

Clipping of arginine-methylated histone tails by JMJD5 and JMJD7

Haolin Liu^{a,b,c}, Chao Wang^{a,b,1}, Schuyler Lee^{a,b,1}, Yu Deng^{d,1,2}, Matthew Wither^{e,1}, Sangphil Oh^{f,1}, Fangkun Ning^{a,b}, Carissa Dege^{a,b}, Qianqian Zhang^g, Xinjian Liu^d, Aaron M. Johnson^e, Jianye Zang^h, Zhongzhou Chen^g, Ralf Janknecht^f, Kirk Hansen^e, Philippa Marrack^{a,b,c,e}, Chuan-Yuan Li^d, John W. Kappler^{a,b,c,3}, James Hagman^{a,b,e,3}, and Gongyi Zhang^{a,b,3}

^aDepartment of Biomedical Research, National Jewish Health, Denver, CO 80206; ^bDepartment of Immunology and Microbiology, School of Medicine, University of Colorado Denver, Denver, CO 80206; ^cHoward Hughes Medical Institute, Denver, CO 80206; ^dDepartment of Dermatology, Duke University, Durham, NC 27710; ^eDepartment of Biochemistry and Molecular Genetics, School of Medicine, University of Colorado Denver, Aurora, CO 80216; ^fDepartment of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104; ^gState Key Laboratory of Agrobiotechnology, China Agricultural University, Beijing 100193, People's Republic of China; and ^hDepartment of Molecular Biology, University of Science and Technology of China, Hefei 900015, People's Republic of China

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Two of the unsolved, important questions about epigenetics are: do histone arginine demethylases exist, and is the removal of histone tails by proteolysis a major epigenetic modification process? Here, we report that two orphan Jumonji C domain (JmjC)-containing proteins, JMJD5 and JMJD7, have divalent cation-dependent protease activities that preferentially cleave the tails of histones 2, 3, or 4 containing methylated arginines. After the initial specific cleavage, JMJD5 and JMJD7, acting as aminopeptidases, progressively digest the C-terminal products. JMJD5-deficient fibroblasts exhibit dramatically increased levels of methylated arginines and histones. Furthermore, depletion of JMJD7 in breast cancer cells greatly decreases cell proliferation. The protease activities of JMJD5 and JMJD7 represent a mechanism for removal of histone tails bearing methylated arginine residues and define a potential mechanism of transcription regulation.

histone tail | arginine methylation | clipping | JMJD5/7

Regulation of gene transcription by epigenetic mechanisms is one of the most important areas of inquiry in modern molecular biology (1). Much epigenetic regulation occurs via post-translational modifications (PTMs) of histone tails and a key assumption is that such modifications should be reversible via enzymes (“erasers”) that remove the marks. However, despite the confirmed importance of methylation of histone arginines in transcriptional regulation (2), the question of whether arginine methylation is reversible is unsolved and controversial. On the other hand, the phenomenon of clipping of histone tails has been reported and has received significant attention in recent years (3–5). However, unlike other epigenetic modifications, no enzyme family has been determined to be responsible for histone clipping, although potential individual enzyme candidates have been implicated in this mechanism (3–5).

Interestingly, proteins of the JmjC domain family have been reported to possess diverse enzymatic activities (6–8). Generally, these functions are related to the JmjC/cupin-like dioxygenase domain that is the hallmark of this protein family. Published activities of various JmjC proteins include demethylation of lysines in histones, demethylation of RNA and DNA, and hydroxylation of various amino acids (6, 8, 9). While many members of the JmjC protein family have been characterized biochemically, the functions of several members, including JMJD4–JMJD8 and HSPBAP1 remain uncharacterized or controversial. For example, JMJD6 has been reported variously to be an arginine demethylase (10, 11), a hydroxylase (12, 13), a single-stranded RNA-binding protein (14), or RNA demethylase (11). Although JMJD6 has been reported to possess histone arginine demethylase activity (10, 11), we and others have been unable to replicate this result (12, 14–17). Thus, the identities of proteins that reverse methylation of arginine residues,

either through conventional demethylation or others, have not been resolved.

Here, we report a mechanism by which mammalian cells remove methylated arginine residues on histone tails: JMJD5 and JMJD7 proteins are proteases that recognize and cleave histone tails with methylated arginine residues. Furthermore, after the initial cleavage, JMJD5 and JMJD7 function as aminopeptidases that progressively digest the C-terminal products. Knockouts of JMJD5 and JMJD7 dramatically increase the content of arginine-methylated histone tails as well as the overall content of histone subunits in cells. Deletion of *JMJD7* genes in human breast cancer cells drastically reduces their growth in soft agar. We conclude that JMJD5 and JMJD7 specifically cleave histone tails containing methylated arginine and progressively digest the cleaved histone product, which might regulate nucleosome stability, replacement, or turnover. Our identification of functions of these JmjC proteins sheds light on regulatory mechanisms that may impact transcription regulation and human diseases in the field of epigenetic gene regulation and tumorigenesis.

Results

c-JMJD5 and c-JMJD7 Remove Methyl Arginine from Histones. Although at least nine arginine methyltransferases have been characterized, no specific enzyme has been conclusively proven to be responsible for removing methyl groups on methylated

Significance

Enzymes responsible for the clipping of histone tails and removal of arginine-methylated histone tails still remain elusive. The underlying mechanism of high histone turnover rate in nonproliferated cells is still a mystery. How RNA polymerase II overcomes nucleosome barriers during transcription is unknown. This article described the discovery of a JmjC domain containing subfamily members JMJD5 and JMJD7, which could be responsible for these unsolved puzzles in epigenetics and transcription fields.

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¹C.W., S.L., Y.D., M.W., and S.O. contributed equally to this work.

²Present address: School of Medicine, Chengdu University, Chengdu 610106, People's Republic of China.

³To whom correspondence may be addressed. Email: kapplerj@njhealth.org, hagmanj@njhealth.org, or ZhangG@NJHealth.org.

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arginine residues within histone tails, although a report showed that some lysine demethylases also have activities against methylated arginines (18). In the hunt for histone arginine demethylase candidates, we hypothesized that one or more orphan JmjC domain-containing proteins, including JMJD4, JMJD5, JMJD7, and JMJD8, may be such a candidate. JMJD5 has been reported to play a critical role in embryonic development (19, 20). The exact functions of JMJD5 remain controversial. JMJD5 has been suggested to possess H3K36 lysine demethylase (21) and/or a hydroxylase activities (22, 23). The possibility remains that JMJD5 and some other "orphan" JmjC proteins target methyl arginines in histones and/or other proteins via different mechanisms.

The overall structural fold and catalytic cores of all members of the JmjC family are well defined (24). Therefore, to investigate the biochemical and biological function of orphan JmjC proteins, the catalytic cores of JMJD5 (residues 183–416, c-JMJD5) and JMJD7 (residues 26–318, c-JMJD7) (both mouse and human cDNA clones, Thermo Scientific) were overexpressed in *Escherichia coli* to produce recombinant JmjC proteins (Fig. S1).

