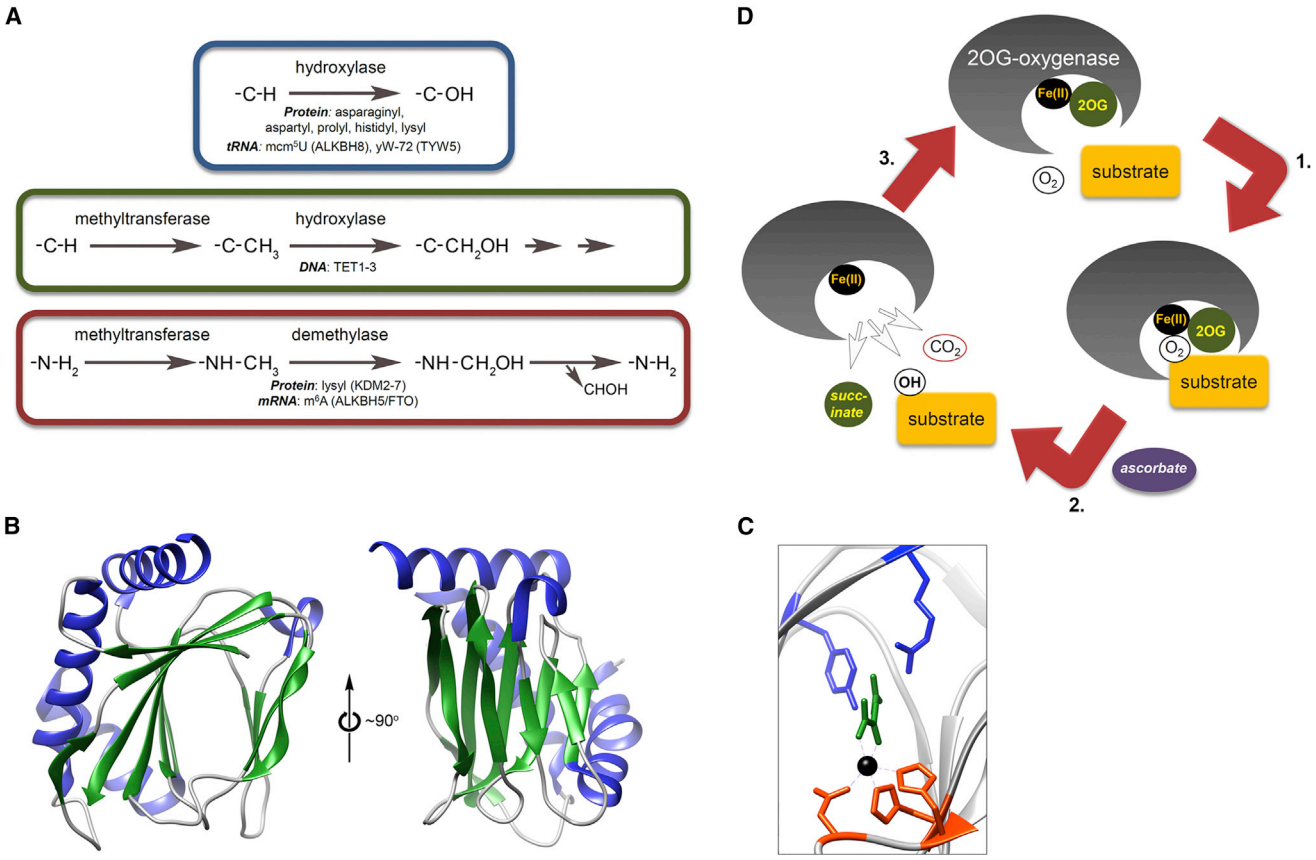


Figure 2. Enzymology of 2-Oxoglutarate Oxygenases: Overview of Reactions, Catalytic Cycle, and Structural Features

(A) Oxidative modifications catalyzed by eukaryotic 2OG oxygenases. Upper panel: Stable hydroxylation of protein and tRNA. Middle panel: Hydroxylation of methyl-cytosine in DNA by TET enzymes (subsequent arrows denote additional oxidations in a demethylation process, see text). Lower panel: demethylation of histones and methyl-adenosine (m^6A) in mRNA. Hydroxylation creates an unstable intermediate that decomposes into the unmethylated form plus formaldehyde (CHOH).

(B) Topology of the 2OG oxygenase catalytic domain. The double-stranded β helix (DSBH, green) forms a barrel-like shape that positions essential residues that ligate co-factors and the prime substrate.

(C) Co-factor binding residues within the DSBH. Magnification of (B) showing Fe(II) (black sphere), Fe(II)-binding residues (orange), 2OG (green), and 2OG-binding residues (blue).

(D) Simplified graphical representation of the catalytic cycle. Hydroxylation is initiated by 2OG binding, followed by the substrate and subsequently oxygen (1). One atom of oxygen is incorporated into the prime substrate and the other into CO_2 with succinate as a byproduct (2). Hence, these enzymes are classed as "dioxygenases". Some 2OG oxygenases require ascorbate for full activity. Structural images in (B) and (C) are derived from PDB: 3OUJ using Chimera.

These products facilitate demethylation in multiple ways, including an active excision and repair pathway (Kroeze et al., 2015). Although often considered an intermediate in demethylation, evidence also supports a direct signaling role for 5-hmC, which can accumulate to substantial levels and is capable of binding "reader" proteins (Mellén et al., 2012).

TET1 is relatively infrequently altered in cancer compared to TET2. Whole-exome sequencing has identified a large number of TET2 cancer mutations, the majority of which cluster in the DSBH domain and impair activity. As such, TET2 is one of the most frequently mutated genes in acute myeloid leukemia, chronic myelomonocytic leukemia, and myelodysplastic syndromes (Ko et al., 2015). However, the role of TETs and 5-hmC in gene expression control and cancer likely extends beyond hematological malignancies. TET mutations have been described in a variety of solid cancers (Huang and Rao, 2014). Furthermore, global 5-hmC levels are commonly reduced in tumors, often in the absence of TET mutations, likely due to reduced TET expression and/or activity (Kroeze et al., 2015). Interestingly, TET activity is altered by co-factor availability and "oncometabolites" in specific tumor contexts (see below).

