Am J Transl Res 2014;6(1):1-15
www.ajtr.org /ISSN:1943-8141/AJTR1311006

*Review Article*

# Histone lysine demethylase (KDM) subfamily 4: structures, functions and therapeutic potential

Roselyne M Labbé, Andreana Holowatyj, Zeng-Quan Yang

*Karmanos Cancer Institute, Department of Oncology, Wayne State University, Detroit, MI 48201, USA*

Received November 18, 2013; Accepted November 25, 2013; Epub December 1, 2013; Published January 1, 2014

**Abstract:** KDM4 histone demethylases catalyze the removal of methyl marks from histone lysine residues to epigenetically regulate chromatin structure and gene expression. KDM4 expression is tightly regulated to insure proper function in diverse biological processes, such as cellular differentiation. Mounting evidence has shown that disrupting KDM4 expression is implicated in the establishment and progression of multiple diseases including cancer. In particular, genomic regions encoding the *KDM4A*, *B* and *C* genes are often amplified, disrupting normal cellular proliferation. Furthermore, KDM4 demethylases are promising druggable targets. In this review, we highlight the latest advances in characterizing the structures and regulatory mechanisms of KDM4 proteins, as well as our current understanding of their alterations and roles in tumorigenesis. We also review the reported KDM4 inhibitors and discuss their potential as therapeutic agents.

**Keywords:** Histone lysine demethylase, KDM4, JmjC domain, cancer

## Introduction

Cell proliferation and cell fate are dynamically controlled through posttranslational histone modifications, including methylation, which is established and tightly regulated by histone methyltransferases and demethylases. These modification marks, which primarily localize to the flexible histone tails, but also to core histone residues, function to alter DNA compaction and to recruit transcription factors and transcriptional machinery [1, 2]. Methylation of lysine and arginine histone side chains and core domains serves to modulate the epigenetic landscape with significance in transcriptional control during embryonic development, genomic imprinting and X chromosomal inactivation [2-4]. The accumulated evidence also links improper histone methylation to the dysregulation of cellular processes underlying several human diseases. For instance, it is now clear that members of the histone lysine demethylase (KDM) subfamily 4 are commonly overexpressed in human cancers, where they have been found to disrupt normal cellular proliferative balance [5, 6]. Here, we aim to review our current understanding of the structures, functions and therapeutic potential of this subfamily of proteins.

## Histone lysine demethylase families

Histone methylation is known to occur on the lysine residues of histones 3 and 4 (H3, H4), and the linker histone H1, isotype 4 (H1.4). On H3, four N-terminal lysine residues (K4, K9, K27, K36) and two structural residues (K56, K79) are able to be methylated [1, 7-10]. The linker histone H1.4, which is associated with intergenic regions of the genome, can also be methylated at lysine 26 (H1.4K26) [11, 12]. At these histone lysine residues, methyltransferases and demethylases can, respectively, add or remove mono- (me1), di- (me2), or trimethyl (me3) marks, the degree of which alters chromatin compaction and gene expression. Methylation of H3K4, H3K36 and H3K79 is generally associated with gene activation, while methylation of H3K9, H3K27, H3K56, H4K20 and H1.4K26 is linked to transcriptional repression [1, 13].

Structurally, the histone lysine demethylases are a diverse group of proteins which can be

broadly categorized under two functional enzymatic families. The first family includes the lysine specific demethylase (LSD1, also known as KDM1A), which, along with the structurally similar KDM1B (LSD2), consist of the flavin adenine dinucleotide (FAD)-dependent amine oxidases, which can remove mono- and dimethyl histone lysine marks [14-16]. These amine oxidases, however, are unable to demethylate trimethyl lysine residues since they require a lone pair of electrons only present on mono- and dimethyl lysine histone residues. The second family of histone demethylases consists of the Jumonji C (JmjC)-domain containing proteins which employ an oxygenase mechanism to demethylate specific histone mono-, di- and trimethyllysine residues. The enzymatic function of the JmjC domain relies on α-ketoglutarate (α-KG), Fe(II), and molecular oxygen as cofactors in the demethylation reaction [13]. An analysis of public protein-domain databases has revealed that humans encode 32 such JmjC-domain containing genes, 24 of which have documented biochemical demethylase activity (**Table 1**). The function of these diverse JmjC-domain containing proteins is further distinguished by combinations of other conserved domains including the PHD, Tudor, CXXC, FBOX, ARID, LRR, as well as JmjN domains. Based on sequence homologies and structural similarities, these 24 JmjC-domain containing demethylases can be categorized into seven functionally divergent protein subfamilies (**Table 1**) [17, 18].

## Genomic and protein structures of KDM4 demethylases

Within the family of JmjC-domain containing demethylases is the large KDM4 subfamily. In the human genome are five functional KDM4 member genes (*KDM4A-E*). Those encoding *KDM4A*, *B* and *C* localize to human chromosomes 1p34.1, 19p13.3, and 9p24.1, respectively. *KDM4D* localizes to human chromosome 11q21, and forms a cluster with two additional intronless *KDM4* genes, *KDM4E* and *KDM4F* [19]. Previously, *KDM4E* and *F* were considered pseudogenes, however *KDM4E* expression has recently been observed, suggesting its role as a functional gene [1, 20, 21]. The KDM4 subfamily is highly conserved, with orthologs of KDM4A, B, and C found among all vertebrates, and orthologs of KDM4D found in placental mammals [21].

