

Regulation of histone arginine methylation/demethylation by methylase and demethylase (Review)

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Abstract. Histone arginine methylation is a universal post-translational modification that has been implicated in multiple cellular and sub-cellular processes, including pre-mRNA splicing, DNA damage signaling, mRNA translation, cell signaling and cell death. Despite these important roles, the understanding of its regulation with respect to certain other modifications, such as phosphorylation and acetylation, is very poor. Thus far, few histone arginine demethylases have been identified in mammalian cells, compared with nine protein arginine methyltransferases (PRMTs) that have been reported. Studies have reported that aberrant histone arginine methylation is strongly associated with carcinogenesis and metastasis. This increases the requirement for understanding the regulation of histone arginine demethylation. The present review summarizes the published studies and provides further insights into histone arginine methylases and demethylases.

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1. Introduction

Post-translational modifications of histone proteins serve key roles in eukaryotic cellular processes, including acetylation,

methylation, phosphorylation and ubiquitination. As epigenetic markers, these modifications provide perspectives on DNA repair, signal transduction, transcriptional regulation, chromatin remodeling and the modulation of protein interactions (1). Enzymes that deposit and remove modifications have been regarded as important drug targets and have been developed to treat various diseases, including cancer. The reversible processes involved in these pathways are mediated by histone methyltransferases and histone demethylases. Modifications of N-terminal tail histones include acetylation, ubiquitination, phosphorylation and methylation of lysine and arginine residues (2), all of which have been addressed in studies involving specific enzymes, including the acetyltransferases, arginine and lysine methyltransferases, ubiquitin ligases and kinases responsible for these modifications. For example, numerous histones have been demonstrated to contain methylarginines (3). Arginine methylation may readily modulate the structure of arginine residues and binding interactions with other proteins, which regulate physiological functions. As abnormal histone arginine methylation is a common phenotype in tumorigenesis, studies of histone arginine methylated substrates associated with cancer are presented in Table I (4-9). Differential methylation modification in histones may either activate or repress gene transcription, depending on the location and nature of these modifications (10). Three distinct types of methylated arginine residues have been reported in eukaryotes. The most prevalent is asymmetric ω -NG, N^G-dimethylarginine (ADMA), in which two methyl groups are replaced on one of the terminal nitrogen atoms of the guanidine group (11). Other derivatives include ω -N^G-monomethylarginine (MMA) and ω -NG, NG-dimethylarginine (SDMA).

The discovery of two enzymes, deaminase enzymes peptidyl arginine deaminase 4 (PAD4) and Jumonji domain-containing protein 6 (JMJD6) changed the consensus of opinion regarding the reversibility of methylation. PAD4 is able to catalyze the demethylation reaction and convert arginine residues into citrulline. JMJD6, an Fe (II)- and 2OG-dependent JumonjiC (JmjC)-domain-containing oxygenase that is also able to catalyze arginine demethylation, was identified as an arginine demethylase. These demethylation techniques have recently led to work by the authors of the present study, focusing on arginine demethylation.

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In the present review, the current perspective on arginine methylases/demethylases, including the controversy around JMJD6, in addition to the novel demethylation activity of 2OG oxygenase family histone lysine demethylases is summarized. Although few arginine demethylases have been identified, any dynamic posttranslational modification mechanism is likely to be involved in carcinogenesis and metastasis, which makes them an important field of study.