ALKB Homologs

In addition to enzymatic methylation, DNA is also subject to methylation damage by endogenous and exogenous agents. Alkylated DNA is repaired in *E.coli* by three repair proteins, one of which (AlkB) is a 2OG oxygenase (Falnes et al., 2007). AlkB repairs N^1-methyladenosine (1-meA) and N^3-methylcytosine (3-meC) by hydroxylation of the methyl group, which, unlike 5-hmC, is highly unstable, rapidly decomposing into the unmethylated and repaired base (with formaldehyde as a byproduct) (Figures 2A and 3B). There are eight human homologs of *E.coli* AlkB (ALKBH1–ALKBH8), and their functions extend beyond DNA repair (see below). Although ALKBH1 shares most similarity to AlkB and was originally reported to demethylate 3-meC in

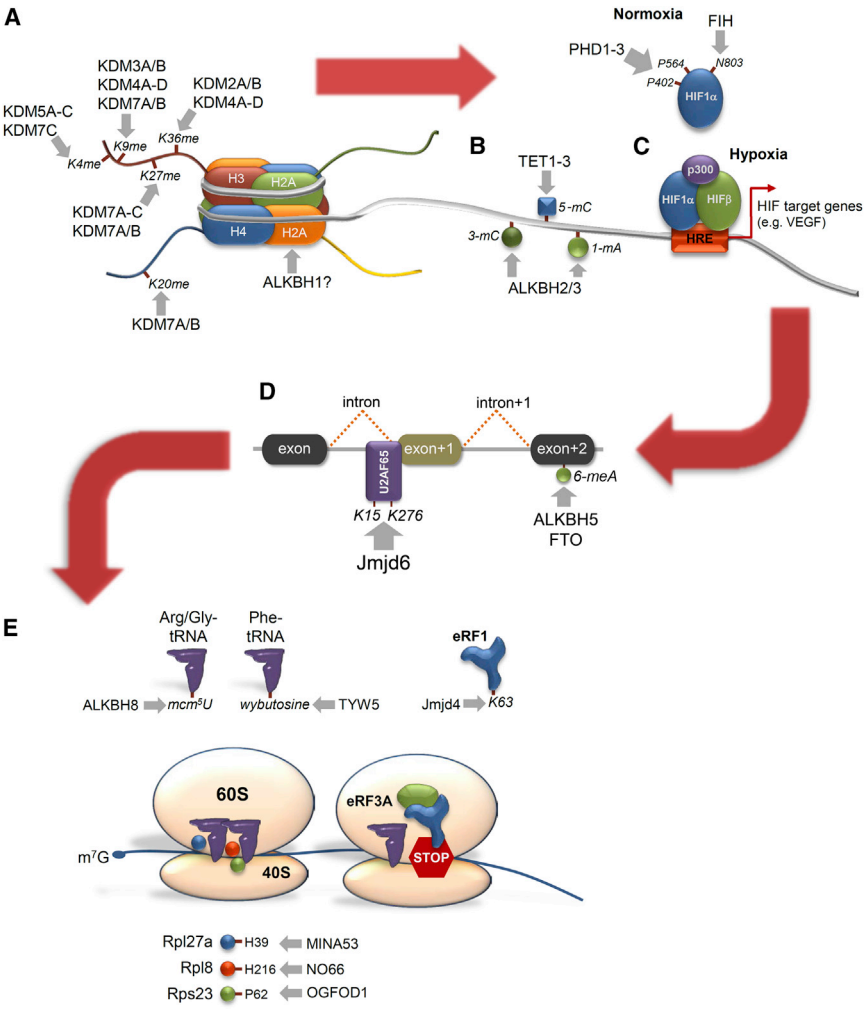


Figure 3. 2-Oxoglutarate Oxygenases Target the Gene Expression Pathway

(A) JmjC histone demethylases. KDM families target the indicated methylated lysyl residues of histones H3 and H4. Histone H2A may support ALKBH1 activity, although a modification has not been proven (designated by "?").

(B) Oxidation of DNA modifications by 2OG oxygenases. TET enzymes hydroxylate 5-methylcytosine (5-mC) in CpG islands. ALKBH2/ALKBH3 demethylate alkylated cytosine (3-methylcytosine, 3-meC) and adenosine (1-methyladenosine, 1-meA).

(C) Hydroxylases control a hypoxia signaling pathway. Prolyl hydroxylases (PHD1–PHD3) modify hypoxia-inducible transcription factor (HIFα) to signal its proteasomal degradation. Factor inhibiting HIF (FIH) hydroxylates and inactivates a transactivation domain. In hypoxia, inhibition of PHDs and FIH stabilizes HIFα, which binds with HIFβ to hypoxia response elements (HREs) to activate transcription of target genes such as vascular endothelial growth factor (VEGF).

(D) 2OG oxygenases in mRNA biology. ALKBH5/FTO demethylates methyl-adenosine (6-meA). Jmjd6 hydroxylates specific lysyl residues in U2AF65.

(E) Translational hydroxylases. ALKBH8 and TYW5 hydroxylate hypermodified nucleotides in the indicated tRNAs (mcm^5U = 5-methoxycarbonylmethyluridine). NO66, MINA53, and OGFOD1 target specific residues of the indicated ribosomal proteins. The colored spheres in the large 60S and small 40S ribosomal subunits indicate the relative position of the hydroxylated residue, not the position of the protein within the ribosomal structure (H39/H216 of Rpl27a/Rpl8 are in disordered loops that extend into the core). Jmjd4 hydroxylates the stop codon-decoding domain of eRF1, the eukaryotic release factor. eRF1 and the GTPase eRF3A mediate stop codon recognition and peptidyl-tRNA hydrolysis. m^7G = 7-methylguanosine cap.