The KDM4A, B and C proteins, which share more than 50% sequence identity, each contain JmjN, JmjC, two plant homeodomains (PHD) and two Tudor domains. KDM4D and KDM4E, in contrast, are considerably shorter proteins which lack the C-terminal region, including the PHD and Tudor domains (**Table 1**). As with all JmjC-domain containing demethylases, the KDM4 JmjC domain bears catalytic function while the JmjN domain interacts extensively with JmjC and provides structural integrity [5, 22]. Recent biochemical studies indicate that KDM4A-C catalyze the removal of H3K9 and H3K36 di- and trimethyl marks, while KDM4D can only demethylate H3K9me3/me2. KDM4E meanwhile, catalyzes the removal of two methyl groups from H3K9me3 and H3K56me3 [23]. Interestingly, the H3K56me3 heterochromatic mark is highly conserved, found also in *C. elegans*, where it regulates DNA replication [23].

Beyond the catalytic core of KDM4A-C, the C-terminal PHD and Tudor domains bear important histone reader functions. Structural and biochemical studies have demonstrated that the Tudor domains of KDM4A can recognize and bind two unrelated histone marks, H3K4me3/me2 and H4K20me3/me2, by means of distinct binding mechanisms. Three aromatic residues in the KDM4A-Tudor domains, F932, W967, and Y973, can form an open cage that accommodates H3K4me3 binding [24]. In contrast, KDM4A binding to H4K20me3 requires the Tudor domains to adopt opposite relative orientations, using the same three aromatic residues which contact different surfaces [25]. In addition, the PHD domains in other histone regulatory proteins have been demonstrated to bind unmodified, methylated, and/or acetylated histone residues on one or more histone tails, offering flexibility in directing epigenetic modifications [26, 27]. However, as of yet, no functional studies or three-dimensional structure of the KDM4A-C PHD domains have been reported, highlighting the need to clarify the molecular function of these domains.

## Expression and physiological functions of KDM4 demethylases

Previous studies have indicated that KDM4A and C are broadly expressed in mouse and/or human tissues, while KDM4D and E are pre-

**Table 1.** Functional classification and histone substrates of the histone lysine demethylases

| Official Symbol | Other Aliases | Gene Location | Gene ID | Protein Domains | Histone Substrates |
|---|---|---|---|---|---|
| KDM1A | LSD1, AOF2, BHC110, KDM1 | 1p36.12 | 23028 | | H3K4me1/me2, H3K9me1/me2 |
| KDM1B | LSD2, AOF1, C6orf193, | 6p22.3 | 221656 | | H3K4me1/me2 |
| KDM2A | JHDM1A, FBXL11, CXXC8, FBL11, FBL7, LILINA | 11q13.2 | 22992 | | H3K36me1/me2 |
| KDM2B | JHDM1B,CXXC2, FBXL10, Fbl10, PCCX2 | 12q24.31 | 84678 | | H3K4me3, H3K36me1/me2 |
| KDM3A | JHDM2A, JHMD2A, JMJD1, JMJD1A, TSGA | 2p11.2 | 55818 | | H3K9me1/me2 |
| KDM3B | JMJD1B, 5qNCA, C5orf7, NET22 | 5q31 | 51780 | | H3K9me1/me2 |
| JMJD1C | KDM3C, TRIP8 | 10q21.3 | 221037 | | H3K9me1/me2 |
| KDM4A | JMJD2A, JHDM3A, JMJD2, TDRD14A | 1p34.1 | 9682 | | H3K9me2/me3, H3K36me2/me3, H1.4K26/me2/me3 |
| KDM4B | JMJD2B, TDRD14B | 19p13.3 | 23030 | | H3K9me2/me3, H3K36me2/me3, H1.4K26me2/me3 |
| KDM4C | GASC1, JMJD2C, JHDM3C, TDRD14C | 9p24.1 | 23081 | | H3K9me2/me3, H3K36me2/me3, H1.4K26me2/me3 |
| KDM4D | JMJD2D | 11q21 | 55693 | | H3K9me2/me3, H1.4K26me2/me3 |
| KDM4E | JMJD2E, KDM4DL | 11q21 | 390245 | | H3K9me2/me3, H3K56me3 |
| KDM5A | JARID1A, RBBP-2, RBBP2, RBP2 | 12p11 | 5927 | | H3K4me2/me3 |
| KDM5B | JARID1B, CT31, PLU-1, PUT1, RBBP2H1A | 1q32.1 | 10765 | | H3K4me2/me3 |
| KDM5C | JARID1C, MRXJ, MRXSCJ, MRXSJ, SMCX, XE169 | Xp11.22-p11.21 | 8242 | | H3K4me2/me3 |
| KDM5D | JARID1D, HY, HYA, SMCY | Yq11 | 8284 | | H3K4me2/me3 |
| KDM6A | UTX, KABUK2, | Xp11.2 | 7403 | | H3K27me2/me3 |
| KDM6B | JMJD3 | 17p13.1 | 23135 | | H3K27me2/me3 |
| JHDM1D | KDM7A | 7q34 | 80853 | | H3K9me1/me2, H3K27me1/me2 |
| PHF8 | KDM7B, JHDM1F, MRXSSD, ZNF422 | Xp11.22 | 23133 | | H3K9me1/me2, H4K20me1 |
| PHF2 | KDM7C, CENP-35, GRC5, JHDM1E | 9q22.31 | 5253 | | H3K9me2 |
| MINA | MINA53, MDIG, FLJ14393, ROX, NO52 | 3q11.2 | 84864 | | H3K9me3 |
| NO66 | ROX, NO66, MAPJD | 14q24.3 | 79697 | | H3K4me1/me3, H3K36me2 |
| KDM8 | JMJD5 | 16p12.1 | 79831 | | H3K36me2 |

