

Review

KDM4/JMJD2 Histone Demethylases: Epigenetic Regulators in Cancer Cells

William L. Berry and Ralf Janknecht

Abstract

Lysine methylation is one of the most prominent histone posttranslational modifications that regulate chromatin structure. Changes in histone lysine methylation status have been observed during cancer formation, which is thought to be a consequence of the dysregulation of histone lysine methyltransferases or the opposing demethylases. KDM4/JMJD2 proteins are demethylases that target histone H3 on lysines 9 and 36 and histone H1.4 on lysine 26. This protein family consists of three ~130-kDa proteins (KDM4A-C) and KDM4D/JMJD2D, which is half the size, lacks the double PHD and Tudor domains that are epigenome readers and present in the other KDM4 proteins, and has a different substrate specificity. Various studies have shown that KDM4A/JMJD2A, KDM4B/JMJD2B, and/or KDM4C/JMJD2C are overexpressed in breast, colorectal, lung, prostate, and other tumors and are required for efficient cancer cell growth. In part, this may be due to their ability to modulate transcription factors such as the androgen and estrogen receptor. Thus, KDM4 proteins present themselves as novel potential drug targets. Accordingly, multiple attempts are under way to develop KDM4 inhibitors, which could complement the existing arsenal of epigenetic drugs that are currently limited to DNA methyltransferases and histone deacetylases. *Cancer Res*; 73(10); 2936–42. ©2013 AACR.

Introduction

Negatively charged DNA wraps around a core of positively charged histones to allow for condensation of our genetic material. The state of compaction changes following specific alterations in histone posttranslational modifications. Acetylation and methylation are the two predominant covalent modifications, where acetylation of a positively charged lysine residue reduces the overall charge of a histone and generally leads to the relaxation of chromatin and thereby enhanced gene transcription. Methylation on arginine or lysine residues, in contrast, does not alter the charge of histones and can have repressive or activating consequences on gene expression, depending on which particular arginine or lysine residue becomes modified (1, 2).

Global as well as local changes in chromatin structure are characteristic for tumors, suggesting that such epigenetic changes are an underlying cause of cancer. Accordingly,

enzymes involved in histone modification and also DNA methylation may be viable drug targets. And indeed, histone deacetylase and DNA methyltransferase inhibitors are already approved by the U.S. Food and Drug Administration for the treatment of cutaneous T-cell lymphoma and myelodysplastic syndrome, respectively. However, targeting enzymes that methylate or demethylate histones has not yet progressed to standard clinical use (3).

JMJD Proteins

Not long ago, histone methylation was considered to be an irreversible mark. This dogma was finally laid to rest upon the discovery of the first lysine-specific demethylase (LSD1) in 2004 (4). Human LSD1 and its only paralog, LSD2, demethylate mono- and dimethylated histone H3 lysine 4 (H3K4) and H3K9 through a FAD-dependent amine oxidation reaction. The second known family of histone demethylases, the JMJD (Jumonji C domain-containing) proteins, is composed of 30 members in humans based on the presence of the roughly 150 amino acid-long JmjC (Jumonji C) domain (5). However, whereas most of the JMJD proteins have been proved to demethylate H3K4, H3K9, H3K27, H3K36, or H4K20, the catalytic activity of several JMJD proteins remains to be uncovered. Notably, some JMJD proteins are predicted to have no catalytic activity at all. Furthermore, it remains controversial whether any JMJD protein can target methylated arginine residues (6).

JMJD proteins use a different reaction mechanism compared with LSD1/2. They act through a dioxygenase reaction mechanism requiring Fe^{2+} , O_2 , and 2-oxoglutarate to demethylate histones. The true catalytic step is the hydroxylation of a lysine

Authors' Affiliation: Department of Cell Biology and Peggy and Charles Stephenson Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma

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Corresponding Author: Ralf Janknecht, University of Oklahoma Health Sciences Center, 975 NE 10th Street, BRC-1464, Oklahoma City, OK 73104. Phone: 405-271 2377; Fax: 405-271 3548; E-mail: ralf-janknecht@ouhsc.edu