DNA and RNA in vitro (Westbye et al., 2008), recent reports suggest a role in binding and/or modifying histone H2A (Korvald et al., 2011; Lando et al., 2012; Ougland et al., 2012). Rather, ALKBH2 and ALKBH3 have the clearest functional homology to AlkB (Aas et al., 2003). Interestingly, the DNA repair activity of ALKBH2 plays a role in gene expression control, enabling the high transcription rate from ribosomal DNA (rDNA) genes by promoting their repair (Li et al., 2013). It will be interesting to determine whether ALKBH2/3 also promotes the expression of other highly transcribed genes in a similar fashion. Furthermore, DNA repair is likely the mechanism by which ALKBH2/3 suppresses cancer arising from chemically induced colitis (Calvo et al., 2012). Conversely, some tumor cells are dependent on overexpression of ALKBH3 to suppress DNA damage and drive tumorigenesis (Dango et al., 2011). Other ALKBH family members have functions that are distinct from ALKBH2/3. Those targeting RNA will be discussed in the relevant sections that follow.

Histone Demethylation

A major level of gene expression control takes place at the N-terminal tails of histones, where a large variety of post-translational modifications occur, including methylation. These dynamic modifications influence chromatin structure and the recruitment of "readers" that regulate transcription. Interest in histone methylation has exploded following the discovery of a sub-family of 2OG oxygenases that catalyzes its reversal (Klose et al., 2006). This family of ~20 enzymes (Figure 1) shares a specific form of the DSBH catalytic fold known as the Jumonji C (JmjC) domain. Of the 2OG oxygenases reviewed here, the family of JmjC histone lysyl demethylases represents the largest and most well characterized with respect to gene expression control and cancer.

JmjC histone demethylases that target each of the common lysyl methylation sites have been identified (Figure 3A). Similar to the hydroxylation of N-methyl marks in DNA by ALKBH2/3, hydroxylation of the methylated ε-amino group in histone lysines is unstable and rapidly decomposes to release formaldehyde and the unmethylated lysyl residue (Figure 2A). JmjC lysine demethylases (KDMs) can be grouped into sub-families (KDM2–KDM7) that share sequence homology within the DSBH, similar substrate specificity, and common domain architecture (Figures 1A and 3A).

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Existing evidence implicates JmjC KDMs in the regulation of transcriptional initiation and elongation. However, rather than acting as powerful transcriptional switches, these enzymes generally act as modifiers. For example, genome-wide chromatin immunoprecipitation and expression studies indicate that although KDMs often bind to a large population of genes, they only regulate the transcription of a subset (Kooistra and Helin, 2012). It is thought that the non-regulated genes maintain KDM occupancy to prevent inappropriate histone methylation. However, this combination of housekeeping and regulatory roles means that KDMs have been widely implicated in fundamental processes, and consequently in many diseases (Johansson et al., 2014). Whereas specific KDMs may be associated with a single disease, the whole family has been implicated in a variety of cancer "hallmarks" (Højfeldt et al., 2013). In fact, KDMs were implicated in cancer prior to their JmjC domain being assigned as having demethylation activity. For example, the GASC1 (KDM4C) gene lies in an amplicon at 9p23-24 that had been observed in a variety of tumor types. The histone demethylase activity of KDM4C was subsequently implicated in suppressing heterochromatin formation and promoting tumor growth (Cloos et al., 2006). The KDM4 sub-family comprises four other members that have also been widely implicated in cancer and comprehensively reviewed elsewhere (Berry and Janknecht, 2013). The H3K27 demethylase KDM6A (UTX) is probably the most frequently mutated KDM across human cancers. Although it displays many of the hallmarks of a tumor suppressor (reviewed in Van der Meulen et al., 2014), mutation can correlate with improved survival in some cases (Kandoth et al., 2013).

To further highlight the role of KDMs in gene expression control and cancer, we will focus on the KDM5 family, which has been widely implicated in tumorigenesis. We direct the reader to other recent reviews for detailed information on the remaining KDM sub-families and cancer (Berry and Janknecht, 2013; Højfeldt et al., 2013; Johansson et al., 2014; Van der Meulen et al., 2014).

KDM5

The KDM5 family consists of four members (KDM5A–KDM5D) that demethylate histone H3 Lys-4 (H3K4) (Figure 3A). KDM5A was recently highlighted as one of 127 significantly mutated genes in over 3,000 tumors from multiple cancer types (Kandoth et al., 2013). KDM5A was originally implicated in the regulation of differentiation and transcription by the Retinoblastoma (Rb) tumor suppressor protein (Benevolenskaya et al., 2005). Consistent with KDM5A being an important target of Rb, loss of KDM5A decreases tumorigenesis in Rb heterozygous knockout mice (Lin et al., 2011). KDM5A is overexpressed in several cancers where it promotes multiple cancer-associated processes (Blair et al., 2011). The deleterious role of KDM5A in cancer appears multi-faceted: a sub-population of highly dynamic tumor cells are rendered "drug-tolerant" by KDM5A overexpression (Sharma et al., 2010). Interestingly, KDM5B may also play a role in tumor heterogeneity. It is required in a sub-population of very slow-cycling melanoma cells to evade drug treatment and support tumor growth (Roesch et al., 2010). KDM5B also promotes breast cancer proliferation by repressing expression of the BRCA1 tumor suppressor (Yamane et al., 2007). Consistent with an important role in this tumor type, KDM5B is commonly amplified in luminal breast cancers, where it is represses a luminal-specific expression program to maintain proliferation (Yamamoto et al., 2014). In contrast to its homologs, KDMC may be tumor suppressive rather than oncogenic. Next-generation sequencing has identified inactivating KDM5C mutations in clear cell renal cell carcinoma (Dalgliesh et al., 2010).