Following expression and purification of c-JMJD5 and c-JMJD7, we tested the proteins' abilities to demethylate arginine substrates bulk histones from calf thymus, which are rich in

posttranslation modifications, including methylated arginine as substrates. Initially, reaction conditions were similar to those reported previously for histone lysine demethylase assays (24–26), but were refined to new conditions later on. Following incubation of recombinant c-JMJD5 or c-JMJD7 and substrates, the reaction products were resolved using SDS/PAGE and Western blotted for detection of methylated arginines or control epitopes using commercial antibodies. c-JMJD5 and c-JMJD7 each decreased detection of histone H3 arginine 2 with dimethylation [H3R2(me2)] (Fig. 1 *A* and *B*) and histone H4 arginine 3 with symmetric dimethylation [H4R3(me2)] (Fig. 1 *C* and *D*) in a dose-dependent manner.

Bulk histones differ from histones *in vivo*, which are assembled as octamer cores and wrapped with DNA in nucleosomes, the basic subunit of chromatin. To test whether c-JMJD5 and c-JMJD7 act similarly on isolated histones and on histones associated with chromatin, chromatin purified from calf thymus was treated with c-JMJD5 or c-JMJD7 in the same way as the bulk histones as described above. Similar to their activities on isolated histones, c-JMJD5 and c-JMJD7 decreased the content of methylated arginine H3R2(me2) within chromatin (Fig. 1 *E* and *F*).

Several mechanisms could account for the loss of the methylated arginines on histone tails. First, methyl groups may be

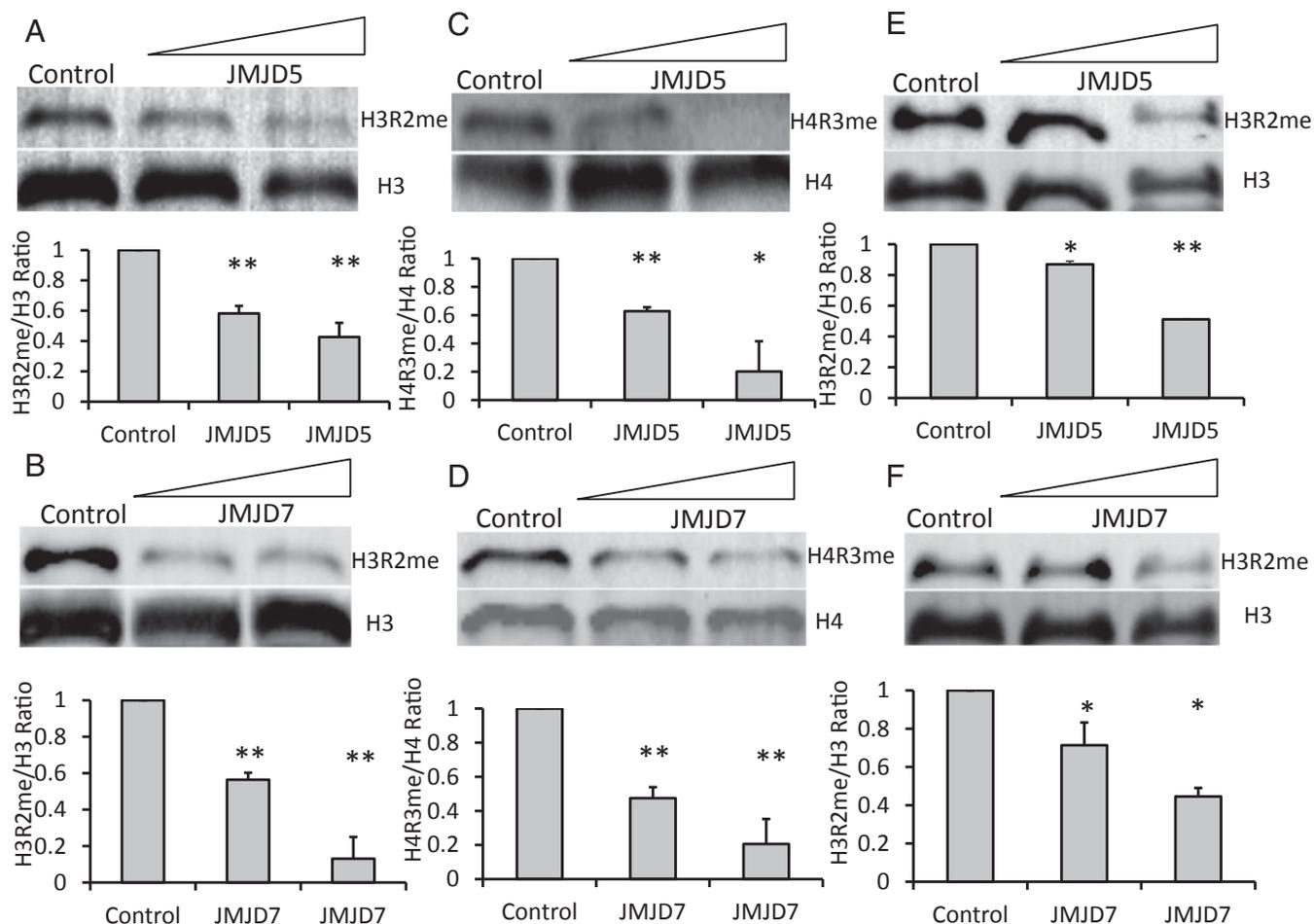


Fig. 1. The reduction of methylated arginine of bulk histones and chromatin by c-JMJD5 and c-JMJD7. Each section contains two parts: (Top) Western blotting; (Bottom) quantified data of the Western blot above. A–D are results of c-JMJD5 and c-JMJD7 on bulk histone. E and F are results of c-JMJD5 and c-JMJD7 on chromatin. (A) c-JMJD5 on H3R2me2; lane 1, control; 2–1 μg, 3–2 μg. (B) c-JMJD7 on H3R2me2; lane 1, control; 2–1 μg, 3–2 μg. (C) c-JMJD5 on H4R3me2, lane 1, control; 2–1 μg, 3–2 μg. (D) c-JMJD7 on H4R3me2; lane 1, control; 2–1 μg, 3–2 μg. (E) c-JMJD5 on H3R2me2 of chromatin; lane 1, control; 2–1 μg, 3–2 μg. (F) c-JMJD7 on H3R2me2 of chromatin; lane 1, control; 2–1 μg, 3–2 μg. **P* < 0.05 compared with control, ***P* < 0.01 compared with control.

removed directly from arginine by demethylation, similar to mechanisms used by JmjC proteins to demethylate methyl lysine (27). Second, methylated arginine may be converted to citrulline, similar to the products of peptidylarginine deiminase (PADI) enzymes (27–30). Third, methyl arginine may be removed by more extensive cleavage of histone tails by proteolysis, thus using a clipping process (3). The following experiments support the third mechanism, in which proteolytic clipping of histone tails removes methylated arginines, and, potentially, the entire tail from targeted histones.