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methyl group, thereby converting it to a hydroxymethyl moiety that spontaneously disconnects from the nitrogen center resulting in the release of formaldehyde. This reaction mechanism allows JMJD proteins in principle to demethylate tri-, di-, and monomethylated lysine residues, whereas LSD1 and LSD2 are prohibited from attacking trimethylated lysines due to the requirement of a free electron pair on the methylated nitrogen (5, 6). One of the largest JMJD subfamilies that has recently attracted much attention is composed of the JMJD2A-D proteins (nowadays preferentially called KDM4A-D, for K demethylase 4 A-D), which are capable of recognizing di- and trimethylated H3K9 and H3K36 as well as trimethylated H1.4K26 as substrates (Fig. 1A and 1B).

In general, H3K9 and H1.4K26 trimethylation are associated with transcription repression or heterochromatin formation, whereas H3K36 methylation has been perceived with activating gene expression (1, 3). However, this situation may be more nuanced, as cross-talking with other histone modifications influences the outcome of H3K9, H3K36, and H1.4K26 methylation (7). Also, H3K36 methylation shifts from mono- to trimethylation from the promoter to the end of transcribed genes. Thus, H3K36 trimethylation perhaps inhibits gene transcription at the start site but facilitates transcription elongation and prevents unwanted transcription initiation within the body of the gene that can negatively interfere with transcription initiation from the

regular start site (Fig. 1B). Moreover, the role of H3K36 methylation (and likely H3K9 and H1.4K26 methylation) is not limited to transcription control but extends to alternative splicing, DNA replication, recombination, and repair (8). Accordingly, modification of the histone methylation status due to changes in KDM4 activity may have profound effects on the transcriptome and other nuclear processes and therefore also on the initiation and progression of cancer. Here, we focus on the potential oncogenic roles of the KDM4 proteins.

The Protagonist: KDM4A/JMJD2A

The most studied member of the KDM4 family is KDM4A/JMJD2A, also sometimes called JHDM3A (JmjC domain-containing histone demethylase 3A). Although KDM4A is capable of demethylating H3K9 and H3K36, qualitative and kinetic measurements indicate that it demethylates H3K9me₃ about 5-fold more efficiently than H3K36me₃. In addition, KDM4A is more efficient in demethylating tri-versus dimethylated H3K9/H3K36 (9–12) and, even more specifically, only demethylates trimethylated H1.4K26 (13). An explanation for its substrate specificity was provided by X-ray crystallographic structure analyses of KDM4A with and without peptide substrates. These studies revealed that amino acids surrounding lysines 9 and 36 on histone H3 primarily determine the binding specificity of KDM4A,