Note: SWIRM: Swi3p, Rsc8p, and Moira domain (pink); Amine Oxidase domain (olive green); Spacer region (light green); CW-type zinc-finger domain (fuchia); JmjC domain (red); CXXC zinc-finger domain (purple); PHD: plant homeodomain (green); FBOX: F-box domain (black); LRR: Leu-rich repeat domain (brown); JmjN domain (blue); Tudor domain (yellow); ARID: AT-rich interacting domain (orange); C5HC2 zinc-finger domain (grey); TPR: tetratricopeptide domain (light blue).

dominantly expressed in the mouse testes [1, 28-30]. To further investigate the expression of human KDM4 demethylases, we conducted meta-analyses of next-generation sequencing profiles for normal tissues using the RNA-Seq Atlas, and for normal and diseased tissues using GENT databases [31, 32]. Generally, *KDM4A*, *B*, and *C* are broadly expressed in normal human tissues, with high expression in the spleen, ovary and colon (**Figure 1**). Based on RPKM (Reads Per Kilobase per Million) values, expression levels of *KDM4A* and *C* are approximately 3-6 fold higher than those of *KDM4B*. For instance, in the spleen, *KDM4A* and *KDM4C* have RPKM values of about 6 and 9 respectively, compared to 1.3 for *KDM4B*. Both *KDM4D* and *E* are predominantly expressed in the human testes. However, the RPKM values of *KDM4E* in human tissues are very low (<0.25) as compared to other *KDM4* genes. The variation in expression levels of the KDM4 subfamily members in human tissues suggest these proteins may be regulated by distinct pathways and have non-overlapping biological functions in different cell types.

To study the physiological function of KDM4, knockout and/or transgenic models have been

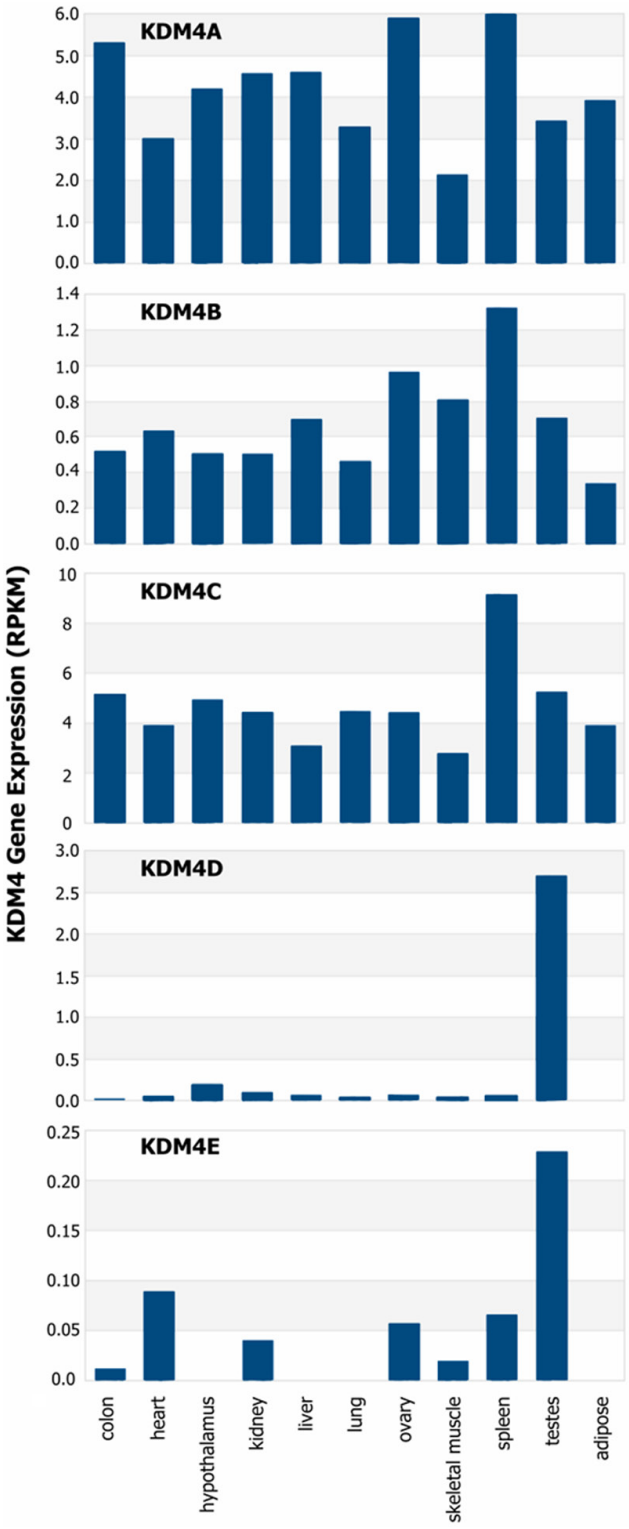


**Figure 1.** Analysis of gene expression levels for KDM4 member genes in normal human tissues using the RNA-Seq Atlas [28]. Next-generation sequencing profiles for each of *KDM4A* (NM_014663), *KDM4B* (NM_015015), *KDM4C* (NM_015061), *KDM4D* (NM_018039) and *KDM4E* (NM_001161630) are presented as Reads Per Kilobase per Million mapped reads (RPKM).

established in model organisms including *Drosophila melanogaster, C. elegans* and mice. Double homozygous mutants of both *Drosophila KDM4* orthologs, *dKDM4A* and *B,* are not viable and die in the second instar larval stage [33]. Depletion of the single *C. elegans KDM4* gene results in germ line apoptosis and slows DNA replication [34]. Studies in mice using conditional heart-specific *KDM4A* knockout as well as transgenic mice have demonstrated that KDM4A promotes cardiac hypertrophy in response to hypertrophic stimuli [35]. Knockout mouse models for *KDM4B* and *D* are viable without gross abnormalities [36]. Conditional knockout of *KDM4B* in mammary epithelial cells exhibit delayed mammary gland development with reduced branching [37]. Though absent in other tissues, KDM4D is highly expressed in spermatocytes and spermatids [30]. Mutant *KDM4D* male mice have globally higher levels of H3K9me3 than control mice [30]. However, adult *KDM4D* mutant mice are as fertile as control mice, possibly through *KDM4B* compensation.