2. Arginine methylases

Arginine residues may be differentially methylated by different types of protein arginine N-methyltransferases (PRMTs) (12), which are classified as type I, II, III or IV enzymes (Fig. 1). Type I and II enzymes regulate gene transcription via methylation of histone proteins. Type I PRMTs (PRMT1, PRMT3, PRMT4 and PRMT6) catalyze ADMA on H3R2 and H4R3, leading to transcriptional activation and ribosomal biosynthesis (13,14). PRMT1 is the principal methyltransferase in human cells, modifying ~90% of methylated arginine residues. PRMT1 generally recognizes arginines within a glycine-arginine-rich region, a motif present in numerous RNA- or DNA-binding proteins (15). PRMT1 is overexpressed in various human cancer types, triggering aberrant hypermethylation of its substrates involved in tumorigenesis. Type II PRMTs (PRMT5 and PRMT7) cause the formation of SDMA on H3R8 and H4R3, resulting in transcriptional repression (16,17). In addition, these enzymes may also regulate other cellular activities by methylating non-histone proteins. In total, >100 non-histone proteins have been reported to contain methylated arginine sites (18). MRE11, p53 and DNA polymerase β , which are associated with DNA replication and DNA damage repair, are reported to be regulated by arginine methylation (11). Most recently, stress granules (SGs) which are cytoplasmic condensates of stalled messenger ribonucleoprotein complexes, have been associated with rapid demethylation on Ras-GAP SH3-binding protein 1 (G3BP1) under stress conditions (19). Type III and type IV PRMTs only catalyze monomethylation. In addition, type IV PRMT has only been identified in yeast (20).

The function of PRMT is associated with multiple cellular activities, including proliferation, transformation and anti-apoptotic processes, all of which are involved in tumorigenesis. PRMT activity may be influenced by expression regulation, PRMT macromolecule formation, substrate accessibility, other forms of posttranslational modification and protein-protein interactions. Alteration of PRMT expression and dysregulation of their enzymatic activity, principally upregulation, has been observed in early embryonic lethality in mice and a number of human cancer types, as described in Table II (8,21-44). PRMT1 is an important methyltransferase with a wide substrate spectrum (15). PRMT5 is critical for the maintenance of pluripotency in mouse embryonic stem cells, as a key suppressor of gene differentiation (45). Due the critical role of PRMT1 and PRMT5, studies have revealed that deletion of PRMT1 or PRMT5 invariably causes embryonic lethality (46,47). Other PRMTs are generally associated with development and aging, including PRMT2, PRMT3, PRMT6 and PRMT8. Mice with deficiencies in these PRMTs may be viable, but display a developmental delay following birth (48).

In summary, disruption of the balance in PRMTs triggering the aberrant methylation of proteins involved in cancer is implicated in promoting oncogenic transformation. Therefore, further investigation of the regulatory mechanisms, the use of model systems, and cellular biological discoveries are required to better understand the function of protein methylation in tumorigenesis.

3. Arginine demethylases

Family members, mechanism and investigation. Methylation at different residues can impact transcriptional and biological functions. Histone demethylase catalyzes the removal of methyl groups both on histone lysine and arginine residues. Histone lysine methylation, a process that was defined as a stable, irreversible chromatin marker, has now been established to be reversible by two families of lysine-specific histone demethylase (KDMs); one is the flavin-dependent KDM1, also termed LSD1, and the other is Fe (II)- and 2OG-dependent JmjC-domain-containing enzymes. The first histone demethylase identified was LSD1, which belongs to the superfamily of the flavin adenine dinucleotide-dependent amine oxidases (49). Subsequently, the first JmjC-containing histone demethylase, KDM2A, was identified (50). By sequence homology searching, two other members of the JmjC-containing histone demethylases were identified. All these observations made the system of methylation and demethylation of lysine a highly dynamic field of study.

As the reversible process of lysine methylation and demethylation has been better understood, the extent to which arginine methylation is dynamic is much less clear. Up to now, there have been no definite reports of specific arginine demethylases. While the arginine methylation may have biological relevance during gene expression and for the function of living organisms, well-balanced arginine methylation, in general, is required for mammalian development and is important for cellular proliferation and differentiation (51,52). Aberrant modification patterns in proteins alter their activity and function, resulting in somatic diseases, including cancer (4-9). Consequently, certain enzymes (PRMTs for example) catalyze arginine methylation modifications, whereas other enzymes remove them. The dynamic cyclical process of methylation and demethylation of arginine reveals has revealed the existence of demethylases that reverse of arginine methylation (53). The existence and function of protein arginine methyltransferases have been known for over a decade (13). To date, two histone arginine demethylases have been identified: PAD4 and JMJD6 (54). PAD4 was deemed to be a demethylase that demethylates histones, converting monomethylated arginine to citrulline. JMJD6 directly converts methylarginine to arginine by removing the methyl group. Previous studies have provided significant insights into the mechanism and function of these demethylases, leaving much unresolved controversy (54). In a recent study, JMJD1B was reported to demethylate H4R3me2s and H3K9me2 to facilitate gene expression for the development of hematopoietic stem and progenitor cells. JMJD1B, previously identified as a lysine demethylase for H3K9me2, mediates the arginine demethylation of H4R3me2s and its intermediate, H4R3me1 (55). Histone lysine demethylases have also been

Table I. Histone arginine methylated substrates associated with cancer.