Protease Activities of c-JMJD5 and c-JMJD7 on Bulk Histones. A major challenge in the study of epigenetic modifications of histone tails is the lack of specificity of some commercially available antibodies (31). Thus, instead of using antibodies, we used a method in which S-[^{14}C -methyl]-adenosyl-L-methionine (^{14}C -SAM) and

recombinant arginine methyl transferases (PRMT1 and PRMT5–7) (32–39) were used to introduce ^{14}C -labeled methyl moieties on bulk histones at specific sites. Methylated products were then assessed for modifications by the c-JMJD proteins. After methylation, the radioactive intensities of the ^{14}C -methylated bulk histones were compared with or without incubation with recombinant c-JMJD5 or c-JMJD7. Changes of ^{14}C signal were quantitated using phosphorimaging.

We assessed the specificities of the recombinant PRMT enzymes. PRMT1 has been reported to methylate H4, while PRMT5 targets H2A, H3, and H4, and PRMT6 is specific for H3. However, in our hands PRMT1 transferred ^{14}C -methyl groups to both H2A and H4, PRMT5 modified H2A and H4 but not H3 and PRMT6 methylated H2A, H3, and H4 (Fig. 2 *A* and *B*, control lanes). Incubation of ^{14}C -containing bulk histones with c-JMJD5 dramatically reduced the amounts of labeled

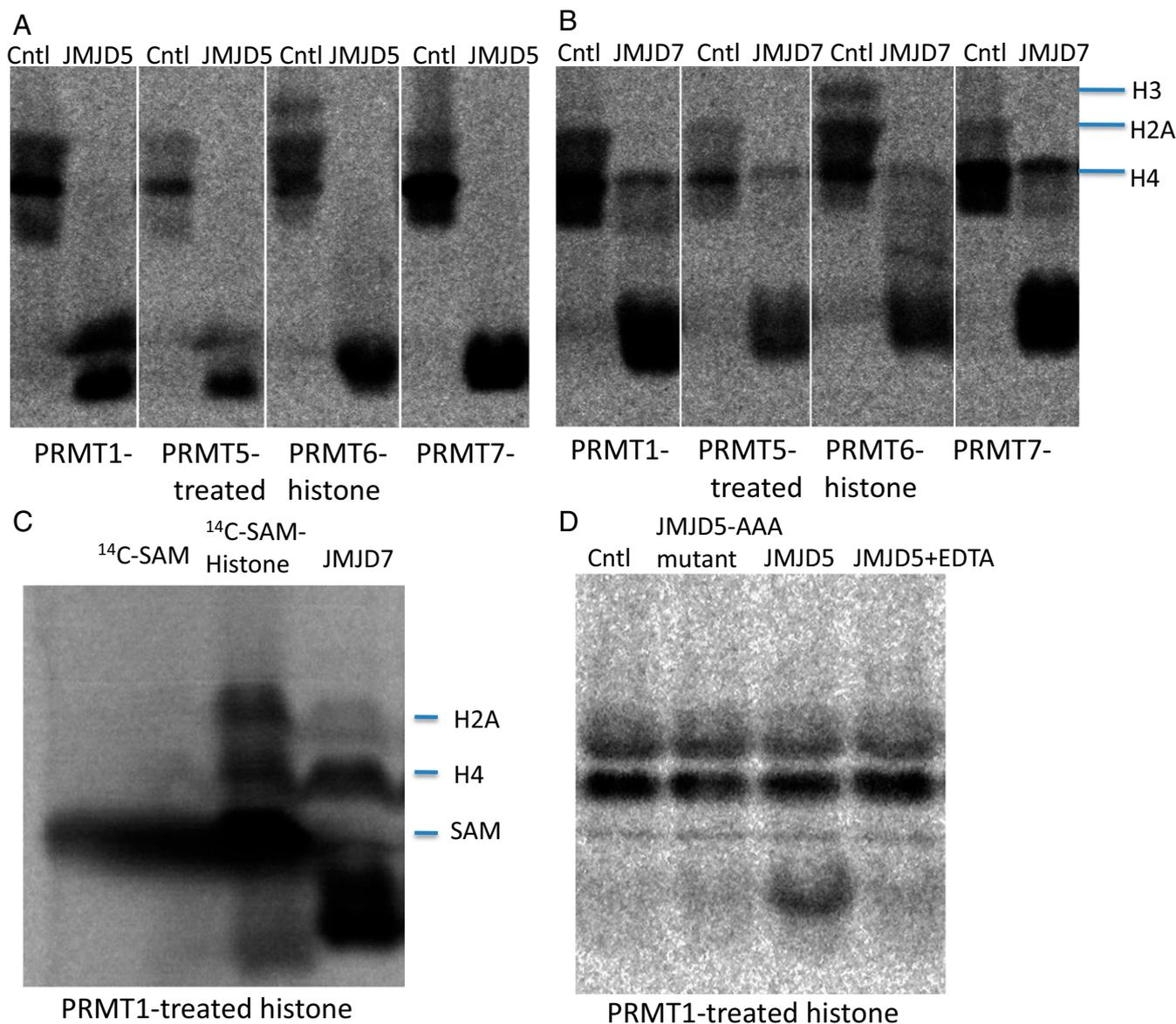


Fig. 2. ^{14}C labeled bulk histone reaction with c-JMJD5 and c-JMJD7. Each section is labeled both on top and bottom. (Top) Controls and enzymes used; (Bottom) ^{14}C -labeled bulk histone generated with individual PRMT proteins. (A) The activities of c-JMJD5 on bulk histone treated with PRMT1, PRMT5, PRMT6, and PRMT7, respectively. (B) The activities of c-JMJD7 on bulk histone treated with PRMT1, PRMT5, PRMT6, and PRMT7, respectively. (C) The ^{14}C -SAM and digested bulk histone. (D) The activities of c-JMJD5, c-JMJD5 with three point of mutations on –HD/E(X)_nH– metal chelating site (HDH → AAA), and c-JMJD5 in the presence of EDTA on bulk histone generated with PRMT1. Cntl, control; H2A, histone subunit 2A; H3, histone subunit H3; H4, histone subunit H4.

histones, regardless of the PRMT used to add methyl groups (Fig. 2*A*, lanes c-JMJD5). c-JMJD7 exhibited similar activity, but demethylated H4 less efficiently (Fig. 2*B*, lanes c-JMJD7). These results occurred no matter whether the histones were labeled with PRMTs that generate symmetric dimethyl- (PRMT5) or asymmetric dimethyl-labeled (PRMT1 and PRMT6) or monomethylated (PRMT1 and PRMT7) arginines.