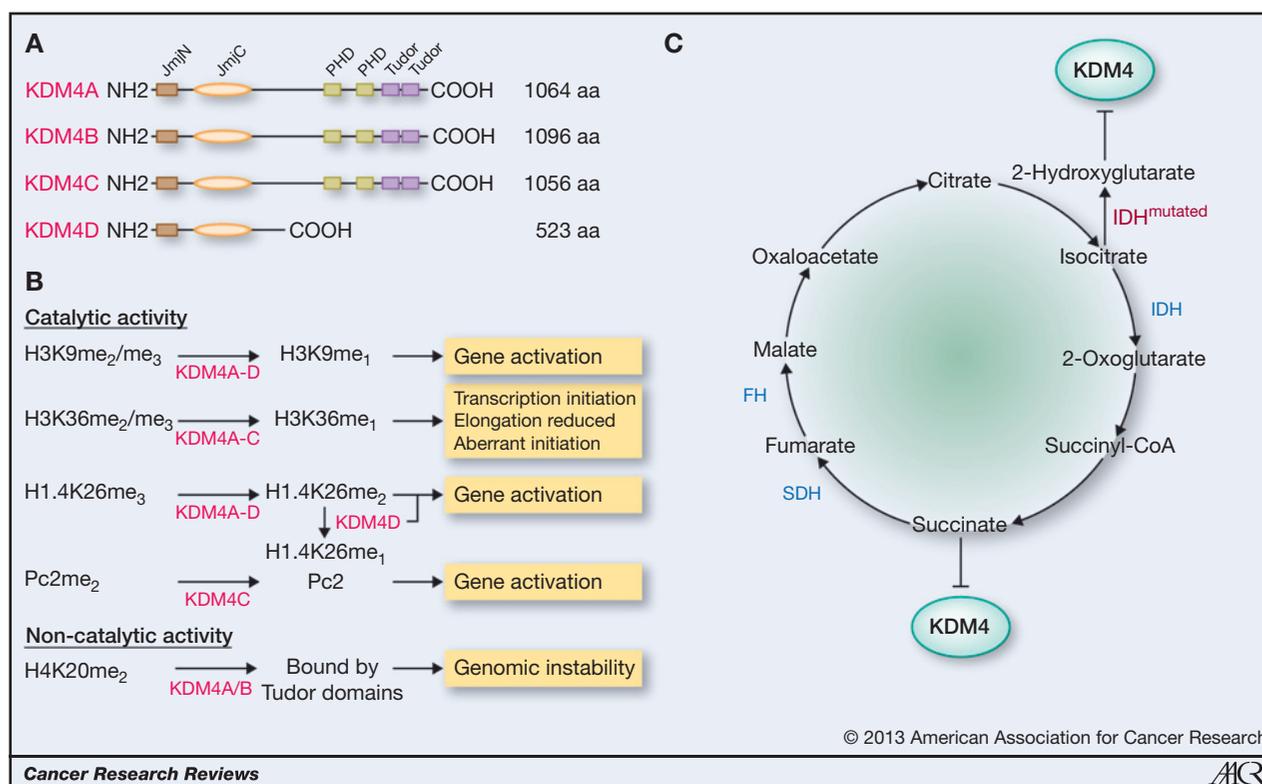


Figure 1. A, schematic structure of the 4 KDM4 proteins. The JmjN domain is required for the activity of the JmjC catalytic center. B, modes of KDM4 function as demethylases or independent of enzymatic activity. C, succinate dehydrogenase (SDH), fumarate hydratase (FH), and isocitrate dehydrogenase (IDH) in the Krebs cycle. Succinate accumulates upon SDH or FH mutation, whereas neomorphic IDH mutations lead to 2-hydroxyglutarate production.

whereas the space and the electrostatic environment in the methyl group-binding pocket allow tri-/di- but not mono-methylated lysine residues to position a methyl group productively toward the iron-containing catalytic center (11, 14–16).

A major study focus on KDM4A has been in transcription regulation, where it may either stimulate or repress gene transcription. The latter function may involve association with the nuclear receptor corepressor complex or histone deacetylases (17, 18) or direct binding to a transcription factor as observed for the p53 tumor suppressor (19), but it remains unresolved whether this repressing function requires KDM4A enzymatic activity. In contrast, KDM4A formed complexes with both the androgen and estrogen receptor (ER) and stimulated their activity, which was dependent on KDM4A catalytic activity (20, 21). Accordingly, depletion of KDM4A in ER-positive T47D breast cancer cells decreased the expression of ER targets such as the c-Jun and cyclin D1 oncogenes and led to reduced cell growth. Equally, JMJD2A knockdown inhibited proliferation of ER-negative MDA-MB-231 and ER-positive MCF7 breast tumor cells (22, 23), suggesting that KDM4A is critical for growth of both ER-positive and -negative breast tumors. Consistent with an oncogenic function of KDM4A, it is overexpressed at the protein level in about 60% of breast tumors (21) and further reports show KDM4A overexpression also at the mRNA level in this cancer (24, 25). Likewise, KDM4A is overexpressed in prostate (26) and lung cancer (ref. 27; see also Supplementary Table S1).