Author, year	Substrate	Function	Refs.
Yang <i>et al</i> , 2010	H4R3	TDRD3 binding	(4)
Gao <i>et al</i> , 2006		Transcriptional activation of ZEB1 (promotes EMT)	(5)
Pal <i>et al</i> , 2007		Represses expression of tumor suppressor genes (NM23)	(6)
Karkhanis <i>et al</i> , 2012	H3R17	Regulates cellular response to DNA damage	(7)
Frietze <i>et al</i> , 2008		Transcriptional activation of E2F1 (promotes cell growth)	(8)
Pal <i>et al</i> , 2004	H3R8	Represses expression of tumor suppressor genes (ST7)	(9)
Pal <i>et al</i> , 2004	H2AR3	Regulates cellular response to DNA damage	(9)

TDRD3, tudor domain containing 3; ZEB1, zinc finger E-box binding homeobox 1; EMT, epithelial-mesenchymal transition.

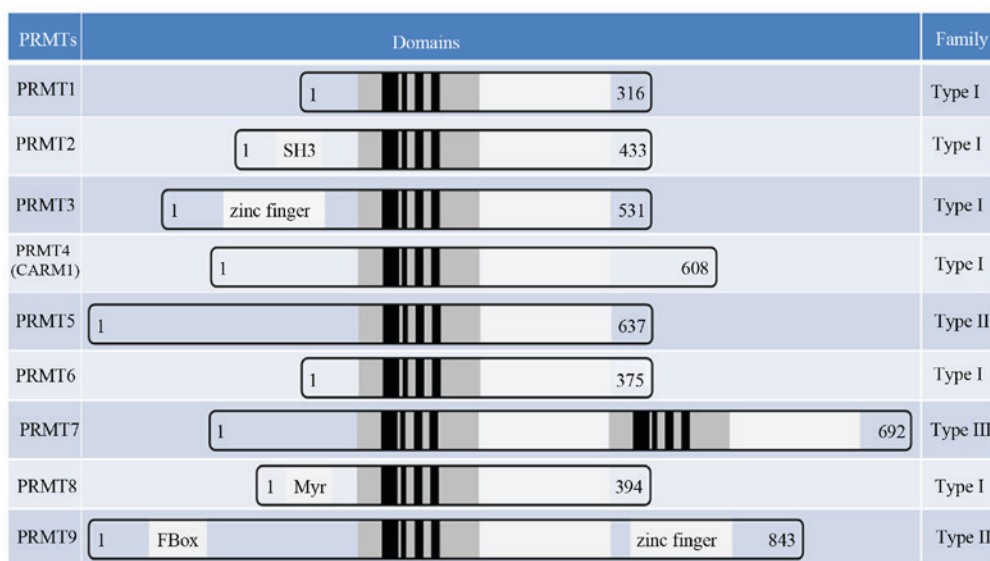


Figure 1. Overview of the human PRMT family. The catalytic methyltransferase domains contain a conserved binding domain (grey) with conserved motifs (black) that are important for the enzymatic reaction. Additional motifs: SH3 domain, zinc finger domain, myristoylation motif, FBox motif. PRMT, protein arginine methyltransferase.

reported to be associated with phenotypic plasticity and mammalian embryonic development (56,57).