During development, several KDM4 members are known to play important roles in maintaining the open chromatin state required in embryonic stem (ES) cells to ensure efficient proliferation and readiness for differentiation [38]. At an epigenetic level, this euchromatic state relies on the absence of H3K9 methylation, which is insured by KDM4 demethylase activity. In mouse development, *KDM4A, B* and *C* are expressed early in the fertilized egg and in undifferentiated ES cells [19, 39]. The functions of KDM4 proteins during development are diverse, as they promote pluripotency in some instances and direct differentiation in others. KDM4A for instance, which is essential for mouse embryonic development, also drives neural crest specification in the chick embryo [40, 41]. In humans, embryonic skeletal, bone and fat cell differentiation depends on KDM4A, KDM4B, and KDM4C, respectively [42-44].

Paradoxically, while KDM4 proteins appear to direct differentiation during embryogenesis, they also participate in maintaining the gene expression signa-

ture typical of undifferentiated stem cells. KDM4 proteins interact with, or prompt the expression of many pluripotency factors including Oct4, Sox2 and c-Myc, which together with Klf4, are sufficient to induce the reprogramming of differentiated cells to a pluripotent state [45]. KDM4A can induce expression of Oct4, which is required for the de-differentiation of adult neural stem cells to induced pluripotent stem (iPS) cells [46]. In undifferentiated human ES cells, KDM4C is conversely induced by Oct4 [19, 28, 39, 47]. Evidence also supports the participation of KDM4C in the Oct-4/Sox2/Nanog expression feedback loop... described by Wagner and Cooney [48]. When KDM4C expression is ablated, Oct-4, Sox2 and Nanog signalling is eliminated [47]. In this context, H3K9me3 demethylation by KDM4C directs the expression of pluripotency factors with critical implications in cellular reprogramming [39, 47]. Together, the interactions between KDM4 proteins with several other molecular regulators likely play important roles for directing stem cell functions during organismal development.

### Regulatory factors of the KDM4 subfamily

Considering the significant biological functions of KDM4 proteins, it is not surprising that cells have developed various mechanisms for controlling their expression, activity and localization. Recent studies have revealed that the abundance of KDM4A in the cell can be regulated by the ubiquitination pathway. For example, KDM4A is mediated by two SCF complexes, SKP1-CUl1-F-Box and FBXO22, which control its turnover and ubiquitination during cell cycle progression [49, 50]. Furthermore, KDM4A and B, but not C or D, are also regulated by ubiquitination in response to DNA damage by the RNF8 and RNF168 complexes [51]. The Hsp90 molecular chaperone also interacts with, and stabilizes the KDM4B protein [52]. Pharmacological inhibition of Hsp90 with geldanamycin consequently leads to ubiquitin-dependent proteasomal degradation of KDM4B, but not KDM4C. A recent study also revealed that the JmjN domain of KDM4D is poly(ADP-ribosyl) ated by PARP-1, affecting its H3K9 demethylation function [53]. It is likely that KDM4A, B and C are regulated by PARP-1 in a similar manner, as the two glutamic acid residues predisposed to poly(ADP-ribosyl)ation are conserved in all KDM4 family members. Very recently, Burton *et al.,* revealed that inositol hexakisphosphate kinase 1 (IP6K1) also interacts with KDM4C and regulates its demethylation function [54]. Over-expression of IP6K1 induces KDM4C dissociation from chromatin and increases H3K9me3 levels [54].

Expression of KDM4B and C are also regulated by several transcription factors in physiological and/or pathological conditions. HIF1, a master regulator of cellular and systemic homeostatic response to hypoxia, can induce KDM4B and C expression in both normoxic and hypoxic conditions [55]. Interestingly, KDM4C selectively interacts with HIF1α, which mediates its recruitment to the HIF1α target gene response elements in breast cancer [56]. KDM4B is also an androgen-regulated demethylase, which can influence AR transcriptional activity not only via demethylation but also by modulation of AR ubiquitination [57]. KDM4B is further a direct transcriptional target of p53 [57].

To fine-tune epigenetic regulation, KDM4 proteins interact with each other as well as with protein complexes, such as those associated with transcriptional activity or DNA mismatch repair. All KDM4 proteins appear to have the capacity to form homodimers, though only KDM4A, B, and C form heterodimers [32]. KDM4 proteins also associate and demethylate non-histone protein substrates such as polycomb 2, the G9a methyltransferase and the chromodomain Y-like protein (CDYL1) [58, 59]. KDM4A, B, and C are known to participate in multiprotein complexes with members of the SWI/SNF chromatin-remodeling complex [36] and can interact with inhibitory complexes including histone deacetylases (HDAC1-3), N-CoR, or the pRb tumor suppressor [17, 40, 60]. Through these interactions, KDM4 demethylases are significant players in directing gene expression in development, homeostasis and disease.