Similar to breast cancer cells, downregulation of KDM4A in multiple colon cancer cell lines resulted in reduced cell proliferation and furthermore increased apoptosis and delayed the G₂-M phase of the cell cycle (19). In U2OS human osteosarcoma cells, KDM4A depletion resulted in G₀-G₁ growth arrest, whereas senescence was induced in normal fibroblasts. On the other hand, overexpression of KDM4A cooperated with the H-Ras oncoprotein in transforming human IMR90 fibroblasts by suppressing Ras-induced senescence (27). However, no growth effect was observed upon KDM4A overexpression or downregulation in HeLa cervical carcinoma or 293T embryonal kidney cells, although S-phase progression was accelerated by wild-type, but not catalytically inactive KDM4A; seemingly, the S-phase acceleration was neutralized by delays in other cell-cycle phases (28). Together, these findings imply that KDM4A overexpression will not generally stimulate tumor growth but will only do so in certain organs or cell types. It remains to be determined which circumstances allow KDM4A to exert a pro-oncogenic function, for instance, the tissue-specific expression of cooperation partners.

Interestingly, overexpression of KDM4A, but not its H188A catalytic mutant, in mouse NIH3T3 cells reduced recruitment of heterochromatin protein 1 (HP1) to pericentric chromatin (10). Moreover, KDM4A overexpression reduced binding of HP1 γ to chromatin during replication in 293T cells concomitant with a more open chromatin structure. On the other hand, depletion of HP1 γ led to increased chromatin accessibility early in S phase, suggesting that KDM4A and

HP1 γ antagonize each other during DNA replication, which may represent a possible mechanism for how KDM4A promotes DNA replication. This antagonism appears to be conserved in the worm *Caenorhabditis elegans*, as loss of its sole KDM4 homolog led to slower DNA replication and this defect could be rescued by depletion of the *C. elegans* HP1 homolog (28).

In the fruitfly *Drosophila*, HP1a associates with KDM4A and stimulates its H3K36 demethylation activity. Unlike mammalian KDM4A, the *Drosophila* homolog appears to be incapable of demethylating H3K9, although this is controversial (29, 30). Moreover, *Drosophila* KDM4A overexpression resulted in HP1a spreading from the chromocenter into the chromosome arms, and HP1a was shown to recruit KDM4A to a subset of heterochromatic genes where it could demethylate H3K36me₃ (29–31). Currently, it is unresolved whether and how the interaction between HP1a and KDM4A affects DNA replication in *Drosophila*. However, it appears that these 2 proteins antagonize each other in the modulation of *Drosophila* gene transcription (32).

KDM4A is endowed with a double plant homeodomain (PHD) as well as a double Tudor domain (Fig. 1A). The latter binds to di- and trimethylated H3K4 and H4K20 (33–35), whereas the function of the KDM4A PHD domains has remained unknown. H3K4 trimethylation is a hallmark of active promoters and is normally mutually exclusive with H3K9 trimethylation, which is a mark of inactive chromatin (3). Thus, KDM4A may be recruited via the Tudor domains to active gene promoters and guarantees that H3K9 and also H1.4K26 become demethylated, which will amplify gene transcription by, for example, counteracting the binding of HP1 to these epigenetic marks. Although not yet studied for KDM4A, KDM4B is also part of the mixed-lineage leukemia 2 complex that has H3K4 methyltransferase activity, pointing at a potentially Tudor domain-independent way that KDM4B and possibly KDM4A concurrently demethylate H3K9 whereas H3K4 becomes trimethylated (36).

An unexpected function of KDM4A (and apparently also KDM4B) independent of its enzymatic activity was revealed in the DNA damage pathway. Here, 53BP1 is recruited into DNA damage foci and required to orchestrate the DNA damage response. Both 53BP1 and the Tudor domains of KDM4A competed for binding to dimethylated H4K20. However, KDM4A became ubiquitinated by the E3 ligases RNF8/RNF168 upon DNA damage and thereby marked for degradation, allowing recruitment of 53BP1 to sites of DNA damage (37). Thus, KDM4A overexpression could impair DNA damage repair and induce genomic instability by suppressing 53BP1 recruitment to DNA damage foci, another mechanism by which KDM4A possibly promotes tumorigenesis. In addition, KDM4A may affect DNA repair by inhibiting the Tip60 acetyltransferase, which is involved in the activation of the ataxia telangiectasia mutated kinase, a key component of the DNA double-strand repair pathway. Interestingly, Tip60 recognizes trimethylated H3K9 at DNA double-strand breaks and is thereby activated, suggesting that in this case, the catalytic activity of KDM4A is required to demethylate H3K9me₃ to exert an inhibitory effect on

Tip60-dependent DNA repair (38). The involvement of KDM4A/KDM4B in DNA damage repair appears to be evolutionary conserved, as loss of *C. elegans* KDM4 led to more DNA damage and altered progression of meiotic DNA double-strand break repair and heterozygous mutation of KDM4B in *Drosophila* resulted in more sensitivity to UV irradiation (9, 28, 39).