Mechanism and function of PAD4. PAD4 was previously identified to be associated with histone citrullination (58-60). The activity of the PAD enzymes is calcium (Ca^{2+})-dependent. Studies have reported that PAD4 is able to demethylate histones *in vitro* and *in vivo* by removing methyl groups from monomethylated arginine, thus regulating histone arginine methylation and gene expression. Modeling experiments suggest that asymmetrically and symmetrically dimethylated arginine cannot be accommodated in the active site of PAD4 due to steric occlusion (61). PAD4 targets multiple sites in histones H3 and H4, including sites methylated by the coactivators histone-arginine methyltransferase CARM1 (H3R17) and PRMT1 (H4R3). By deaminating and demethylating histones, PAD4 is able to regulate endogenous pS2 gene expression stimulated by estradiol, and thereby affect chromatin structure and function. Demethylation is not a 'true' demethylation reaction; it removes, but does not reverse methylation. Neutral citrulline is produced, which has considerably different

chemical properties compared with unmethylated arginine, and this process is not sufficient to maintain arginine circulation (62,63). In addition, as PAD4 is also able to catalyze non-methylated arginine, there is dispute as to whether PAD4 is a strict histone demethylase (64). The detailed mechanism of action of PAD4 is depicted in Fig. 2.

Mechanism and function of JMJD6. JMJD6 was previously described as a phosphatidylserine receptor of the plasma membrane of macrophages and dendritic cells (64). Chang *et al* (54) reported that JMJD6 functions as an arginine demethylase, which is an Fe (II)- and 2-oxoglutarate-dependent dioxygenase. The catalytic mechanism of JMJD6 is presented in Fig. 3. Like other JmjC domain-containing histone demethylases reported to be involved in histone lysine demethylation, JMJD6 shares extensive sequence and predicted structural homology with asparaginyl hydroxylase (54). Using conventional biochemical methods, it was reported that JMJD6 was able to demethylate H3R2me2 and H4R3me2 by removing methyl groups (54). Further studies demonstrated that JMJD6 demethylated R260 of estrogen receptor α , thereby regulating estrogen

Table II. Dysregulation of PRMTs and cancer.

Author, year	PRMT	Effect of dysregulation in cancer	Refs.
Goulet <i>et al</i> , 2007, Zou <i>et al</i> , 2012, Elakoum <i>et al</i> , 2008, Shia <i>et al</i> , 2012, Mathioudaki <i>et al</i> , 2008, Mathioudaki <i>et al</i> , 2011, Mathioudakis and Salvatori, 2008	PRMT1	Overexpressed in breast, prostate, non-small cell lung, colon and bladder cancer and leukemia	(21-27)
Papadokostopoulou <i>et al</i> , 2009, Zhong <i>et al</i> , 2012, Zhong <i>et al</i> , 2011	PRMT2	Overexpressed in breast cancer and associated with ER α -positive tumors	(28-30)
Papadokostopoulou <i>et al</i> , 2009, Takahashi <i>et al</i> , 2012, Singh <i>et al</i> , 2004	PRMT3	Higher levels of PRMT3 activity in breast tumors	(28,31,32)
Hong <i>et al</i> , 2004, Majumder <i>et al</i> , 2006, Kim <i>et al</i> , 2010, Habashy <i>et al</i> , 2013	PRMT4	Overexpressed in breast, prostate and colorectal cancer, non-small cell lung carcinomas. Early expression in early stages of hepatocarcinogenesis	(33-36)
Pal <i>et al</i> , 2007, Wang <i>et al</i> , 2008, Bao <i>et al</i> , 2013, Powers <i>et al</i> , 2011	PRMT5	Overexpressed in gastric, colorectal, bladder, colon and lung cancer, and lymphoma and leukemia	(6,37-39)
Yoshimatsu <i>et al</i> , 2011	PRMT6	Overexpressed in bladder and lung cancer cells. Downregulated in invasive breast ductal carcinoma	(40)
Thomassen <i>et al</i> , 2009, Yao <i>et al</i> , 2014, Baldwin <i>et al</i> , 2015	PRMT7	Overexpressed in primary breast cancer and breast cancer lymph node metastasis	(41-43)
Yang and Bedford, 2013	PRMT8	Somatic mutations were found in ovarian, skin and large intestine cancer	(44)

PRMT, protein arginine methyltransferase.