JmjC-Only 2OG Oxygenases

JmjC KDM enzymes share several features in common that allow them to be considered as an independent group within the 2OG oxygenase family. These include the presence of a variety of functional motifs that mediate protein:protein interactions, DNA-binding, and targeting to specific histone tail modifications (Klose et al., 2006). They also share similarities in sequence and structure within the JmjC domain, which underpins their common function (McDonough et al., 2010). Importantly, however, the JmjC domain is not limited to this sub-family of 2OG oxygenases and is not restricted to catalyzing lysine demethylation. A phylogenetically discrete group of 2OG oxygenases also contains JmjC catalytic domains, but in the absence of other functional motifs (at least based on primary sequence analyses) (Johansson et al., 2014; Klose et al., 2006). Some of these "JmjC-only" 2OG oxygenases have been reported to have KDM activity.

Jmjd5 is a JmjC-only 2OG oxygenase that was originally characterized in breast cancer cells, where it was suggested to promote proliferation via H3K36me2 demethylation and transcriptional activation of the cyclin A1 locus (Hsia et al., 2010). A role for Jmjd5 in regulating chromosomal segregation was also proposed to involve de-repression of Jmjd5 H3K36me2 demethylation activity by RCCD1 (Marcon et al., 2014). However, structural studies indicated that the JmjC domain of Jmjd5 is unlikely to accommodate a methylated lysyl residue, consistent with its limited homology to that of the JmjC KDM family (Del Rizzo et al., 2012; Wang et al., 2013).

Two other members of the JmjC-only family, MINA53 and NO66, have been reported to possess KDM activity. MINA53 was originally described as a nucleolar-localized Myc target gene whose expression is commonly deregulated in cancer (Eilbracht et al., 2005; Tsuneoka et al., 2002). Subsequently, MINA53 was reported to catalyze H3K9me3 demethylation and to regulate the transcription of rDNA (Lu et al., 2009). NO66 shares significant sequence and structural homology to MINA53 and has also been implicated in cancer and transcriptional regulation (Eilbracht et al., 2004; Sinha et al., 2010; Suzuki et al., 2007). Despite this homology, NO66 was assigned a distinct and unusual H3K4/K36 demethylase activity (Sinha et al., 2010). Similar to Jmjd5, recent structural analyses suggest that the catalytic pocket within the JmjC domain of MINA53 and NO66 is unlikely to accommodate a methylated lysine (Chowdhury et al., 2014), which may be consistent with biochemical analyses showing a lack of demethylase activity in vitro (Williams et al., 2014). Unbiased proteomic screens recently led to the discovery that MINA53 and NO66 are histidyl hydroxylases with substrates in protein translation (see below) (Ge et al., 2012; Williams et al., 2014). Therefore, the targets of these enzymes involved in transcriptional regulation may require further investigation.

Considering that JmjC KDMs likely evolved from JmjC-only hydroxylases (Chowdhury et al., 2014), it is possible that they share some targets in common. This would raise the possibility of chromatin hydroxylation by JmjC-only enzymes, which could partly explain some confusion in the assignment of JmjC activities. For example, Jmjd6 was described as a histone argininyl demethylase (Chang et al., 2007) before proteomic screens identified it as a lysyl hydroxylase of splicing factors (see below) (Webby et al., 2009). Perhaps consistent with similarities in the primary sequence of these splicing factors and histones, Jmjd6 has also been reported to catalyze low-level histone hydroxylation (Unoki et al., 2013).

Regulation of Transcription Factor Activity

By far the most famous example of gene expression control by hydroxylation, with wide-reaching implications for tumor biology, is that of HIF. HIF is a heterodimeric bHLH transcription factor complex consisting of a dynamically regulated α subunit (of which three isoforms exist, 1α, 2α, and 3α) and a constitutively expressed β subunit (Ratcliffe, 2013). HIFα is expressed at very low levels in well-oxygenated cells due to proteasomal targeting by three prolyl hydroxylases (PHD1–PHD3/EGLN1–EGLN3). PHD2 (EGLN1) is considered to be the dominant HIF hydroxylase in most tissues, whereas PHD3 (EGLN3) is induced by HIF as part of a negative feedback loop in vivo (Jaakkola and Rantanen, 2013). Hydroxylation by PHDs creates a recognition motif for the pVHL E3 ubiquitin ligase subunit, a tumor suppressor gene frequently deleted in renal cell carcinoma (Shen and Kaelin, 2013). Reduced PHD activity in hypoxia blocks HIFα degradation (the oxygen dependence and sensing role of these enzymes will be discussed below), resulting in dramatic induction of HIFα protein, which becomes fully activated following inhibition of a fourth hydroxylase called factor inhibiting HIF (FIH) (Figure 3C). FIH suppresses HIF activity in normoxia by hydroxylating an asparaginyl residue in a critical HIFα transactivation domain, which ablates its binding to the transcriptional co-activator p300/CBP (Lisy and Peet, 2008).

HIF activation initiates a transcriptional program that regulates hundreds of genes. ChIP-seq analysis has identified more than 500 binding sites for HIF in the human genome (Schödel et al., 2013), both at promoters and more distal regions, which can also be functionally relevant. For example, a SNP associated with susceptibility to renal cell carcinoma located in a distal enhancer of the cyclin D1 gene allows HIF2α to bind and drive overexpression in VHL-deficient RCC (Schödel et al., 2012). HIF target genes also regulate cell survival, invasion, angiogenesis, metabolism, differentiation, and epigenetics (Ratcliffe, 2013; Shen and Kaelin, 2013). Although this program has evolved to allow survival and adaption in response to physiological hypoxia, the same programs are hijacked in hypoxic and "pseudo-hypoxic" tumor cells. As such, the HIF pathway is widely implicated in malignancy (Ratcliffe, 2013; Semenza, 2010; Shen and Kaelin, 2013).