The Close Relatives: KDM4B/JMJD2B and KDM4C/JMJD2C

KDM4B and KDM4C are structurally very similar to KDM4A (Fig. 1A) and have the same target specificity and comparable enzymatic activities *in vitro* (12, 13). However, earlier reports indicated a much lower catalytic activity of KDM4B than other KDM4 proteins (9, 40); it is currently unclear what the cause of this discrepancy is, for instance, the use of differently sized recombinant KDM4B proteins or dissimilar substrates to measure catalytic activity. Phylogenetically, KDM4B and KDM4C are present like KDM4A in all vertebrates studied, whereas *Drosophila* has only 2 KDM4 homologs and *C. elegans* just one (12).

Similar to KDM4A, KDM4B and KDM4C mRNA levels are upregulated in breast tumors. Interestingly, KDM4B expression is higher in ER-positive than in ER-negative breast tumors, whereas the opposite holds true for KDM4C (41–43). In addition, KDM4B appears to be overexpressed in triple-negative breast tumors (ref. 25; see also Supplementary Table S1). Like KDM4A, KDM4B formed complexes with ER and stimulated transcription of ER target genes (36, 42). Downregulation of KDM4B in ER-positive MCF7 or T47D cells reduced cell proliferation and tumor formation in nude mice, whereas no changes were reported for ER-negative MDA-MB-231 cells upon KDM4B knockdown (36, 42, 44). Further, KDM4C downregulation led to reduced proliferation of ER-negative HCC1954 and Colo24 breast cancer cells, whereas its overexpression in nontransformed MCF10A cells caused colony formation in soft agar and increased mammosphere formation, an indicator of cancer stem cells (41). Notably, KDM4C is a target gene of the pluripotency factor Oct4, and KDM4C downregulation induced the differentiation of embryonic stem cells (45), suggesting that KDM4C overexpression is particularly important in cancer stem cells. Collectively, KDM4C overexpression may contribute to tumor formation in ER-negative breast tumors, whereas KDM4B mediates neoplastic transformation of ER-positive cells. The fact that KDM4B is an ER target gene (36, 42, 44) lends further support to the view that KDM4B is involved in the genesis of ER-positive tumors.

Also similar to KDM4A, KDM4C formed complexes with and stimulated androgen receptor and promoted androgen-dependent growth of prostate cancer cells, thus implicating KDM4C in prostate tumorigenesis (46). Evidence has emerged that KDM4B and KDM4C are overexpressed in prostate tumors and medulloblastomas (26, 47); in addition, KDM4B is overexpressed in gastric, bladder, lung, and colorectal cancer and required for proliferation, colony formation ability, invasion, or survival of respective cell lines

(refs. 48–50; see also Supplementary Table S1). Moreover, the KDM4C gene was found to be translocated in mucosa-associated lymphoid tissue lymphoma, resulting in its overexpression (51). While all this furthers the notion of KDM4B and KDM4C as oncoproteins, such evidence must be regarded with caution, as KDM4B/C overexpression in tumors may be a consequence rather than a cause of tumorigenesis and changes of *in vitro* physiology upon modulation of KDM4B or KDM4C levels in cancer cells are no proof that KDM4B/C overexpression initiates or supports tumor formation in the human body.