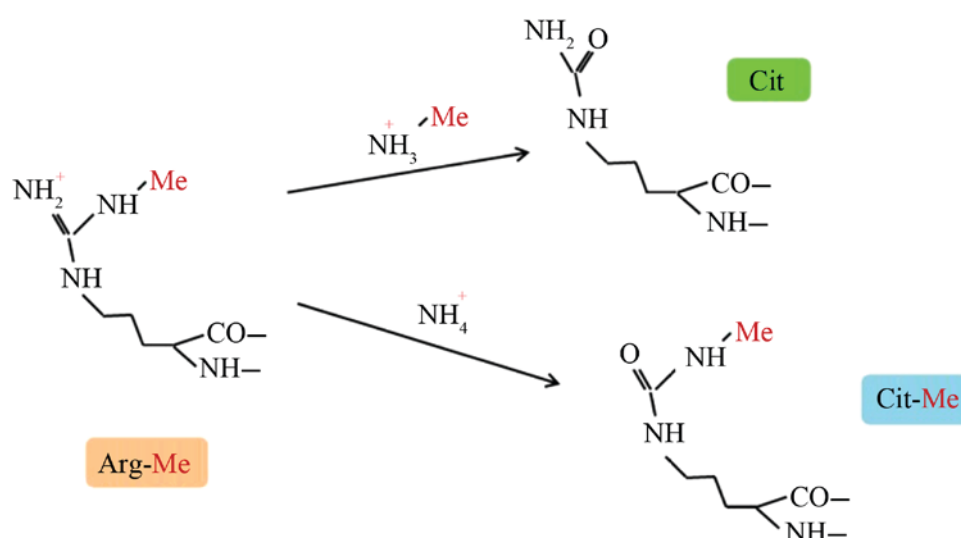


Figure 2. Mechanism and demethylase activity of PAD4. PAD4 demethylates H3 and H4. The two possible mechanisms of the PAD4 reaction with methylated arginine in a protein substrate are presented. PAD4, peptidyl arginine deaminase 4; Cit, citrulline.

nongenomic signaling (65,66). Previously, Lawrence *et al* (67) reported that JMJD6 was able to demethylate RNA helicase A in cells infected with foot-and-mouth disease virus, which facilitates viral replication. Gao *et al* (68) observed that JMJD6 was able to demethylate the heat-shock 70 kDa protein (HSP70) on the R469 residue *in vitro*. Factually, it was confirmed that the level of methylated HSP70 increased when JMJD6 was knocked

down in cells, and the demethylation reaction following transfection with recombinant JMJD6 protein was assayed by mass spectrometry. More recent work reported that G3BP1 methylation was able to represses SG formation, and JMJD6 was demonstrated to interact with G3BP1 complexes and to function in G3BP1 demethylation to promote SG formation (69). Plant Jumonji homologs have also been reported to serve an essential

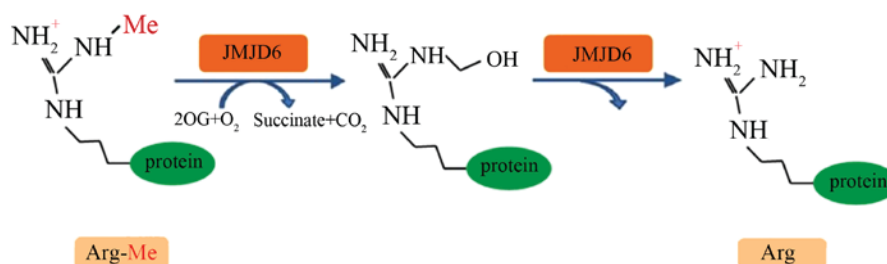


Figure 3. Mechanism and demethylase activity of JMJD6. The two demethylation steps catalyzed by JMJD6 are presented. Firstly, JMJD6 hydroxylates the methyl group consuming oxoglutarate (2OG), and a subsequent deformylation reaction produces formaldehyde (CH_2O). JMJD6, Jumonji domain-containing protein 6.