Incubation with either of the c-JMJD5/7 proteins generated additional bands that migrated faster on SDS/PAGE, suggesting conversion of the methylated moieties to products with reduced mass (Fig. 2*A* and *B*). However, we did not observe the faster migrating bands in the Western blotting experiments shown in Fig. S2. To account for this paradox, we posit that the fragments released from these histone tails were too small to be detected using standard SDS/PAGE gels and/or because small fragments were below the resolution of Western blotting. Based on the relative location of the products (Fig. 2*C*), we suspected that the smaller fragments of the histone tails were too small for antibody recognition in our previous assays (Fig. S2).

Our data suggest that c-JMJD5 and c-JMJD7 are both proteases, because they cleave off histone tails with all possible variations of methyl arginine, including monomethyl-, asymmetric dimethyl-, and symmetric dimethyl arginine. To estimate the approximate size of the cleavage products, we electrophoresed ¹⁴C-SAM (molecular weight of 398.44 Da) by itself or in a mixture of ¹⁴C-SAM and bulk histones as a control with and without c-JMJD7 (Fig. 2*C*). The bands generated by c-JMJD7 migrated faster than SAM alone (Fig. 2*C*). This suggests that these small radioactive bands probably contain only one, two, or three amino acid residues.

Overall, these results suggest that c-JMJD5 and c-JMJD7 both remove methylated arginines, whether they are monomethylated (products of PRMT1 and PRMT7), symmetrically (products of PRMT5) or asymmetrically (products of PRMT1 or PRMT6) dimethylated, from histones by proteolytic cleavage.

To further characterize and confirm the proteolytic activities of c-JMJD5 and c-JMJD7, we ruled out protease contamination of the proteins. Several approaches were used. First, a mixture of protease inhibitors was added to all reactions. Second, if c-JMJD5 and c-JMJD7 were the responsible enzymes, they should be metalloproteases, given the dependence of other members of this family on divalent cations. To find out whether the proteolytic activity we observed was from metalloproteases, we added EDTA to the assays. The activities of both the c-JMJD5 and c-JMJD7 preparations were dependent on divalent cations (Fig. 2*D*). Third, to determine whether the iron chelating motif -HXD/E(X)_nH-, which is conserved in all JmjC proteins, is required for activity, the two His residues and the Asp residue of the motif were mutated to Ala in c-JMJD5 (c-JMJD5-AAA, triple mutation). The activity of c-JMJD5-AAA was almost completely abolished (Fig. 2*D*).

Cleavage Sites of c-JMJD5 and c-JMJD7 on Histone Tails. Our detection of the proteolytic activities of c-JMJD5 and c-JMJD7 on methylated histone tails begs the question as to which sites are recognized specifically by these proteins. Due to the nonspecific reactions (or cross-reactions) of antibodies and the multiple potential methylation sites of PRMT1, PRMT5, PRMT6, and PRMT7, neither approach described above identified the specific sites recognized by c-JMJD5 or c-JMJD7. It was unclear whether c-JMJD5 and c-JMJD7 recognize methylated H4R3(me2), H3R2(me2), and/or other sites. To define the specificities of c-JMJD5 and c-JMJD7, synthetic histone peptides marked with pH3R2(me2s) were used as substrates in cleavage assays. After the reactions, the mixtures were subjected to mass spectrometry analysis on a MALDI-TOF/TOF analyzer. Both c-JMJD5 and c-JMJD7 generated a series of peaks from pH3R2(me2s)-containing H3 tail peptide (Fig. 3). Of particular interest, we

detected a peak with molecular weight of 2,494.30 Da, which is 255.17 less than the original peptide (MW2749.47 Da) (peak A, Fig. 3*A*). This cleavage product is predicted to result from cleavage of the first two N-terminal residues of pH3R2(me2s). We found a similar result of c-JMJD7 (peak A, Fig. 3*B*). This suggests that both c-JMJD5 and c-JMJD7 cleave the H3 tail just after the dimethylated arginine residue (Fig. 3). This cleavage product is consistent with results of the ¹⁴C-labeled bulk histone assays, which generated peptide fragments smaller than the ¹⁴C-labeled SAM itself after digestion with c-JMJD5 or c-JMJD7 (Fig. 2*D*).

In addition to the 2,494.30 peak, we also observed a series of peaks corresponding to the C-terminal fragments generated by cleavage at T3(peak B), K4(peak C), Q5(peak D), T6(peak E), and A7(peak F) (Fig. 3). Together, these peaks indicate aminopeptidase activities of c-JMJD5 and c-JMJD7, with removal of one residue at a time progressively from the N termini of the peptides following their initial cleavage after R2. One possible explanation is that, after removal of the first two residues, both c-JMJD5 and c-JMJD7 continue to digest the remaining C-terminal products of the peptides via aminopeptidase activity. This raises the possibility that these enzymes possess aminopeptidase activity for the original unmethylated peptide. However, we did not observe a product resulting from the loss of Ala1 (MW 2,678.4 Da). The data confirm that both c-JMJD5 and c-JMJD7 cleave the peptides preferentially following the methylated arginine residues, followed by progressive digestion of C-terminal products. To confirm that both endopeptidase and exopeptidase activities is originally from c-JMJD5/JMJD7 proteins, point mutations of three ion chelating residues within the catalytic center of JMJD5 (c-JMJD5-AAA)/JMJD7 (JMJD7-AAA) do not show obvious cleavage activity (Fig. 3).

Specificity of c-JMJD5 Toward pH3 Peptide with Different Modifications.

Interestingly, c-JMJD5 also showed weak activity toward pH3 peptide without modification (Fig. S3), although its activity was undetectable on pH3K4me2 (Fig. S4). Although c-JMJD5 and c-JMJD7 showed both endopeptidase and exopeptidase activities toward arginine-methylated pH3 peptide, the question here is whether c-JMJD5 or c-JMJD7 has different binding affinity, depending on the methylation status of arginine. In this regard, fluorescence polarization experiments were performed to characterize the specific binding between c-JMJD5 and pH3 peptides with different modification. In a c-JMJD5-containing mixture, a random peptide does not show any change (Fig. 4), native form pH3 peptide yields measurable binding affinity ~7 μM (Fig. 4), while pH3R2me2a binds with surprisingly high affinity ~0.112 μM (Fig. 4), or ~70 times that of pH3. This binding affinity is similar to those between PHD domains and methylated lysines (0.16–30 μM; ref. 40), but is more than one order of magnitude higher than affinities between Tudor domains and methylated arginines (~4 μM; ref. 41). This is also true compared with pH3K4me2, where the binding affinity between c-JMJD5 and pH3K4me2 is ~4 μM (Fig. 4), or ~40 times lower than that of pH3R2me2a (Fig. 4). These results showed that c-JMJD5 discriminates pH3R2me2a from pH3 or pH3K4me2 by exhibiting a much higher binding affinity.