### Alterations and roles of the KDM4 subfamily in cancer

It is now well established that alterations in the expression of both methyltransferases and demethylases trigger the progression of cancer. Though only recently apparent, mounting evidence points to the role of histone demethylases in disrupting the proliferative balance, survival and metastatic potential of cells from multiple tissues. Many histone demethylases

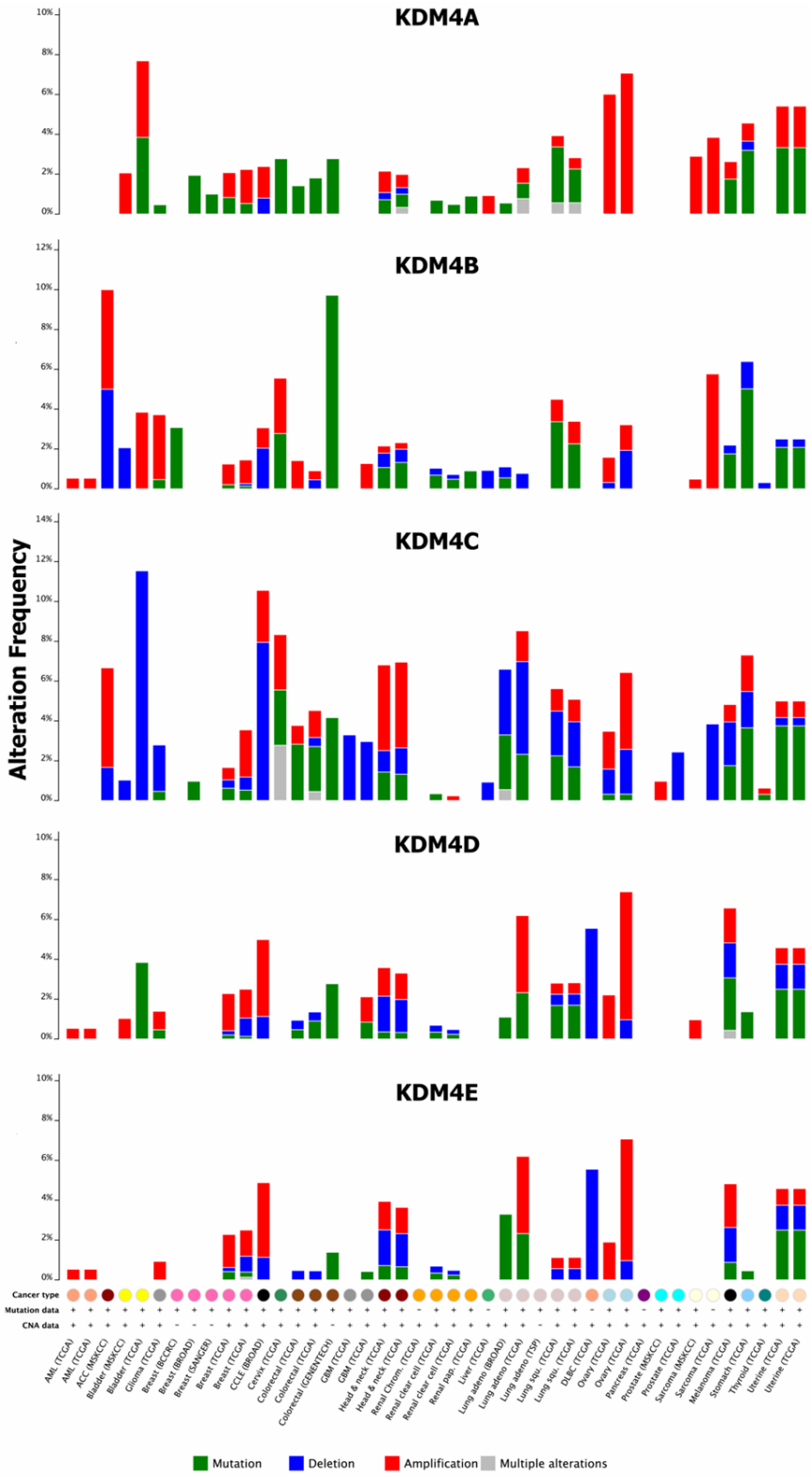


**Figure 2.** Alteration frequencies of *KDM4* subfamily genes identified in human tumors of multiple origins reported across 52 databases held in the Cancer Genomics cBioPortal [67, 68]. Alteration frequencies are displayed for each of four categories, including: genetic amplifications (red), deletions (blue), mutations (green) or multiple alterations (grey).

are dysregulated in cancer, where the effect is either to activate expression of oncogenes, repress expression of tumor suppressors, alter DNA mismatch repair, disrupt chromosomal stability, or interact with key hormonal receptors which control cellular proliferation [61-63]. Previous studies have demonstrated that *KDM4* genes are amplified and overexpressed in various tumor types, including lung, breast, esophageal, prostate cancers and lymphoma [28, 57, 64-66]. To establish a comprehensive profile of genomic alterations for *KDM4A-E* in human cancer, we conducted a large-scale meta-analysis of the genetic amplifications, deletions and mutations reported across 52 databases in the Cancer Genomics cBioPortal [67, 68]. An overview of this data reveals that *KDM4A-E* are altered across many tumor types (**Figure 2**). This data is complemented by a recent analysis of 4,934 cancer copy number profiles from The Cancer Genome Atlas (TCGA) Pan-Cancer data set, which has revealed significant amplifications of the *KDM4C* genomic region in human cancer cells [69]. The involveme-

nt of KDM4 proteins in cancer is further supported by findings of several independent research groups.