Of the three HIF prolyl hydroxylases, PHD2 and PHD3 have been most widely implicated in cancer. PHD2 may have opposing effects on tumorigenesis through tumor cell-intrinsic functions and effects on the microenvironment. Partial loss of endothelial PHD2 reduces metastasis by normalizing tumor vasculature via HIF-induced VEGF receptor (Mazzone et al., 2009), whereas tumor cell PHD2 is reported to inhibit growth and angiogenesis in xenograft assays (Bordoli et al., 2011; Su et al., 2012). Consistent with a tumor-suppressive role of PHD2 in some contexts, germline mutations have been reported in paraganglioma patients, with loss of heterozygosity in the tumor (Ladroue et al., 2008; Yang et al., 2015). However, such mutations are considered rare (Astuti et al., 2011).

Several lines of evidence also implicate PHD3 activity in tumor suppression. PHD3 promotes neuronal apoptosis in response to limited nerve growth factor (NGF) signaling during development (Schlisio, 2009). Mutations in NF1, c-RET, SDH, or VHL are thought to repress PHD3 expression or function, leading to reduced neuronal apoptosis and pheochromocytoma. Consistent with this, loss of PHD3 increases the number of neurons in the superior cervical ganglion (Bishop et al., 2008). Furthermore, activation of PHD3 induces apoptosis and tumor suppression in an A375 melanoma cell xenograft model (Tennant and Gottlieb, 2010). The role of PHD3 in apoptosis is dependent on specific HIF isoforms in some contexts but is HIF independent in others. Therefore, other pathways may play a role downstream of PHD3 in specific biological contexts. In relation to this, other substrates of the HIF PHDs have been proposed that may be unrelated to HIF biology (reviewed in Wong et al., 2013; Zheng et al., 2014). Although further characterization of these substrates may be required, it is of interest that they include proteins involved in gene expression control, metabolism, and cancer (Wong et al., 2013; Zheng et al., 2014).

Regulation of RNA Biology

In addition to a major role in transcriptional regulation through modification of DNA and histones, 2OG oxygenases also control gene expression at the level of RNA. Here they have been shown to directly target mRNA and tRNA and to regulate mRNA splicing.

Splicing

Jmjd6 proteomic screens identified a variety of associated proteins involved in RNA metabolism, particularly arginine-serine (RS)-rich domain proteins (Webby et al., 2009). Consistent with this, Jmjd6 regulates splicing of reporter genes and endogenous mRNAs (Boeckel et al., 2011; Webby et al., 2009) (Figure 3D). Although these effects may be independent of enzyme activity (Heim et al., 2014), Jmjd6 does catalyze low-level lysyl hydroxylation of the U2AF65 splicing factor (Webby et al., 2009). Interestingly, it has been proposed that, through its effects on splicing, Jmjd6 promotes angiogenesis by suppressing expression of the soluble form of the VEGF receptor Flt-1 (Boeckel et al., 2011). Consistent with multiple roles in cancer, Jmjd6 also regulates growth, migration, and invasion and is associated with poor prognosis in lung and breast cancer (Lee et al., 2012). Whether these attributes reflect Jmjd6's role in splicing control is not yet known.

N^6-Methyladenosine Demethylases

Methylation of adenosine to N^6-methyladenosine (6-meA) is a common nucleotide modification present in approximately 25% of mRNAs and is generally located in the 5′ UTR or in the proximity of the stop codon (reviewed in Meyer and

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Jaffrey, 2014). 6-meA has been implicated in mRNA splicing, translation, and stability. Similar to methylated DNA and histones, 6-meA is a dynamic modification. It is deposited by a specific methyltransferase complex and reversed by at least two 2OG oxygenases, FTO and ALKBH5 (Figure 3B).

ALKBH5 is a nuclear enzyme of the ALKBH family. Interestingly, ALKBH5 is a HIF target gene (Thalhammer et al., 2011), which may place its expression under the indirect control of the HIF hydroxylases. Similar to the mechanism of histone lysine demethylation, hydroxylation of 6-meA by ALKBH5 or FTO creates an unstable intermediate, which decomposes into adenosine plus formaldehyde (Meyer and Jaffrey, 2014) (Figure 2A). Unlike ALKBH5, FTO is significantly expressed in the cytoplasm. Therefore, it has been proposed that ALKBH5 and FTO are unlikely to be functionally redundant. Interestingly, sequence variants within introns 1 and 8 of the FTO gene are associated with increased risk of breast cancer and melanoma, respectively (Garcia-Closas et al., 2013; Iles et al., 2013).

tRNA Hydroxylases

In addition to mRNA, RNA modifications are also found in other RNA classes, including tRNA. A variety of modifications have been described in the anticodon stem and loop region of tRNAs and are proposed to regulate translational fidelity and tRNA folding (El Yacoubi et al., 2012). For example, phenylalanine tRNA contains a hypermodified base, hydroxywybutosine, which is synthesized in a biosynthetic pathway involving the hydroxylase TYW5 (Figure 3E) (Noma et al., 2010). Similarly, 5-methoxycarbonylmethyluridine (mcm^5U) is a hypermodified base within the anticodon loop of Arg- and Gly-tRNA, which is hydroxylated by a member of the ALKBH family, ALKBH8 (van den Born et al., 2011) (Figure 3E). ALKBH8 knockdown suppresses tumor xenograft growth due to cell cycle arrest, increased apoptosis, and reduced angiogenesis and invasion (Shimada et al., 2009).