Maybe a more convincing case for KDM4C as an oncogene is present in primary mediastinal B-cell lymphoma and Hodgkin lymphoma, where amplification of the 9p24 chromosomal region has been found. KDM4C and the tyrosine kinase JAK2 are encoded within this amplicon, and both proteins are capable of epigenetic modulation, including activation of the *c-Myc* oncogene. A potential mechanism is the eviction of HP1 from the *c-Myc* promoter, as binding of HP1 to trimethylated H3K9 or H1.4K26 would be inhibited by KDM4C-dependent demethylation as well as by JAK-mediated phosphorylation of H3Y41 that also suppresses HP1 binding to chromatin. Moreover, inhibition of JAK2 and KDM4C synergized to kill primary mediastinal B-cell and Hodgkin lymphoma cells (52). These genetic and *in vitro* data combined provide a strong argument that, in addition to JAK2, KDM4C is pro-oncogenic and a potential drug target in these lymphomas. Notably, 9p24 amplification has also been found in esophageal squamous cell carcinomas, which coined the older name GASC1 (gene amplified in squamous cell carcinoma 1) for KDM4C (53), as well as in sarcomatoid carcinoma of the lung and desmoplastic medulloblastoma (54, 55), suggesting that KDM4C may cooperate with JAK2 in many different tissues to contribute to tumor formation.

Overexpression of KDM4A-C in the same cancer (e.g., prostate cancer) and similar physiologic functions in cancer cells suggest that KDM4 proteins may conduct overlapping functions. Moreover, the absence of obvious pathologic phenotypes in KDM4B- as well as KDM4D-knockout mice hints at redundancy within the KDM4 family (42, 56). However, KDM4 proteins can also behave differently. For instance, hypoxia induced KDM4B and, to a lesser extent, KDM4C transcription, whereas the KDM4A gene appeared to be unaffected (57, 58). In fact, KDM4B was required for increased transcription of many hypoxia-inducible genes in colorectal cancer cell lines. Also, KDM4B was overexpressed in colorectal cancer specimens that concurrently were positive for carbonic anhydrase 9, a marker of hypoxia, and KDM4B overexpression was correlated with larger tumor size and advanced clinical stage (50). In addition, KDM4C bound to and stimulated hypoxia-inducible factor-1 α , the key transcription factor mediating the cellular response to hypoxia (59). Therefore, KDM4B and/or KDM4C may help tumors to thrive in a hypoxic environment. Another example of differences among KDM4 proteins comes from the study of the polycomb 2 protein (Pc2), which is associated with heterochromatin formation and epigenetic silencing. Pc2

was shown to be dynamically methylated, and KDM4C was the only member of the KDM4 family capable of demethylating Pc2. This demethylation of Pc2 led to the activation of growth control genes, providing a distinct mechanism for how KDM4C could facilitate tumorigenesis independent of histone demethylation (60).

The Outlier: KDM4D/JMJD2D

KDM4D is unique within the KDM4 family in that it lacks both the PHD and Tudor domains and thus is only half the size of KDM4A-C (Fig. 1A). Phylogenetically, KDM4D evolved recently, as it has only been found in placental mammals (12). Bioinformatical analyses revealed the presence of 2 further human genes, *KDM4E* and *KDM4F*, whose gene products are very similar to KDM4D. However, *KDM4E* and *KDM4F* are most likely pseudogenes (9, 61). Furthermore, in contrast with KDM4A-C, KDM4D has a different substrate specificity: It does not demethylate H3K36 owing to several differences in its substrate binding cleft yet has gained the ability to attack dimethylated in addition to trimethylated H1.4K26 (9, 13, 62). Also, KDM4D attacks H3K9me₂ with similar efficiency as H3K9me₃ and may, albeit inefficiently, even demethylate H3K9me₁ (11, 12, 63). Another difference of KDM4D is manifest in its association behavior: Whereas KDM4A and KDM4C homo- and heteromerize among each other, KDM4D is only capable of forming homomers (63).

Like KDM4A and KDM4C, KDM4D functioned as a co-activator of the androgen receptor (20). Also, like KDM4A, KDM4D was required for colon cancer cell proliferation and survival. However, in contrast with KDM4A being a repressor of p53 transcriptional activity, KDM4D stimulated p53-dependent gene expression (64). Whereas the stimulation of the androgen receptor and colon cancer cell growth points to a pro-oncogenic function, the role of KDM4D in activating p53-dependent gene transcription would suggest the opposite, highlighting that further research is needed to resolve the net effect, if any, of KDM4D in tumorigenesis.