Activities of JMJD5 and JMJD7 in Vivo. It was reported that JMJD5 is a negative regulator of cell cycle repressors, including p21 and p53, which control the transition from G1 phase to S phase (20, 42). However, how JMJD5 regulates the expression of p21 and p53 remains elusive. The cleavage activity of c-JMJD5 on methylated histone tails in vitro indicates that JMJD5 may modify histones similarly in vivo, which in turn controls the transcription of genes encoding repressors of the cell cycle. To confirm this hypothesis, we generated mouse fibroblast cells with conditionally deleted *JMJD5* alleles. Levels of arginine

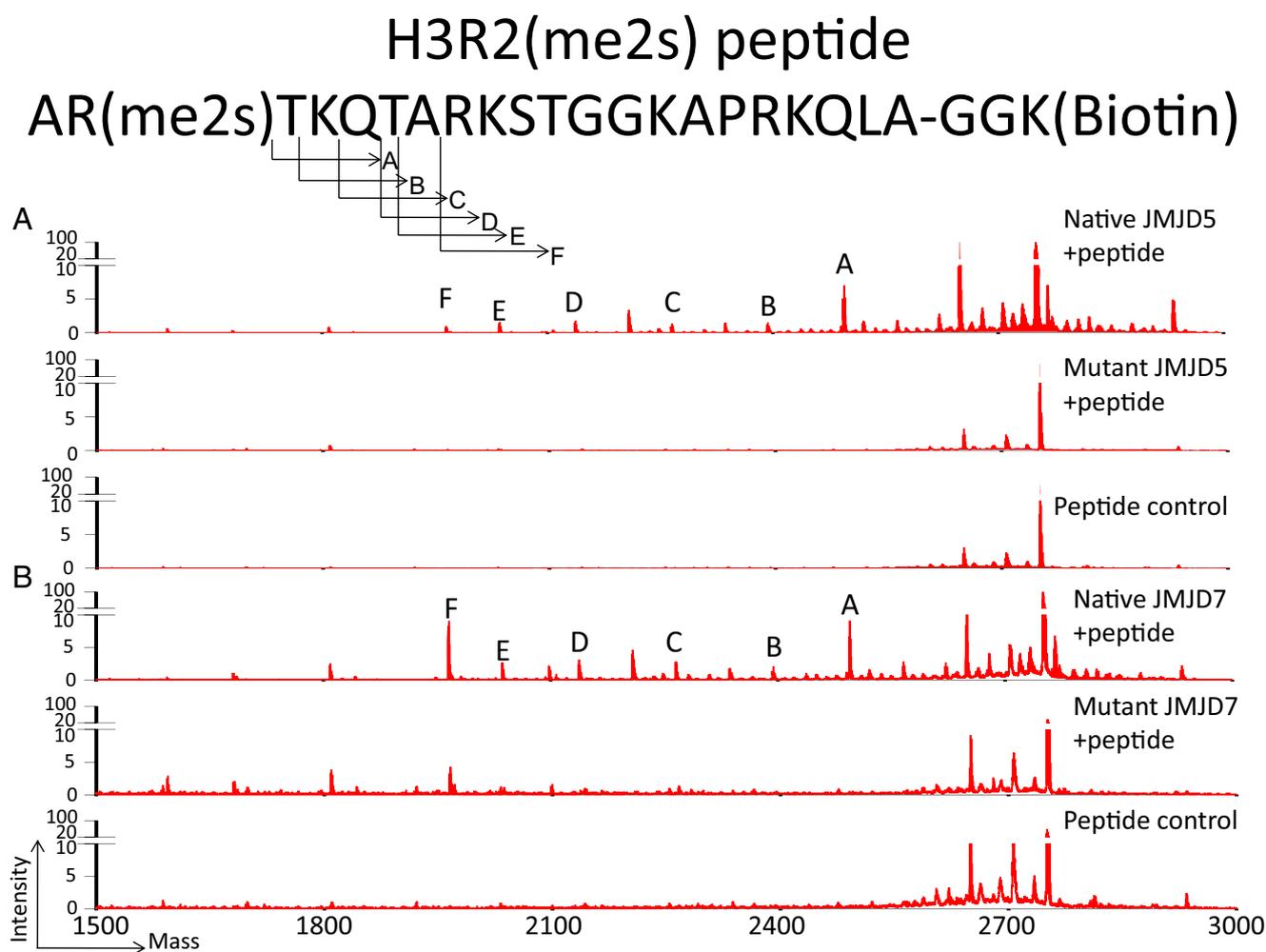


Fig. 3. The endopeptidase and exopeptidase activities of c-JMJD5 and c-JMJD7 on synthesized histone tails. (A) c-JMJD5 and mutant c-JMJD5 cleavage on pH3R2me2. The *Top* portion is the sequence of the pH3R2me2 peptide with symmetric dimethylation on R2 with molecular weight of 2,749.47 Da. After cleavage, a major band of molecular weight of 2,494.3 (peak A) is the product of peptide with the first two residues missing. (B) c-JMJD7 generated a similar profile.

methylation in fibroblasts with knockout of JMJD5 were compared with levels in normal fibroblasts. We found that the arginine methylation of H3R2 was dramatically increased in the JMJD5-deficient cells (Fig. 5A). This was also true for H4R3. However, it is surprising that the overall content of both H3 and H4 was also dramatically increased (Fig. 5A). This result may indicate that cleavage of methylated histone tails by JMJD5 renders the remaining nucleosomes prone to degradation, either through the aminopeptidase function of JMJD5 or by other proteases. This is consistent with the aminopeptidase functions of c-JMJD5 and c-JMJD7, which continue to clip off histone tails after the first cleavage at the methylated arginine site.

The exact functions of JMJD7 have not been determined *in vivo*. One report suggests a critical role of JMJD7 in the development of autism (43). A particularly puzzling aspect of JMJD7 is its readthrough transcription into the *Phospholipase A2* gene, which generates a putative JMJD7-PLA2G4B fusion with predominant localization within the cytoplasm (44). We hypothesize that JMJD7 may function similarly to JMJD5, but may recognize different forms of histone tails and/or nonhistone proteins. In this regard, we used the CRISPR/Cas9 system to knock out JMJD7 in a breast cancer cell line, MDA-MB231. The content of arginine methylation of both H3R2/H4R3 and overall H3/H4 was increased with the knockout of JMJD7 (Fig. 5B),

although not as dramatically as JMJD5 (Fig. 5A). Most importantly, the colony-forming abilities of JMJD7-deficient MDA-MB231 cells were greatly decreased (Fig. 6), which is similar to the inhibition of breast cancer cell growth upon down-regulation of JMJD5 (21). To avoid potential errors introduced by CRISPR/CAS9 and to cross-confirm whether the phenotype of JMJD7-deficient breast cancer cells is indeed caused by reducing JMJD7, either the native form of JMJD7, or an enzymatically inactive version of JMJD7 (JMJD7-AAA) defective in divalent cation binding, were introduced back into JMJD7-deficient cells. The native form of JMJD7 rescued normal proliferation in JMJD7-deficient cells, while JMJD7-AAA did not in two independent knockout cell lines (Fig. 6). It will be of great interest to determine the identities of genes regulated by JMJD7 as well as how JMJD7 is regulated and localized to the nucleus.

