

# Lysyl 5-Hydroxylation, a Novel Histone Modification, by Jumonji Domain Containing 6 (JMJD6)\*

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**Background:** JMJD6 hydroxylates U2AF65, but its role in histone modification has been obscure.

**Results:** Our analysis of histones purified from JMJD6 knock-out mouse embryos reveals that JMJD6 hydroxylates histone lysyl residues.

**Conclusion:** JMJD6 mediates histone lysyl 5-hydroxylation, which is a novel histone modification.

**Significance:** Our study identifies a new function for Jumonji family proteins in epigenetic modification of histones.

JMJD6 is reported to hydroxylate lysyl residues of a splicing factor, U2AF65. In this study, we found that JMJD6 hydroxylates histone lysyl residues. *In vitro* experiments showed that JMJD6 has a binding affinity to histone proteins and hydroxylates multiple lysyl residues of histone H3 and H4 tails. Using JMJD6 knock-out mouse embryos, we revealed that JMJD6 hydroxylates lysyl residues of histones H2A/H2B and H3/H4 *in vivo* by amino acid composition analysis. 5-Hydroxylysine was detected at the highest level in histones purified from murine testis, which expressed JMJD6 at a significantly high level among various tissues examined, and JMJD6 overexpression increased the amount of 5-hydroxylysine in histones in human embryonic kidney 293 cells. These results indicate that histones are additional substrates of JMJD6 *in vivo*. Because 5-hydroxylation of lysyl residues inhibited *N*-acetylation and *N*-methylation by an acetyltransferase and a methyltransferase, respectively, *in vitro*, histone 5-hydroxylation may have important roles in epigenetic regulation of gene transcription or chromosomal rearrangement.

Jumonji domain containing 6 (JMJD6),<sup>2</sup> which possesses high binding affinity to single-stranded RNA, is reported to hydroxylate lysyl residues of an RNA splicing factor, U2AF65

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<sup>2</sup> The abbreviations used are: JMJD6, Jumonji domain containing 6; qRT-PCR, quantitative RT-PCR; HAT, histone acetyltransferase; E14.5, embryonic day 14.5; AdoMet, S-adenosyl-L-methionine.

(1, 2). JMJD6 contains a JmjC domain that catalyzes lysyl hydroxylation of proteins in the presence of 2-oxoglutarate, Fe(II), and ascorbate. Proteins belonging to the JmjC family are classified into 2-oxoglutarate oxygenases (3). Among the known 2-oxoglutarate oxygenases, PLOD3 (procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3) mediates hydroxylation of unmodified lysyl residues, yielding 5-hydroxylysine (4). Most JmjC family members catalyze hydroxylation of *N*-methyl groups at the  $\epsilon$ -amino group of lysyl residues and generate hydroxymethyl groups, which are immediately processed to formaldehyde molecules, resulting in demethylation of methylated lysyl residues (5). However, JMJD6 does not add a hydroxyl group to the *N*-methyl group but adds it to one of the backbone carbons in a lysyl side chain and generates a stable 5-hydroxylysine (1). JMJD6 knock-out mice exhibited severe anemia, growth retardation, and a delay in terminal differentiation of the kidney, intestine, liver, and lung during embryogenesis, resulting in perinatal lethality (6, 7).

In this study, we first identified JMJD6 as a novel UHRF1 (ubiquitin-like with PHD and RING finger domains 1) interacting protein. UHRF1 has important roles in transferring DNA methylation status and recognizes histone modification status (8). Therefore, we thought that JMJD6 might hydroxylate histone molecules through interaction with UHRF1. Using JMJD6 knock-out mice, we revealed that JMJD6 hydroxylates histone lysyl residues and generates 5-hydroxylysine *in vivo*. 5-Hydroxylation is a novel histone lysyl modification. Because it interfered with *N*-acetylation and *N*-methylation by an acetyltransferase and a methyltransferase, respectively, the modification may regulate transcription through these interactions with other histone modifications.