Interestingly, the pro-inflammatory TNF- α induced KDM4D expression in dendritic cells and macrophages. Moreover, the ability of KDM4D to demethylate H3K9 was shown to be involved in the TNF- α response (65). Therefore, KDM4D may influence tumorigenesis not only in cancer cells but also potentially in the tumor microenvironment and immune cells by mediating inflammatory responses elicited by cytokines such as TNF- α .

Therapeutic Implications and Perspective

Several lines of evidence suggest that enhanced catalytic activity of KDM4 proteins is associated with cancer. If KDM4 proteins are indeed drivers of tumorigenesis, they represent viable novel targets for cancer therapy. Many efforts are under way or were already undertaken to design specific KDM4 inhibitors, but their specificity and use as anticancer drugs require rigorous preclinical testing in the future (66–68). As a cautionary note, however, genetically engineered

mouse models are needed to definitively prove that overexpression of KDM4 proteins is an underlying cause of tumor formation. Also, such mouse models would represent an invaluable tool for eventually testing KDM4 inhibitors *in vivo*.

Apart from being histone demethylases, KDM4 proteins are predicted to demethylate nonhistone proteins, and Pc2 was the first such example (60). Many more nonhistone proteins that are targeted by KDM4 proteins will likely be discovered and thereby provide a deeper understanding of how KDM4 proteins can contribute to cancer formation. Not in the least, KDM4 proteins are dioxygenases and could therefore hydroxylate lysine or asparagine residues, as already shown for other JMJD proteins (6). Thereby, KDM4 proteins may regulate the function of a much broader spectrum of proteins by affecting different kinds of post-translational modifications. Also, as shown for the role of KDM4A/B in the DNA damage response pathway (37) and for many KDM4 target genes in *Drosophila* (32), there are instances when KDM4 proteins act independently of their enzymatic activity and thus inhibitors targeting their catalytic center would not completely shut down KDM4 biologic activities. Another open question concerns the function of the PHD domains in KDM4A-C: Do they, like the Tudor domains, bind to specific histone modifications as observed for other proteins (69), and how does this relate to the function of KDM4 proteins?

Tumors often develop in a hypoxic environment, which is a major limiting factor to their survival and proliferation. Especially KDM4B is induced by hypoxia and involved in the upregulation of hypoxia-inducible genes (44, 50, 57, 58). Would inhibition of KDM4B therefore aggravate the hypoxic tumor microenvironment, which could lead to tumor necrosis? Also, as KDM4 proteins are O₂-dependent enzymes, is their catalytic activity suppressed by hypoxia in tumors, and what physiologic consequences would that have?

Finally, KDM4 proteins are 2-oxoglutarate-dependent enzymes, so any perturbation of the endogenous 2-oxoglutarate pool will affect KDM4 activity. Or does overexpression of KDM4 deplete 2-oxoglutarate and thereby inhibit other 2-oxoglutarate-dependent enzymes? It is known that mutations of succinate dehydrogenase (SDH) and fumarate hydratase (FH) found in various cancers impair the Krebs cycle (Fig. 1C), leading to accumulation of succinate that is an end-product inhibitor of JMJD proteins, including KDM4A and KDM4D (70, 71). Moreover, cancer-associated mutations in isocitrate dehydrogenase (IDH) not only reduce the formation of 2-oxoglutarate but also have gained a new catalytic activity of converting isocitrate to 2-hydroxyglutarate (Fig. 1C), which is an inhibitor of KDM4 proteins (72). Thus, could KDM4 overexpression be required in tumors to balance the inhibition of their catalytic activity upon SDH, FH, or IDH mutation? Regardless, KDM4 activity appears to be intricately controlled by the cancer metabolome, but an understanding of how this will affect KDM4 function and cancer cells requires more study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: W.L. Berry, R. Janknecht

Writing, review, and/or revision of the manuscript: W.L. Berry, R. Janknecht

Study supervision: R. Janknecht

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William L. Berry and Ralf Janknecht

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