Translational Hydroxylases

Protein synthesis is becoming increasingly recognized as an important level of gene expression control that is heavily regulated by growth factor signaling and nutrient-sensing pathways. Unsurprisingly, therefore, the loss of translational control is often described in disease (Le Quesne et al., 2010). Indeed, enhanced ribosome biogenesis and deregulated protein synthesis are considered hallmarks of cancer (Silvera et al., 2010). If 2OG oxygenases are key mediators of gene expression control and tumorigenesis, then one might predict that protein synthesis is an important target. The role of ALKBH8 and TYW5 in tRNA hypermodification, together with that of ALKBH2 in maintaining rDNA transcription, would be consistent with this possibility. In this section we review recent reports that have identified additional hydroxylase targets in protein synthesis.

Ribosomal Oxygenases

Three 2OG oxygenases have recently been identified as ribosomal protein hydroxylases. OGFOD1 is a prolyl hydroxylase of the small subunit protein Rps23, where it modifies Pro-62, a highly conserved residue located within the decoding center of the ribosome (Katz et al., 2014; Loenarz et al., 2014; Singleton et al., 2011) (Figure 3E). Perhaps consistent with a fundamental role in translation, loss of OGFOD1 activity caused altered translational accuracy and reduced protein synthesis associated with increased autophagy and reduced growth.

The two remaining ribosomal oxygenases identified thus far are MINA53 and NO66, which were introduced earlier in relation to reported KDM activities and roles in transcription. These 2OG oxygenases were recently shown to be histidyl hydroxylases that target large ribosomal subunit proteins: MINA53 hydroxylates His-39 of Rpl27a, while NO66 modifies His-216 of Rpl8 (Ge et al., 2012) (Figure 3E). In both cases the modified residue is located within a disordered loop that extends deep into the ribosome. Interestingly, the modification site in Rpl8 is close to the peptidyl-transferase center. Although the function of these modifications in translation is not yet clear, MINA53 and NO66 have been implicated in a variety of malignant phenotypes (Komiya et al., 2010; Suzuki et al., 2007; Tsuneoka et al., 2002). Further work is required to determine the relative contribution of transcriptional versus translational control to the role of these enzymes in tumorigenesis.

Translation Factor Hydroxylation

Like MINA53 and NO66, Jmjd4 is a JmjC-only 2OG oxygenase. Although Jmjd4 is most closely related to the carbon-5 lysyl hydroxylase Jmjd6, it does not interact with the RS domain-splicing factors mentioned earlier. Rather, proteomic analyses identified Jmjd4 as a carbon-4 lysyl hydroxylase that targets eRF1 (Feng et al., 2014) (Figure 3E), the eukaryotic translational termination factor. Jmjd4 hydroxylates Lys-63 within the stop codon-decoding domain of eRF1, a residue previously implicated in contacting the invariant uridine in stop codons. Indeed, Jmjd4 activity and eRF1 Lys-63 hydroxylation are required for optimal translation termination in vivo and in vitro (Feng et al., 2014). Interestingly, copy number variations in the Jmjd4 gene indicate it is amplified in a variety of tumor types, most frequently in breast and liver carcinomas (14% and 11%, respectively) (http://www.cbioportal.org). This may be consistent with the fact that genes regulating translation are commonly overexpressed in rapidly proliferating tumor cells in order to sustain elevated protein synthesis and cell growth (Silvera et al., 2010).

2-Oxoglutarate Oxygenases Act at the Interface of Nutrient Availability and Metabolism

Together, the research described above supports gene expression control as a major function of 2OG oxygenases. There they act at the very foundations of the genetic code through to the final stages of protein synthesis. It is perhaps unsurprising that 2OG oxygenases are so heavily implicated in tumorigenesis, considering the central role of gene expression in cell growth and division and the deregulation of these processes in cancer. The basis for gene expression control by 2OG oxygenases may relate to their unique position at the interface of nutrient availability and metabolism (Figure 4). The activity of 2OG oxygenases depends on fundamental nutrients and an essential Kreb's cycle intermediate. Has evolution harnessed the chemistry of 2OG oxygenases to link the availability of these nutrients and metabolites to control of gene expression, growth, and homeostasis? To explore this further, we discuss the evidence supporting a role for 2OG oxygenases as nutrient-responsive enzymes. Although the majority of work in this area currently relates to

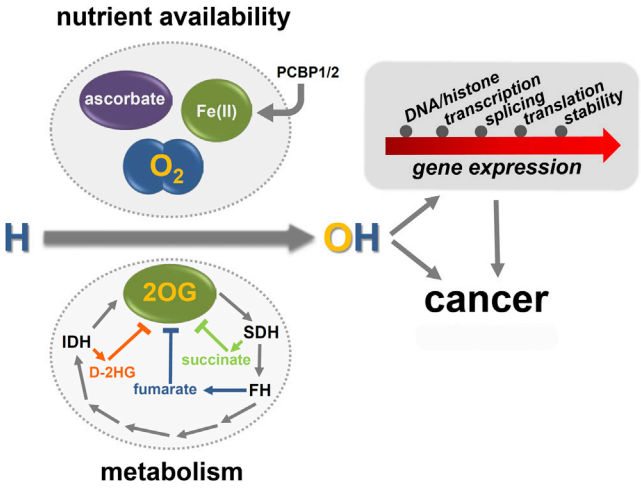

Figure 4. 2-Oxoglutarate Oxygenases as Sensors of Nutrient Availability and Metabolism in Gene Expression Control and Cancer

Nutrient availability: The availability of fundamental nutrients (ascorbate, Fe(II), and oxygen) and an important Kreb's cycle intermediate (2OG) can determine the activity of some 2OG oxygenases and, in turn, their ability to regulate gene expression and cancer-associated process. Fe(II) loading of some 2OG oxygenases by chaperones of the PCBP family regulates their activity. Metabolism: Elevated levels of D-2-hydroxyglutarate (D-2HG), succinate, and fumarate result from mutations in isocitrate dehydrogenase (IDH), succinate dehydrogenase (SDH), and fumarate dehydrogenase (FH), respectively. High levels of these "oncometabolites" (indicated by colored arrows) compete with 2OG and inhibit oxygenase activity in cancer. The Kreb's cycle is depicted by gray arrows.

the HIF hydroxylases, there is a growing literature describing other 2OG oxygenases regulated by co-factor availability.