Discussion

Over three decades ago, Allis et al. reported the cleavage of H3 histone tails and its potential critical role in transcriptional regulation (45). Furthermore, cathepsin L was identified as an enzyme that cleaves histone tails (46). Kouzarides and colleagues also reported that an endopeptidase in *Saccharomyces cerevisiae* cleaves the first 21 residues from H3 (47). A number of groups have

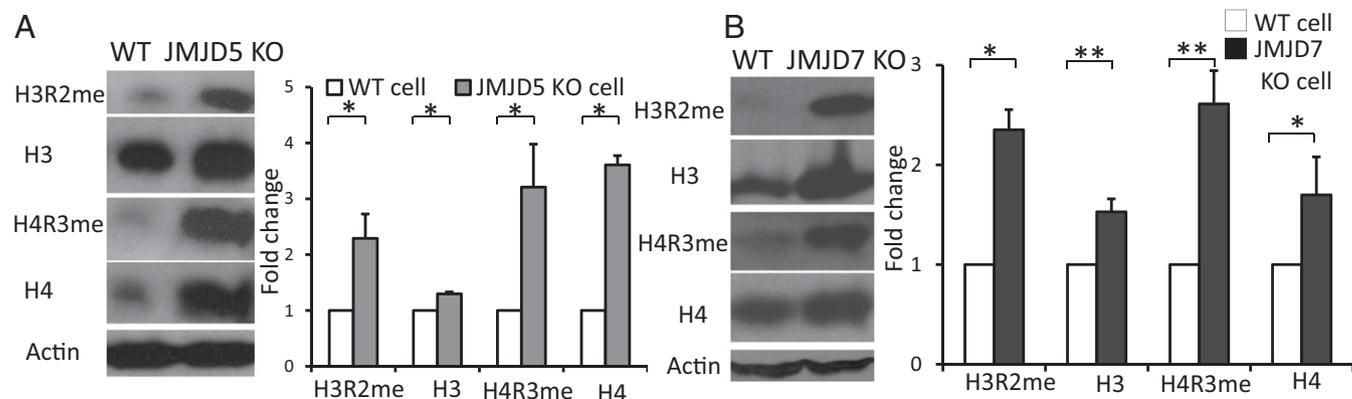


Fig. 5. JMJD5 knockout dramatically increased the content of both arginine methylated and total H3 and H4 in mouse embryonic fibroblast (MEF) cells. JMJD7 knockout has similar effect. *A* and *B* contain two parts: (*Left*) Western blot of interested target and (*Right*) quantified results of corresponding Western blot. (*A*) The arginine methylation of H3 and H4 is increased in JMJD5 knockout MEFs. However, the overall content of H3 and H4 increases as well, suggesting degradation of H3 and H4 in the presence of JMJD5. (*B*) The knockout of JMJD7 in breast cancer cell lines leads to the increase in arginine-methylated H3 and H4, as well as the total H3 and H4. * $P < 0.05$ compared with WT cell, ** $P < 0.01$ compared with WT cells.

of the peptide backbone adjacent to methyl arginine residues on histone tails by c-JMJD5 and c-JMJD7. These results are further confirmed by our mass spectrum data on synthetic methylated peptides. We also determined that c-JMJD5 and c-JMJD7 function as both endopeptidases and aminopeptidases (exopeptidases) to further trim histone tails, where we detected removal of individual amino acids, including Ala, Gly, Ser, Thr, Pro, Arg, Lys, and Gln residues from the H3 and H4 tails (Fig. 3). The unique nonspecific activities of these enzymes represent a mechanism of catalysis. It is worthwhile to characterize this activity further, including the full range and mechanism of amino acid recognition. These discoveries suggest that the methylated arginines removed from histone tails are neither recycled nor reversed, but rather are completely destroyed through degrada-

tion. This result is consistent with the turnover rate of histones in nonproliferating cells (49).

JMJD5 has been reported to be critical for the cell cycle (20, 21, 42). Epigenetic modifications are closely related to various cancers and are promising drug targets, notably, JMJD5 is highly expressed in cancer patients and is critical for proliferation of cancer cells (21, 50). Our knockout of JMJD7 in breast cancer cells dramatically impacts soft agar growth of MDA-MB231 cells. These results suggest that JMJD5, JMJD7, and possibly other orphan JmjC proteins contribute to tumorigenesis.

The clipping phenomenon of histone tails has been known for several decades but is largely understudied, possibly because the permanent removal of histone tails is not in line with the notion that the reversibility of epigenetic marks is critical for epigenetic modification. What could be the effect of removing the highly