*KDM4A*

*KDM4A* amplification and overexpression is highly prevalent in ovarian cancer and in squamous cell carcinoma [6, 62]. More importantly, the overexpression of KDM4A in tumors specifically triggers highly localized chromosomal instability, consisting of site specific copy gains at 1q12, 1q21 and Xq13.1 [62]. *KDM4A* knockdown has been shown to not only impact cell growth but also metastasis *in vitro* and in mouse models [6]. KDM4A interacts with the activating protein 1 (AP1) transcription factors which control cell proliferation, apoptosis and differentiation [6]. KDM4A histone demethylation can induce the expression of AP1 genes including *JUN* and *FOSL1*, which promote cell growth and metastasis [6]. It also directly facilitates AP1 complex binding to *JUN* and *FOSL* promoters, creating a positive feedback loop which maintains AP1 activation. Furthermore, it is reported that KDM4A promotes cellular transformation by blocking senescence through transcriptional repression of the CHD5 tumor suppressor [70].

*KDM4B*

Of the demethylases that mediate nuclear receptor responsiveness in breast and prostate cancer, much is known about the role played by KDM4B. KDM4B is highly expressed in estrogen receptor (ER)-positive, aggressive subtypes and can be induced by the ER in an estrogen-dependent manner in breast cancer [36, 71]. KDM4B can bind to the ER, which together demethylate repressive H3K9me3 marks and recruit members of the SWI/SNF-B and MLL2 chromatin remodeling complexes to induce gene expression in an estrogen dependent manner [36]. Targets of the KDM4B-ER complex include not only oncogenic MYB, MYC and CCND1, which induce proliferation, but also the ER and KDM4B themselves, resulting in an activating feedback loop [71, 72]. Conversely, knockdown of KDM4B greatly inhibits estrogen dependent gene expression, and stabilizes p53 which halts breast tumor cell proliferation [73]. In prostate cancer cells, KDM4B expression, which positively correlates with the severity of cancer, can cooperate with the AR to induce the AR transcriptional response [57]. KDM4B also stabilizes the AR through inhibiting its ubiquitination and degradation. Knockdown of *KDM4B* results in a near complete depletion of AR protein levels [57]. Together, the interaction between KDM4B and nuclear receptors in prostate and breast cancers consist of major drivers that can dictate the aggressiveness of disease.

KDM4B also appears to contribute to metastasis and hypoxia. Overexpressed in colorectal cancer, KDM4B can induce expression of the plasma membrane signaling protein, PRL-3, which triggers lymph node metastasis [74]. KDM4B also promotes a pro-survival gene expression response in renal cancer cells through the accumulation of HIF1α [75]. Consequently, KDM4B mediates hypoxic conditions, frequently associated with highly proliferative and therapeutically refractory cancer cells.

*KDM4C*

KDM4C, also referred to as GASC1 (Gene Amplified in Squamous Cell Carcinoma), is overexpressed in numerous cancers including esophageal squamous cell carcinoma, breast and prostate cancers, medulloblastoma, metastatic lung sarcomatoid carcinoma, in primary mediastinal B-cell lymphoma and Hodgkin's lymphoma, and in acute myeloid leukemia [22, 28, 64, 65, 76-79]. In a high-resolution SNP analysis of 212 medulloblastoma genomes, KDM4C was among several histone modifying enzymes aberrantly expressed, specifically enriched in a significant 15% fraction of genomes [78]. Accordingly, high level chromosome 9 gains observed correspond to hypomethylation of H3K9 residues in medulloblastoma tumors, supporting the substantial role played by the methylome in aberrant gene transcription [76, 78]. Recurring evidence supports that *KDM4C* overexpression results from aberrant amplification of chromosome 9 at the 9p23-24 foci [65]. It is also aberrantly expressed as a fusion partner to the immunoglobulin heavy chain gene (IGH) in mucosa-associated lymphoma, following 9p translocation [66].

On a functional basis, KDM4C can act to promote tumorigenesis through several mechanisms. It activates expression of oncogenes