Oxygen

A paradigm for nutrient-sensing by 2OG oxygenases is provided by the HIF hydroxylases. PHD activity is reduced under conditions of limited oxygen, which prevents proteasomal degradation of HIFα. The affinity of PHDs for oxygen may not be dissimilar to some other hydroxylases, however, suggesting that other factors also likely contribute to the potential of a 2OG oxygenase to act as an oxygen sensor (Ratcliffe, 2013). Interestingly, the activity of FIH toward HIF is less sensitive to hypoxia than the PHDs in some contexts (Lisy and Peet, 2008). This difference may allow the HIF pathway to fine-tune the magnitude and repertoire of target gene activation in response to a range of oxygen tensions.

Outside of the HIF system, there has been comparatively little analysis of potential oxygen sensing by other 2OG oxygenases, except for some preliminary analyses on the KDM family. Increased histone methylation during hypoxia is thought to be due to a combination of an increase in the activity of specific methyltransferases toward some sites and the inhibition of JmjC KDMs that target others (reviewed in Melvin and Rocha, 2012). Indeed, proof-of-principle experiments indicate that KDM4E activity is regulated by oxygen in a physiologically relevant range in vitro and that KDM4E reacts with oxygen slowly, similar to PHD2 (Sánchez-Fernández et al., 2013). Furthermore, a global induction of H3K4me3 in hypoxia may be due to inhibition of KDM5A (Zhou et al., 2010). Interestingly, a role for KDMs in the hypoxia response is supported by their general transcriptional regulation by HIF (reviewed in Shmakova et al., 2014).

The activity of recently characterized protein hydroxylases (Jmjd4, MINA53/NO66, OGFOD1) has also been tested in hypoxia. However, even in severe hypoxia (0.1%), these translational hydroxylases retain significant activity, similar to FIH (Feng et al., 2014; Ge et al., 2012; Singleton et al., 2014). This may highlight the likelihood that not all 2OG oxygenases will act in oxygen-sensitive signaling modules. However, the potential for "range-finding" raises the possibility that a 2OG oxygenase could be more oxygen sensitive in a specific context (Ratcliffe, 2013). For example, it is interesting to note that eRF1 hydroxylation is more sensitive to hypoxia in cells that express lower levels of Jmjd4 (Feng et al., 2014). Furthermore, the expression of translational hydroxylases may be modestly suppressed by hypoxia (Pollard et al., 2008).

Fe(II)

2OG oxygenases may have the potential to sense Fe(II) availability, since binding of Fe(II) to the active site of these enzymes is relatively labile. Indeed, early studies on HIF hydroxylases illustrated that supplementation of tissue culture media with Fe(II) was sufficient to promote hydroxylase activity (Knowles et al., 2003). More recently it was reported that PHDs are inhibited by reducing the expression of specific iron chaperones (PCBP1/PCBP2) that are responsible for delivering Fe(II) to their active sites (Nandal et al., 2011) (Figure 4). Although these examples may simply be consistent with the requirement of 2OG oxygenase activity for Fe(II), they also highlight the potential for changes in Fe(II) availability to modulate hydroxylase activity. Consistent with this possibility, a prolyl hydroxylase in *Pseudomonas* has a relatively high K_m for Fe(II) and is implicated in Fe(II) metabolism (Scotti et al., 2014).

Ascorbate

As discussed in the Introduction, the concept that loss of 2OG oxygenase activity due to nutrient deprivation could cause disease was illustrated by scurvy, where reduced collagen hydroxylase activity results from ascorbate deficiency. Indeed, other 2OG oxygenases may also be sensitive to ascorbate levels: supplementing tissue culture media with physiological levels of ascorbate results in HIFα downregulation due to increased PHD activity (Knowles et al., 2003). Ascorbate availability has also been linked to the activity of other 2OG oxygenases, particularly TET and KDMs. For example, ascorbate promotes reprogramming of induced pluripotent stem cells by inducing the activity of the KDM2 family (Wang et al., 2011). Similarly, ascorbate promotes TET activity and 5-hmC levels to regulate stem cell differentiation (Blaschke et al., 2013; Chen et al., 2013). Not all 2OG oxygenases require ascorbate for activity, however, raising the possibility that other reducing agents may also play a role in some circumstances.

2-Oxoglutarate

The possibility that a 2OG oxygenase could act as sensor of Kreb's cycle function via 2OG is intriguing. It has been proposed that the HIF PHDs may indirectly sense amino acid availability via associated changes in 2OG levels (Durán et al., 2013). Furthermore, increasing intracellular 2OG in hypoxia may be sufficient to activate HIF PHDs in some contexts (Tennant et al., 2009). Importantly, self-renewal of embryonic stem cells can

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be enhanced by increasing levels of 2OG, via a mechanism involving activation of H3K27me3 demethylases and TET enzymes (Carey et al., 2015). Competitive inhibition of 2OG-binding by specific tumor-associated metabolites is discussed below.

Oncometabolites Target 2OG Oxygenases in Cancer

The dependence of 2OG oxygenase activity on a fundamental Kreb's cycle intermediate may present itself as an "Achilles' heel" in cancer. Mutation of key metabolic enzymes generates high levels of intermediates ("oncometabolites") that, due to their structural similarity to 2OG, competitively inhibit a variety of 2OG oxygenases (Figure 4).