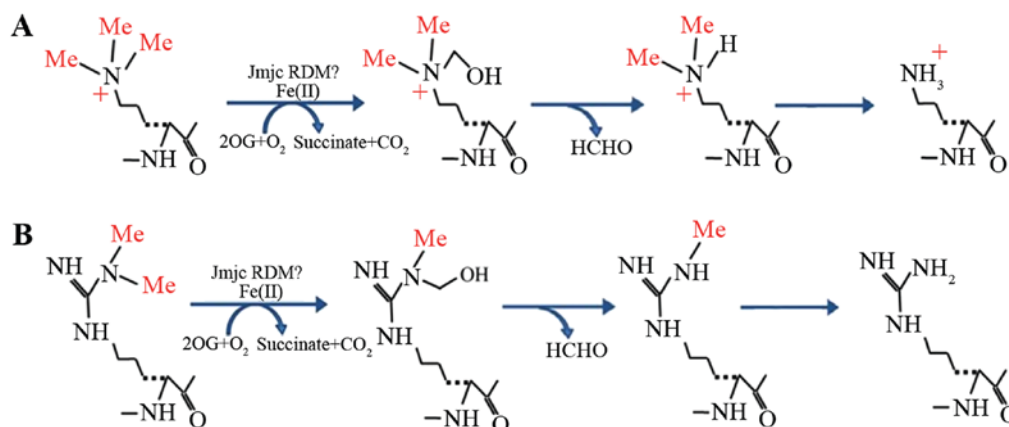


Figure 4. Demethylation mechanism of lysine and arginine. (A) Mechanism for JmjC-catalyzed lysine demethylation. JmjC lysine demethylases catalyze oxidative decarboxylation of 2OG to form succinate, carbon dioxide and a reactive iron(IV)-oxo intermediate. Fragmentation of the hemiaminal releases formaldehyde and the unmethylated lysine residue. (B) Proposed mechanism for JmjC-catalyzed arginine demethylation, a reaction which is analogous to lysine demethylation. JmjC, JumonjiC; KDM, lysine-specific histone demethylase; RDM, arginine-specific histone demethylase.

role in histone modification, and have important functions in epigenetic processes, gene expression and plant development (70). In *Arabidopsis spp.*, two JmjC domain-containing proteins, JMJ20 and JMJ22, function as histone arginine demethylases on H4R3 that act as positive regulators of seed germination in the phytochrome B-phytochrome interacting factor 3-like 5-somnus pathway (71,72).

Nevertheless, JMJD6 was considered display two distinct functions, arginine demethylase and lysyl-hydroxylase (73). Webby *et al* (73) reported that arginine-rich (RS) domains synthesized with dimethylated arginine residues could be JmjC substrates, such as U2AF65 and LUC7-like2 (LUC7L2). Using mass spectrometry analyses, this study did not observe dimethyl arginine-demethylation in U2AF65 or LUC7L2, nor in histones. Rather, it was detected that the hydroxylation of U2AF65 was significantly increased with JMJD6 overexpression. Combined with the essential role of JMJD6 in mRNA splicing, it was confirmed that JMJD6 catalyzed the lysyl hydroxylation of U2AF65, serving a specific role in the regulation of RNA splicing (73). Previously, Heim *et al* (74) reported that JMJD6 interacts with the RS-domains of specific SR-like proteins and is involved in RNA-protein complexes, which has an effect on the splicing of reporter genes. Therefore, there is a degree of doubt regarding whether JMJD6 contains a focal role in demethylation arginine enzyme activity (75). Further studies should also clarify whether there are other enzymes that function as specific arginine demethylases on histones and non-histones.

Mechanisms of action and perspectives on JmjC histone lysine demethylases. A subset of Fe (II)- and 2OG-dependent JmjC containing oxygenases, histone lysine demethylases, has been reported to be able to catalyze arginine demethylation (76,77). Studies have indicated that arginine and lysine demethylation have similar mechanisms including oxidation of the arginine methyl group, whose reaction is analogous to that of lysine demethylation as described in Fig. 4. Therefore, the Fe (II)- and 2OG oxygenases may represent good candidates arginine methylases. Notably, a previous study revealed that JmjC KDMs are able to catalyze oxidation reactions, which is required for the oxidation of a methylarginine group (78). This research inspired further work into the additional function of KDMs in arginine demethylation.