## EXPERIMENTAL PROCEDURES

*JMJD6 Wild-type and Knock-out Mice*—Details of the JMJD6 knock-out mice were described elsewhere (6). C57BL/6 mice

## JMJD6 Hydroxylates Histone Lysyl Residues

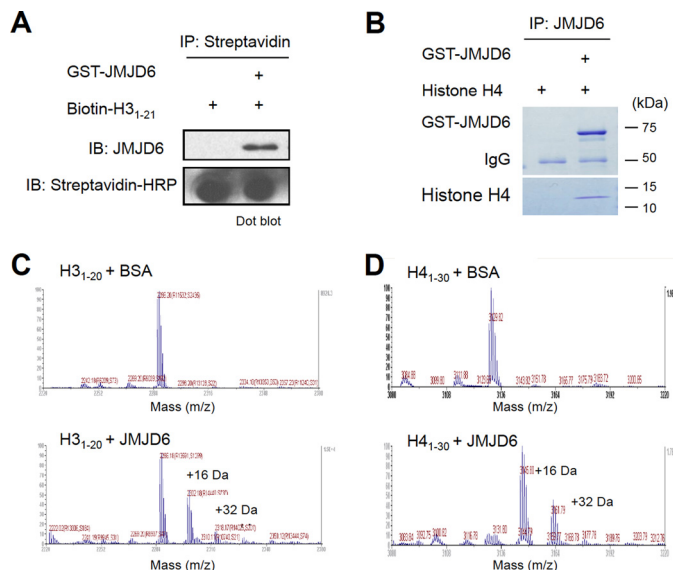
were used as wild-type control mice. JMJD6 knock-out embryonic day 14.5 (E14.5) embryos were obtained by crossing heterozygous JMJD6 mutant mice.

**Antibodies, Plasmids, and Cell Lines**—The following antibodies were used: anti-JMJD6 rabbit polyclonal antibody (ab10526, Abcam), and anti- $\beta$ -actin mouse monoclonal antibody (GTX26276, GeneTex). Human JMJD6 cDNA was cloned into pGEX-6p-1 (GE Healthcare) and pcDNA5/FRT/TO (Invitrogen). Doxycycline (Dox)-inducible JMJD6 stable cells were generated using the Flp-In T-REx system (Invitrogen) according to the manufacturer's instructions. JMJD6 expression was induced by Dox (final concentration, 0.5  $\mu$ g/ml; TaKaRa, Tokyo, Japan). J1 mouse ES cells were obtained from ATCC (Manassas, VA) and maintained in DMEM with 15% fetal bovine serum (FBS), nonessential amino acids, 2-mercaptoethanol, and leukemia inhibitory factor. Flp-In T-Rex 293 cells were obtained from Invitrogen and the Dox-inducible JMJD6 stable 293 cells were maintained in DMEM with 10% FBS, 10% tetracycline-free FBS, hygromycin B (100  $\mu$ g/ml), and blasticidin S (15  $\mu$ g/ml).

**Quantitative RT-PCR**—For qRT-PCR reactions, specific primers and probes for mouse JMJD6 (forward, 5'-GACCCG-GCACAACACTACTACG-3'; reverse, 5'-CTCTTGTGCATTG-AGCAGAAC-3') and mouse GAPDH (forward, 5'-CCATGT-TTGTGATGGGTGTG-3' and reverse, 5'-ACTGTGGTC-ATGAGCCCTTC-3') were used. PCR reactions were performed using the TaKaRa Thermal Cycler Dice® Real Time System Single following the manufacturer's instructions. Amplification conditions were 30 s at 95 °C and then 40 cycles each consisting of 5 s at 95 °C and 30 s at 60 °C.

**Purification of GST-JMJD6 and *In Vitro* Binding Assay**—Recombinant GST-JMJD6 was expressed in BL21-CodonPlus DE3-RIL cells. The transformed bacteria were incubated in L-Broth media with 0.1 mM isopropyl 1-thio- $\beta$ -D-galactopyranoside at 16 °C overnight. Following this, the bacteria were lysed in sonication buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 10% glycerol, 1% Triton X, and 0.8 mg/ml lysozyme) by sonication. GST-JMJD6 was purified using glutathione-Sepharose 4FF (GE Healthcare) and eluted by glutathione. The purified proteins were incubated with biotin-labeled histone H3<sub>1-21</sub> peptides (12–405, Millipore, Billerica, MA) or recombinant full-length histone H4 (14–697, Millipore) in 0.1% Nonidet P-40 lysis buffer (150 mM NaCl, 0.1% Nonidet P-40, and 50 mM Tris-HCl (pH 8.0)) for 1 h at 4 °C. The biotin-labeled histone H3<sub>1-21</sub> peptides were pulled down with interacting proteins by streptavidin Sepharose (S951, Invitrogen). Full-length histone H4 was immunoprecipitated with anti-JMJD6 rabbit polyclonal antibody (ab10526, Abcam), which was also used for Western blotting.