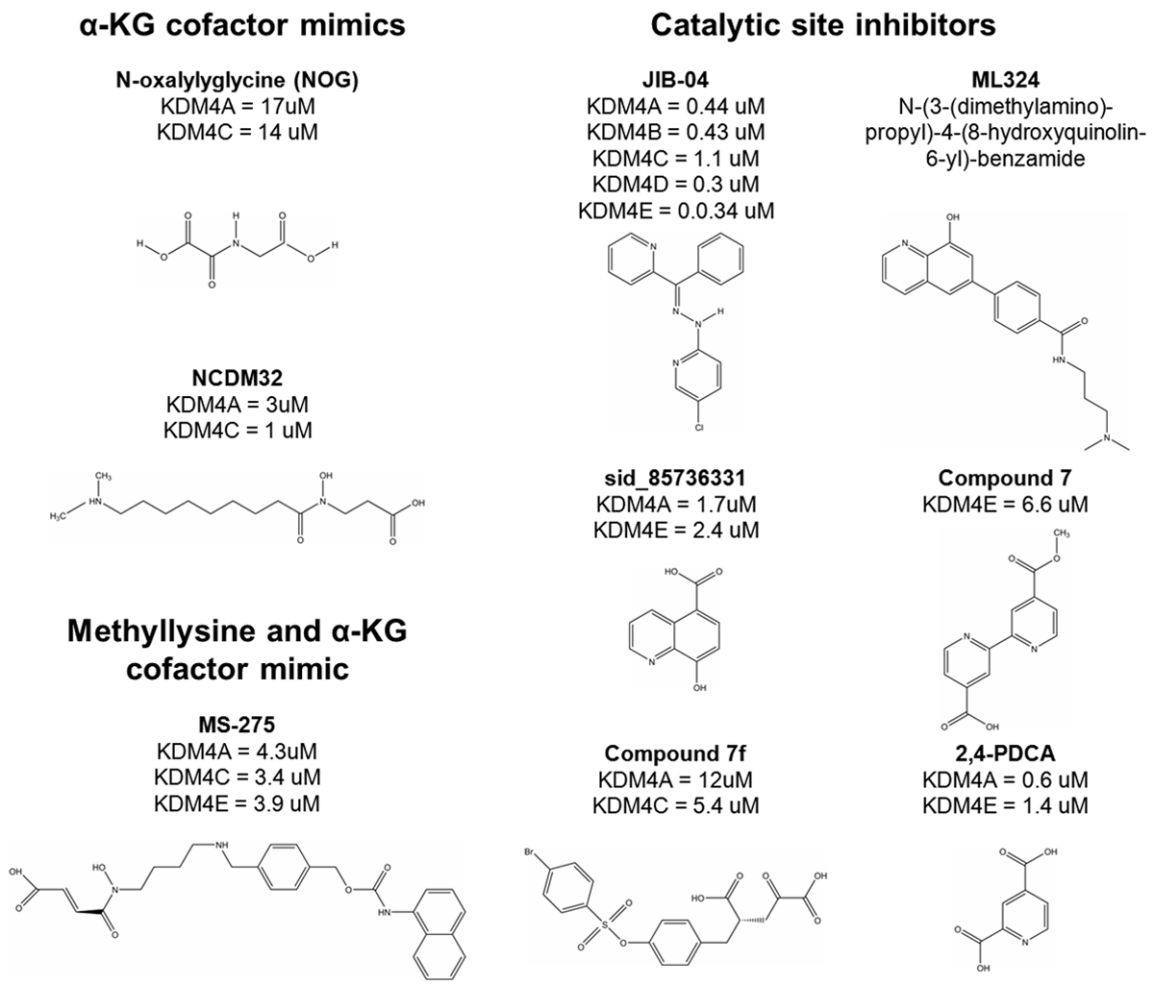


**Figure 3.** Chemical structure and half maximal inhibitory concentration (IC50) for representative KDM4 inhibitors.

such as MDM2, a regulator of p53, and binds to the AR to stimulate androgen dependent gene expression and tumour cell proliferation [80, 81]. In breast cancer, KDM4C amplification and overexpression are prevalent in aggressive basal-subtypes. Recent studies indicate that KDM4C is a transforming breast oncogene: stable KDM4C overexpression in non-tumorigenic cells induces transformed phenotypes, whereas KDM4C knock down inhibits tumor proliferation and metastasis [56, 82]. KDM4C overexpression also confers stem cell-like characteristics such as the ability to form mammospheres in culture and induces expression of NOTCH1, a pro-survival factor in breast cancer stem cells [65, 83]. Such KDM4C mediated genetic programs in cancer cells reiterate its functions in ES cells, supporting the hypothesis that it functions in establishing stem cell-like transcriptional programs in cancer cells [65].

*KDM4D and KDM4E*

In contrast to other KDM4 members, KDM4D and E are structurally divergent proteins, lacking both C-terminal PHD and Tudor domains, which may reason why no conclusive evidence exists of their contribution to cancer establishment or progression. However, as with KDM4A, KDM4D can interact with nuclear receptors such as the AR, suggesting it may function to regulate gene expression in tissues such as the prostate [84]. The mechanism of KDM4D binding to the AR is distinct from KDM4A, which binds at its C-terminus. Yet, the roles of KDM4D and E in cancer remain unclear and require further investigation.

**KDM4 subfamily in other diseases**

Beyond the role of KDM4 proteins in cancer, their dysregulation can severely disrupt normal

cellular functions in other diseases [73, 85]. Aberrant KDM4A expression has been linked to cardiac failure, cardiac hypertrophy, to the progression of viral infections, as well as disorders such as alopecia areata [29, 86-88]. SNPs in KDM4C genes are also associated with autism and alcohol withdrawal symptoms [89, 90]. Together, these instances demonstrate the breadth of KDM4 protein functions in establishing disrupted gene expression programs.

## KDM4 inhibitors

Considering the significant implication of KDM4 demethylases in the development of various diseases, a thorough understanding of their molecular mechanism and effective therapeutic inhibition is of considerable interest. On the basis of the three-dimensional structures available and studies of their catalytic mechanisms, a number of KDM4 inhibitors have been identified and reported. These inhibitors can be categorized into three major groups: α-KG cofactor mimics and disruptors, metal cofactor mimics, as well as histone substrate analogs (**Figure 3**). Here, we describe the historical development of KDM4 inhibitors and describe novel molecules recently proven to have good efficacy and specificity in both biochemical and cellular assays.

### *α-KG cofactor mimics and disruptors*

The vast majority of KDM4 inhibitors currently consist of α-ketoglutarate (α-KG) or 2-oxoglutarate (2-OG) cofactor competitive inhibitors which bind the iron Fe(II) molecule in the catalytic site (reviewed in [91]). All JmjC-domain containing demethylases require α-KG as a cofactor in the demethylation reaction. Thus, α-KG cofactor mimics appear to inhibit multiple members of the histone lysine demethylases. Hamada *et al.* first explored the inhibitory potential of α-KG analogues including N-oxalylglycine (NOG) and subsequently presented hydroxamate analogues such as NCDM32, which has a 500 fold better KDM4C inhibitory activity compared to NOG (**Figure 3**) [92, 93]. Other KDM4 subfamily cofactor disruptors include the α-KG analog 2,4-pyridindicarboxylic acid (PDCA), the PDCA derivative, compound 15c and a 4-carboxylate containing 2,2-bipyridyl derivative compound 7 [94, 95]. Following report on these inhibitors, Rose *et al.* used crystallographic analyses to discover a sub pocket within the KDM4 active site which was significantly larger and more open than in other oxygenases [96]. This sub pocket also extends into the substrate binding groove. Accordingly, a series of N-oxalyl-D-phenylalanine derivatives, thought to occupy this sub-pocket, were developed with the intention of selectively inhibiting KDM4 proteins among all cellular oxygenases. This effort led to the identification of molecules such as compound 7f (**Figure 3**).