To test whether the catalytic domains of the JmjC KDMs are able to act as arginine-specific histone demethylases (RDMs), recombinant proteins containing the six identified human JmjC KDM subfamilies [KDM2A, KDM3A, KDM4E, KDM5C, KDM6B and PHF8 (also known as KDM7B0)] were produced (79-81). Demethylation activity was tested with matrix assisted laser desorption ionization-time of flight mass spectrometry. It was observed that KDM3A, KDM4E, KDM5C and KDM6B exhibited demethylation catalytic activity. In addition, KDM4E and KDM5C were demonstrated to catalyze the demethylation of histone peptides methylated at H3R2, H3R8, H3R26 (KDM4E, but not KDM5C) and H4R3, and also certain non-histone peptide sequences. Moreover,

Table III. JmjC KDMs catalyzing arginine demethylation.

Type	Subtype	Hydroxylase	KDM	RDM	Novel RDM	Unknown
KDM2	KDM2A	-	+	-	-	-
	KDM2B	-	+	-	-	-
KDM3	KDM3A	-	+	-	+	-
	KDM3B	-	+	-	-	-
	KDM3C	-	+	-	-	-
KDM4	KDM4A	-	+	-	+	-
	KDM4B	-	+	-	-	-
	KDM4C	-	+	-	-	-
	KDM4D	-	+	-	-	-
	KDM4E	-	+	-	+	-
KDM5	KDM5A	-	+	-	-	-
	KDM5B	-	+	-	-	-
	KDM5C	-	+	-	+	-
	KDM5D	-	+	-	-	-
KDM6	KDM6A	-	+	-	-	-
	KDM6B	-	+	-	+	-
KDM7	PHF2	-	+	-	-	-
	KDM7A	-	+	-	-	-
	PHF8/KDM7B	-	+	-	+	-
JmjC only	MINA53	+	+	-	-	-
	NO66	+	+	-	-	-
	JMJD8	-	-	-	-	+
	JMJD6	+	-	+	-	-
	JMJD4	+	-	-	-	-
	FIH	+	-	-	-	-
	JMJD5/KDM8	-	+	-	-	-
	HSPBAP1	-	-	-	-	+
	JMJD7	-	-	-	-	+

Human JmjC oxygenases grouped according to the sequence analysis of their catalytic domains, indicating their assigned/proposed functions and novel functions identified in a previous study (76); certain assignments remain controversial. JmjC, JumonjiC; KDM, lysine-specific histone demethylase; RDM, arginine-specific histone demethylase.

KDM3A was verified to function as a key epigenetic factor for Janus kinase 2-signal transducer and activator of transcription 3 activation in cancer cells (82). Therefore, based on these observations, certain JmjC KDMs do have RDM activity, as described in Table III. Nonetheless, the reaction of JmjC oxygenases that catalyzes arginine demethylation has been detected *in vitro* (76). Considerable efforts are required to accurately investigate arginine demethylation, in addition to true specific arginine demethylases, with respect to RDM activity in cells.

Examination of arginine demethylation. Given the ability of recombinant JmjC oxygenases to catalyze the demethylation of arginine residues, as reported by Walport *et al* (76), the authors of the present study were interested to investigate whether certain JmjC enzyme were also able to catalyze arginine demethylation under normal conditions. To isolate the effective component, Hela nuclear extracts was separated

by $(\text{NH}_4)_2\text{SO}_4$ precipitation (83). Based on the proposed mechanism for histone demethylation (Fig. 5, unpublished), the enzymatic reaction conditions that were used to analyze the Fe(II) and α -KG dependent dioxygenase family of histone demethylases were adopted. Notably, significant demethylation activity on symmetrical histone 4 methylated arginine 3 was observed at a 30-50% $(\text{NH}_4)_2\text{SO}_4$ concentration, but not at 10-30%, suggesting that a degree of arginine demethylase or enzymatic function on arginine demethylation may exist in this component. In order to isolate and identify arginine demethylase, gel electrophoresis and mass spectrometry analysis are required in the future.