Isocitrate Dehydrogenase

Specific mutations in the catalytic pocket of isocitrate dehydrogenase 1 (IDH1) and IDH2 are neomorphic in that they switch activity from reversible conversion of isocitrate to 2OG, to oxidation of 2OG to D-2-hydroxyglutarate (D-2HG) (Morin et al., 2014; Yang et al., 2013b). These mutations have been identified in a variety of cancers, including gliomas and acute myeloid leukemia, where accumulating levels of D-2HG is thought to be a fundamental driver of tumorigenesis (Morin et al., 2014). Consistent with TET enzymes being targets of D-2HG (Figueroa et al., 2010; Koivunen et al., 2012; Xu et al., 2011), IDH and TET2 mutations are mutually exclusive (Figueroa et al., 2010; Gaidzik et al., 2012), and DNA from IDH mutant tumors is frequently hypermethylated (Ko et al., 2010; Turcan et al., 2012). In fact, deregulated epigenetic homeostasis by D-2HG extends beyond DNA methylation. Histone methylation marks are also elevated in IDH mutant cells, consistent with competitive inhibition of KDMs by D-2HG (Chowdhury et al., 2011; Xu et al., 2011). The effects of IDH mutation on the activity of HIF PHDs appears complex. Although an initial report suggested that IDH mutation was associated with HIF stabilization (Zhao et al., 2009), subsequent studies reported that D-2HG can promote PHD in vitro and reduce hypoxic HIFα induction in vivo (discussed in Losman and Kaelin, 2013). The ability of D-2HG to stimulate the in vitro activity of PHDs results from its non-enzymatic conversion into 2OG (Tarhonskaya et al., 2014). Whether this mechanism contributes to the effects of IDH mutation and D-2HG on HIFα regulation in vivo remains unclear.

Fumarate Hydratase and Succinate Dehydrogenase

Loss-of-function mutations in fumarate hydratase (FH), an enzyme that catalyzes conversion of fumarate to malate, are associated with elevated fumarate levels and hereditary leiomyomatosis and renal cell cancer (Yang et al., 2013b). Similarly, hereditary paraganglioma and pheochromocytoma is associated with inactivating mutations in the four succinate dehydrogenase (SDH) subunits (A–D), which prevents the conversion of succinate into fumarate, leading to massive succinate accumulation (Morin et al., 2014). Exactly how fumarate and succinate drive tumorigenesis is an area of active investigation. Several molecular mechanisms have been proposed, which may not necessarily be mutually exclusive (Morin et al., 2014; Zheng et al., 2015). One such potential mechanism is the inhibition of 2OG oxygenases via competition with 2OG. Indeed, abnormal levels of fumarate and succinate inhibit PHDs, causing HIF activation in normoxia (Morin et al., 2014). This "pseudo-hypoxia" may be a tumor "driver" in some circumstances. For example, inhibition of HIFα expression in tumor cells with stable SDHB knockdown reduces their growth as xenografts (Guzy et al., 2008). In contrast, HIFα inhibition does not prevent the formation of hyperplastic cysts in renal tubules following FH deletion (Adam et al., 2011). Other mechanisms may therefore contribute to tumorigenesis driven by FH (and SDH) mutation, including perhaps the inhibition of other 2OG oxygenases. Interestingly, histone and DNA methylation are elevated in cells with SDH loss-of-function, consistent with inhibition of KDM and TET enzymes (Morin et al., 2014; Yang et al., 2013b). Treating cells with fumarate or succinate induces histone methylation marks, which is phenocopied by knockdown of FH and SDH (Xiao et al., 2012). Importantly, basal- and TET-induced 5-hmC production is also inhibited under these conditions.

Concluding Remarks

Here we have described 2OG oxygenases as novel enzymes that catalyze hydroxylation and demethylation of DNA, RNA, and protein to regulate multiple steps in gene expression. We have highlighted their potential as nutrient-responsive signaling enzymes and discussed the expanding role of 2OG oxygenases in cancer. Despite these recent insights into the function of 2OG oxygenases, this enzyme family still remains relatively poorly characterized. Several enzymes remain completely unstudied and others have only been partially characterized (Figure 1). It is tempting to speculate that these enzymes may in the future also be implicated in the emerging themes reviewed here. It remains a distinct possibility that they might catalyze unique oxidative modifications to control additional steps in gene expression.

We have also reviewed the existing evidence supporting the 2OG oxygenase family as a potentially new class of nutrient sensor. Well-characterized nutrient-responsive signaling modules include those centered around the AMPK and mTOR kinases, which signal reductions in the ATP:AMP ratio and amino acid availability, respectively, to regulate metabolism, nutrient uptake, and autophagy (Yuan et al., 2013). Crosstalk between such pathways allows signal integration and fine-tuning of multiple complex biological responses. Therefore, one might predict the existence of additional layers of crosstalk between these pathways and members of the 2OG oxygenase family.

As discussed, the reliance of these enzymes on 2OG makes them vulnerable to competitive inhibition in the context of oncometabolite-driven cancers. Although initial studies have characterized the PHD, KDM, and TET families as targets of oncometabolites, it is possible that inhibition of other 2OG oxygenases may also play a role in the pathogenesis of the associated cancers. For example, it will be interesting to explore whether the activity of RNA oxygenases and translational hydroxylases is regulated by oncometabolites. Inhibition of these 2OG oxygenases by D-2HG, fumarate, or succinate could contribute to deregulated gene expression control in the context of IDH, FH, or SDH cancer mutations (Figure 4). As summarized above, characterized 2OG oxygenases have also been associated with cancer due to their involvement in hypoxia signaling and epigenetic regulation and their altered expression, amplification, and mutation. Indeed, cancer genetics databases indicate that the importance of these enzymes in cancer is probably

underestimated. Considering the significant potential of 2OG oxygenases as a novel class of drug targets (Rose et al., 2011), future work investigating the role of these enzymes in gene expression control and cancer is warranted.

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