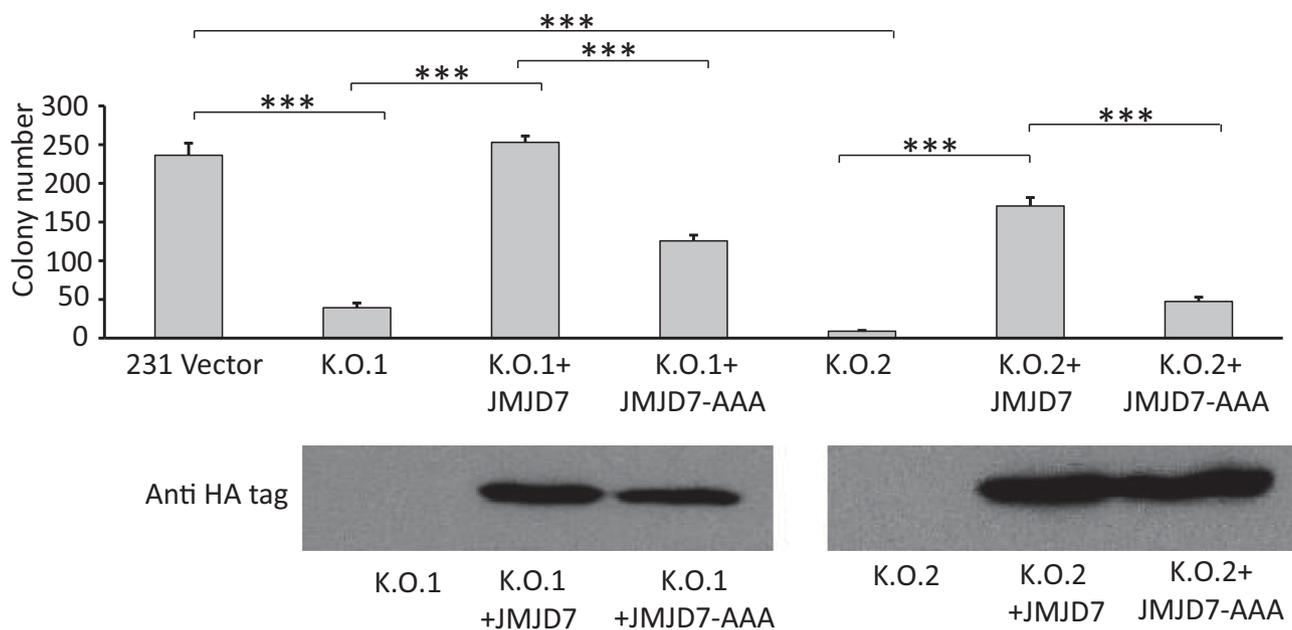


Fig. 6. (Top) Knockout of JMJD7 in MDA-MB231 cells significantly attenuate the soft agar forming abilities of two independent JMJD7-knockout populations (K.O.1 and K.O.2). Introduction of wild-type JMJD7 rescues the phenotypes of both knockout cell lines (K.O.1 + JMJD7 and K.O.2 + JMJD7) but not when enzymatic-inactive JMJD7 was introduced (K.O.1 + JMJD7-AAA and K.O.2 + JMJD7-AAA). (Bottom) Western blot analysis of JMJD7 or JMJD7-AAA expression. *** $P < 0.001$.

positively charged histone tail, in conjunction with the variety of modifications present on the transcription machinery Pol II (RNA polymerase II)? For a relatively simple bacterial system, recruitment of RNA polymerase (RNAP) to the promoter region of a target gene by transcription factors triggers transcription initiation (51, 52). However, for a eukaryotic system, the nucleosome, which contains eight subunits of histone proteins, provides another layer of structural complexity, which prevents Pol II from accessing promoters, transcription initiation, and elongation. There is abundant evidence showing that ATP-dependent chromatin remodelers, such as switch/sucrose nonfermentable (SWI/SNF), act to alter nucleosome or chromatin architecture via histone subunit exchange, ejection, and spatial reorganization (i.e., by sliding) (53–55). Based on our findings, we propose a possible mechanism in which the cleavage of the highly charged

histone tails lead to weaker association between DNA and histone subunits so as to the final depletion of histone subunits from nucleosome and creation of nucleosome-free regions (Fig. 7).

The aminopeptidase activities of both JMJD5 and JMJD7 suggest that both enzymes will continue to digest histones after the first specific cleavage at the methylated arginine residues. A scenario may be the following: Arginine methylations of histone tails act as a mark of histone degradation beyond ubiquitination. Nucleosomes or chromatin, upon truncation of histone tails, which dramatically decrease the stability of nucleosomes, will eventually be degraded. This is evidenced by our observation of the dramatic increase in H3 and H4 levels in the JMJD5 knockout mouse fibroblast cells and JMJD7 knockout human breast cancer cells (Fig. 5) and confirmed by rescue experiments (Fig. S6). The degradation of histone tails could be a part of the

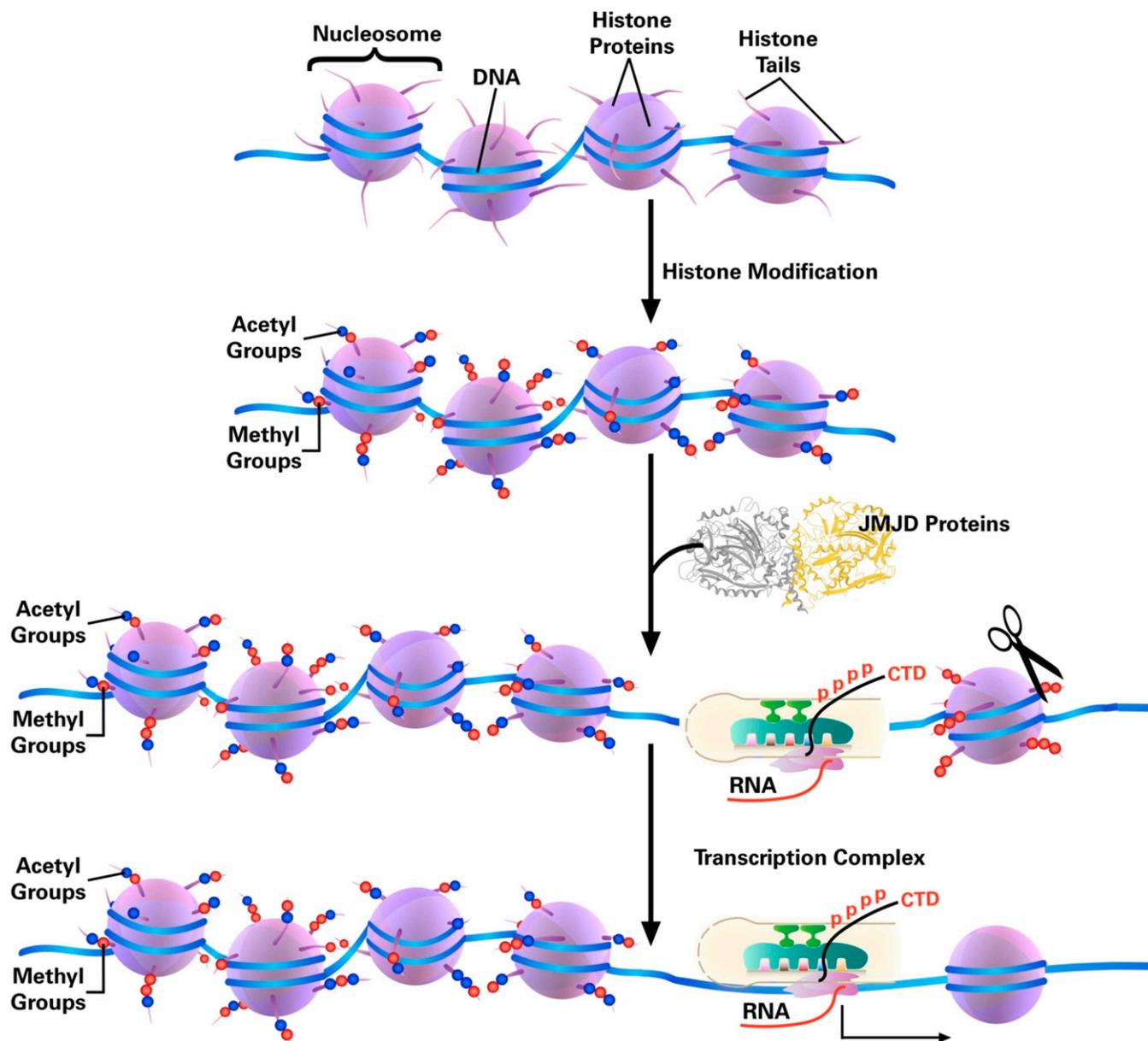


Fig. 7. Transcription elongation model. Compared with a prokaryotic system, eukaryotic DNA is wrapped around histones and not accessible for Pol II. The highly positively charged histone tails play critical roles in the packing of chromatin. Acetylation of the tails could loosen up the tight packing while cleavage of histone tails by JMJD5, JMJD7, and possibly others could lead to complete depletion of nucleosomes for the accessibility of Pol II, or Pol II is able to overcome the tailless nucleosomes as that in Archaea.