4. Conclusions and future perspectives

Post-translational modifications are at the core of the field of epigenetics and are involved in the majority of signal transduction, initiating or amplifying dynamic signals. Arginine

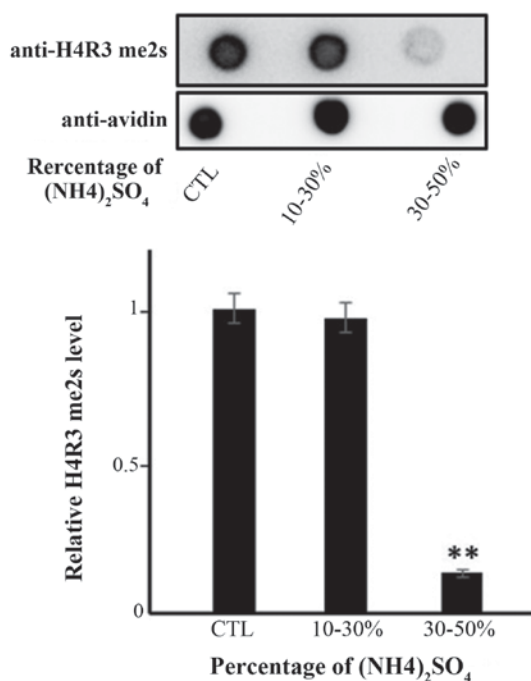


Figure 5. Demethylation activity exists at a 30-50% (NH₄)₂SO₄ precipitation of HeLa nuclear extract. Dot immunoblot analysis of H4R3 me2s following incubation with nuclear extract. Avidin served as a loading control. Western blot analysis represents the results of one experiment, representative of three independent experiments. Student's t-test was performed. **P<0.01 vs. CTL. CTL, control.

methylation is a key post-translational modification involved in tumorigenesis. Recently, arginine methylation regulation has become a rapidly growing field that carries great promise for extending the understanding of pathological processes. Dysregulation of arginine methylation or the abnormal expression of enzymes responsible for post translational modifications may be key events in numerous diseases, including cancer or other diseases. While it is estimated that arginine methylation is as common as other post translational modifications, only nine PRMTs, but hundreds of kinases and E3 ubiquitin ligases for phosphorylation and ubiquitination, have been reported (84). Despite the research into methylation, it is not possible to conclude that arginine methylation regulation is not as dynamic as other modifications.

Various reports have described the hydroxylation and RDM activities of the JmjC oxygenase JMJD6 (54,65,73,74). Meanwhile, JMJD6 is a molecule of interest for its controversial arginine demethylase activity on histones and other non-histone proteins (75), and a recent study demonstrated that other 2OG-dependent JmjC oxygenase KDMs do have RDM activity (including KDM3A, KDM4E, KDM5C and KDM6B) (76). Studies have demonstrated that certain enzymes extend their functions independently of their own activities. As a histone demethylase, LSD2 was reported to act as an E3 ubiquitin ligase and to inhibit cancer cell proliferation through a histone demethylase-independent pathway (85). Therefore, a hypothesis may be drawn as to whether true specific arginine demethylases exist, or whether other enzymes perform this function as a 'part-time' job. However, negative results cannot rule out the existence of a potential specific arginine demethylase. For our examination of arginine demethylase, we

found there was demethylase activity at a 30-50% (NH₄)₂SO₄ concentration of nuclear extracts. Therefore, a novel method required to identify novel enzymes that solely demethylate methylarginines and to understand the dynamic nature of histone demethylases. Finally, the identification and characterization of histone demethylase enzymes may improve the understanding of arginine methylation, and open a new frontier in the study of dynamic epigenetic regulation. Furthermore, molecular investigations in addition to physiological analyses are required to gain a better understanding of the association between the dysregulation of arginine methylation and cancer biology.

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Availability of data and materials

Not applicable.

Authors' contributions

JZ contributed to the literature retrieval, experiments and manuscript preparation. LJ and MHL contributed to the cell culture and sample treatment in addition to manuscript modification. LFH and ZGG supervised the present study, assessed experiment data and contributed to the approval of the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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