In addition to these inhibitors, compounds structurally unrelated to α-KG were also found to bind and inhibit the KDM4 catalytic site. Among 236,000 compounds assayed in a high throughput screen by King *et al.,* were 8-hydroxyquinoline derivatives such as sid_85736331 [97]. Further cellular assays confirmed that these compounds potently inhibited H3K9 demethylation in HeLa cells. Within this novel class of inhibitors, Liang *et al.* showed that the related compound, ML324 effectively inhibited intermediate early viral gene replication mediated by KDM4A in herpes virus infected cells [88, 97]. These experiments stand as proof of principle for the development of therapeutically active compounds against KDM4 proteins *in vivo*.

### *Metal cofactor disruptors*

Disruption of iron and zinc cofactors also inhibits KDM4 protein catalytic activity, and can be accomplished by both non-iron metals and organic molecules. Non-iron metals such as nickel have the potential to disable the catalytic activity of KDM4A and C through occupancy of the iron binding pocket [98]. Structural and bioinformatics analyses have also revealed a Zn(II) Cys3-His binding site in the KDM4A catalytic domain, which is absent in other α-KG dependent oxygenases [94]. In KDM4A, the $Zn2^{+}$ ion, required for its catalytic activity, is specifically ejected through the binding of disulfiram, and ebselen [94]. These metal cofactor disruptors offer an alternative inhibitory mechanism which may be used to selectively target KDM4 demethylases.

### *Histone substrate competitive inhibitors*

Thus far, few methyllysine histone substrate mimics have been designed or tested, with the exception of WAG-003 and a derivative of the well characterized histone deacetylase (HDAC)

inhibitor, MS-275 [99, 100]. WAG-003 is a Tudor domain inhibitor analogous to the antiarrhythmic drug amiodarone, which modestly inhibits KDM4A *in vitro*. The MS-275 derivative, in contrast, was designed as a methyllysine cofactor mimic linked to an α-KG mimic, inhibiting both key sites of KDM4 proteins (**Figure 3**). *In vitro* assays have demonstrated that while this molecule and its prodrug, methylstat, potently inhibit KDM4A, C and E, its inhibition of non-target oxygenases is much weaker [100]. Thus, the development of comparable dual targeting molecules has the advantages of disrupting multiple KDM4 domain functions while offering good selective inhibition.

To date, only one other structurally distinct KDM4 subfamily inhibitor, JIB-04, identified in an unbiased cellular screen, effectively and specifically inhibits KDM4 activity *in vivo* as well as *in vitro*. In biochemical assays, JIB-04 potently inhibited the catalytic activity of KDM4 member proteins including KDM4A, B, C and E [101]. Furthermore, JIB-04 has an unprecedented capacity to specifically inhibit KDM4 protein function in cancer cells, as well as in tumors *in vivo* [101]. JIB-04 is not a competitive inhibitor of α-KG, and the exact molecular mechanism is unclear. Yet, JIB-04 does not appear to affect the function of other α-KG-dependent enzymes, nor alter transcriptional growth programs in normal cells. As such, this inhibitor stands as an important breakthrough in the field of epigenetic drugs research, which will likely serve as a model in the development of analogs with excellent *in vivo* potency and specificity.

## Conclusions

KDM4 demethylases function extensively in multiple cellular events throughout organismal development and homeostasis. Despite the recent discovery that the KDM4 subfamily plays an essential role in regulating gene expression and chromatin architecture via H3K9 and H3K36 demethylation, there is still much to learn about how KDM4 proteins are recruited to genomic loci, how they modulate histone demethylation and subsequently activate specific downstream targets in different cell types. Moreover, it is clear that KDM4 proteins cooperate in similar macromolecular complexes and processes, yet the redundancies and interactions between them are still not well understood. Considering the enormous potential of these epigenetic master regulators in modulating gene transcriptional programs, it is not surprising that their alterations are implicated in human diseases, particularly in cancer. However, the molecular mechanisms by which KDM4-dependent chromatin regulation translates into oncogenicity and cancer progression remain poorly understood. Thus, deeply understanding the biology and mechanism of KDM4 demethylases will be a significant component of future research.

Considering that epigenetic changes are reversible and histone demethylases are druggable, KDM4 proteins are promising therapeutic targets. However, one caveat remains that most KDM4 inhibitor scaffolds are borrowed from studies of structurally or mechanistically related enzymes and are often also active against related non-target proteins. In addition, most inhibitors are cofactors and/or substrate mimics and so far have only very limited or undetermined specificity for the KDM4. It is thus anticipated that the next decade of KDM4 demethylase research will intensely focus on developing specific and effective small molecule inhibitors for experimental and therapeutic applications.

## Acknowledgements

This work was partially supported by grants from the Karmanos Cancer Institute Angelika Burger Joint Postdoctoral Training Grant to Dr. R.M.L and Dr. Z-Q.Y, the Department of Defense Breast Cancer Research Program award (BC083945) and Prostate Cancer Research Program award (PC110481), the Mary Kay Foundation Cancer Research Grant Program and the Karmanos Cancer Institute-SRIG to Dr. Z-Q.Y.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Zeng-Quan Yang, Karmanos Cancer Institute, Department of Oncology, Wayne State University, 4100 John R Street, HWCRC 815, Detroit, MI 48201, USA. Tel: 313-576-8339; Fax: 313-576-8029; E-mail: yangz@karmanos.org

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