larger regulatory mechanism. Interestingly, it was well known that Archaea contain histone-like proteins and form nucleosome-like structures but without tails or chromatin remodeling complex (56–58). It seems that RNA polymerases alone in Archaea could overcome tailless nucleosomes during transcription since there is no known chromatin remodeling complex in Archaea (58). It may be true for Pol II to overcome nucleosomes after tail cleavage by JMJD5 and JMJD7 (Fig. 7). However, we cannot rule out if it needs the help of chromatin remodeling complexes.

Materials and Methods

Cloning, Expression, and Purification. The N-terminal 26-residue truncated mouse *JMJD7* was cloned into the pGEX 4T-2 plasmid, which was transformed into Rosetta competent cells. A tobacco etch virus (TEV) protease cut site was engineered between the GST tag and c-JMJD7. When the cell density reached $OD_{600} = 0.8$, protein expression was induced by 1 mM IPTG at 18 °C overnight. GST–c-JMJD7 protein was purified using glutathione beads, and c-JMJD7 was excised from the fusion protein using the TEV protease. c-JMJD7 protein was further purified using a sizing column. Cloning, expression, and purification of human c-JMJD5 and mouse c-JMJD5 followed a similar procedure as that of JMJD7 (Fig. S1). Only the catalytic core of human JMJD5 (183–416) is used for activity assays.

Detection of Arginine-Methylated Histone Assay Using Calf Bulk Histone. A total of 2 μ g calf bulk histone (Sigma) was dissolved in buffer [20 mM Tris-Bis (pH ~ 6.5–7.0), 150 mM NaCl, 50 mM $(NH_4)_2Fe(SO_4)_2/ZnCl_2$, 1 mM α -ketoglutarate, and 2 mM ascorbic acid]. Different amounts of c-JMJD5 and c-JMJD7 were added to the tubes in the presence of protease inhibitor mixture (Roche). The reaction was carried out at 37 °C for 3–4 h. The sample was separated by SDS/PAGE, transferred to nitrocellulose membrane, and detected with H3R2me2 (Millipore) and H4R3me2a antibodies (Active Motif). After the first blotting, the bound antibody was washed away using Restore Western Blot Stripping Buffer (Thermo Scientific). H3 and H4 total antibody (Active Motif) was then probed as the loading control.

Calf Thymus Chromatin Preparation. Calf thymus was kindly provided by L. Wysocki's laboratory. In brief, the thymus was minced using a razor blade and homogenized with a Dounce homogenizer in sucrose/Tris/PMSF buffer (10 mM Tris, 5 mM $MgCl_2$ and 0.3 M sucrose pH7.2 with 1 mM PMSF added before use). The homogenate was then passed through cheesecloth and pelleted by centrifugation. Nuclei were released from cells in TKM buffer (50 mM Tris, 50 mM KCl and 15 mM $MgCl_2$) and resuspended in 1/2 TKM buffer with 1% Triton X-100. The mixture was aliquoted into 15-mL conical tubes and the cell suspension was underlaid with 60% sucrose with 1/2 TKM buffer, and centrifuged at 1,000 \times g for 10 min with no break. Nuclei were collected at the interface and resuspended in 1 mM EDTA for the lysis of nuclei. The chromatin was collected by centrifugation.

Radiolabeling of Bulk Histone by PRMTs Using ^{14}C -SAM. A total of 100 μ g calf bulk histone was dissolved in methyl transfer buffer [50 mM Tris-HCl

(pH 8.0), 2 mM EDTA, 1 mM DTT]. Ten micrograms of PRMT1, PRMT5, PRMT6, or PRMT7 was added to separate tubes in the presence of protease inhibitor mixture. As a radioactive methyl donor, 1 μ Ci adenosyl-L-methionine, S-[methyl- ^{14}C] (PerkinElmer) was used. The reaction was carried out at 30 °C for 2 h. The excess ^{14}C -SAM was removed by centrifugation using Amicon centrifugation tubes with molecular weight cutoff (MWCO) of 10 kDa. The c-JMJD5 and c-JMJD7 reaction was performed as described above, differing only in that the duration time was 4 h at 37 °C. After the reaction, the sample was separated by SDS/PAGE gel, which was then dried using a vacuum pump. The ^{14}C radioactive signal was detected using a Typhoon 9500 imager (GE Healthcare).

Mass Spectrum Analysis of Reaction Mixture. Briefly, 1–5 μ g of purified c-JMJD5 or c-JMJD7 was incubated with 1–5 μ g of dimethyl pH3R2me2s (symmetric and asymmetric) in a reaction buffer [20 mM Tris-Bis (pH ~ 6.6–7), 20 mM NaCl, 50 μ M $(NH_4)_2Fe(SO_4)_2$, 1 mM α -ketoglutarate, and 2 mM ascorbic acid] for 1–2 h at 37 °C or room temperature. The reaction was stopped by adding 0.1% trifluoroacetic acid (TFA). Then the reaction mixture was diluted 10 times with water before adding 10 mg/mL α -cyano-4-hydroxycinnamic acid MALDI matrix in 70% acetonitrile/0.1% TFA. The mixed material was spotted on a plate to evaporate the solvent and co-crystallize the peptide/matrix. Samples were analyzed by a MALDI-TOF/TOF analyzer (4800 Plus; SCIEX) at the University of Colorado Health Sciences Center. Data were processed using Data Explorer software.

Fluorescence Polarization Experiment. All of the regular and methylated peptides were synthesized by AnaSpec. A total of 10 μ M c-JMJD5 was titrated and equilibrated with fixed concentrations of each peptide, respectively (pH3, pH3R2me2a, pH3K4me2, and random peptide), incubated at 25 °C for 30 min between each titration interval, and subject to fluorescence measurement. The buffer used in the fluorescence quenching assay was 20 mM Tris-HCl pH 6.5. The excitation wavelength of 280 nm and the emission wavelength of 342 nm were used for data collection and recorded with a Fluoromax-3 spectrometer. Data were normalized and the dissociation constant (K_d) for each peptide was calculated by fitting to a four-parameter sigmoidal dose–response curve with SigmaPlot v11.0.

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