***In Vitro* Hydroxylation Assay**—To perform the enzyme assay, GST-JMJD6 was prepared as described above. Extracted GST-JMJD6 was concentrated using a 50 K column (Millipore), and its buffer was replaced with 50 mM Tris-HCl (pH 7.5) by dialysis using EasySep (TOMY, Tokyo, JAPAN). Purity of GST-JMJD6 was assessed by Coomassie Brilliant Blue staining. The enzyme assay was performed in 50 mM Tris-HCl (pH 7.5) buffer containing 500  $\mu$ M  $\alpha$ -ketoglutarate, 100  $\mu$ M L-ascorbate, 100  $\mu$ M Fe(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10  $\mu$ M GST-JMJD6, and 20  $\mu$ M histone peptides.



**FIGURE 1. JMJD6 interacts with and hydroxylates histone H3 and H4 *in vitro*.** A and B, *in vitro* pull-down assay. Biotin-labeled histone H3<sub>1-21</sub> peptides (A) or recombinant histone H4 (B) were incubated with or without GST-JMJD6, pulled down by streptavidin-Sepharose, and detected by dot blot using streptavidin-HRP (A) or Coomassie Brilliant Blue (CBB) staining (B). Pulled down GST-JMJD6 was detected by Western blotting using anti-JMJD6 antibody (A) or Coomassie Brilliant Blue staining (B). C and D, enzymatic activity of GST-JMJD6 was measured by MS analysis. Histone H3<sub>1-20</sub> (C) and H4<sub>1-30</sub> (D) peptides were served as substrates. BSA was used as a negative control. IB, immunoblot.

Protein purification and the enzyme assay were performed on the same day to avoid reduction of enzymatic activity of JMJD6.

**MS Analysis**—Peptides treated with JMJD6 were acidified with trifluoroacetic acid (TFA; final concentration, 0.5%) and absorbed with ZipTipC18. The captured peptides were washed with 0.1% TFA with 2% acetonitrile once and eluted with 0.5  $\mu$ l of the matrix solution (4 mg/ml cyano-4-hydroxycinnamic acid, 0.1% TFA, 70% acetonitrile) onto the MALDI target plate (AB Sciex, Foster City, CA). The spotted samples were analyzed with the reflectron mode of 4800 plus MALDI-TOF-TOF mass spectrometer (AB Sciex).

**Purification of Histones and Detection of 5-Hydroxylysine by Amino Acid Composition Analysis**—Histones H2A/H2B and H3/H4 were separately purified from tissues or culturing cells using a histone purification kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. The extracted histones were separated by SDS-PAGE, transferred to a membrane (Immobilon-P<sup>SQ</sup>, Millipore), and stained by Coomassie Brilliant Blue. The transferred histones were used for amino acid composition analysis to detect 5-hydroxylysine.

The JMJD6-treated peptides or the purified histones were hydrolyzed in 6 N HCl vapor at 110 °C for 20 h. The acid hydrolysates of the peptides were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, and 6-aminoquinolylcarbamyl amino acid thus obtained was quantified by ion-pair chromatography using tetramethylammonium bromide on a C18-reversed phase column (L-column 2, 3.0 mm, inner diameter  $\times$  250 mm, 3  $\mu$ m, CERI, Tokyo, Japan) (9). Each amino acid was separated by HPLC. The acid hydrolysates of the histones were purified on a graphitic carbon column (Hypercarb, 2.1 mm, inner diameter  $\times$  100 mm, 3  $\mu$ m, Thermo Fisher Scien-

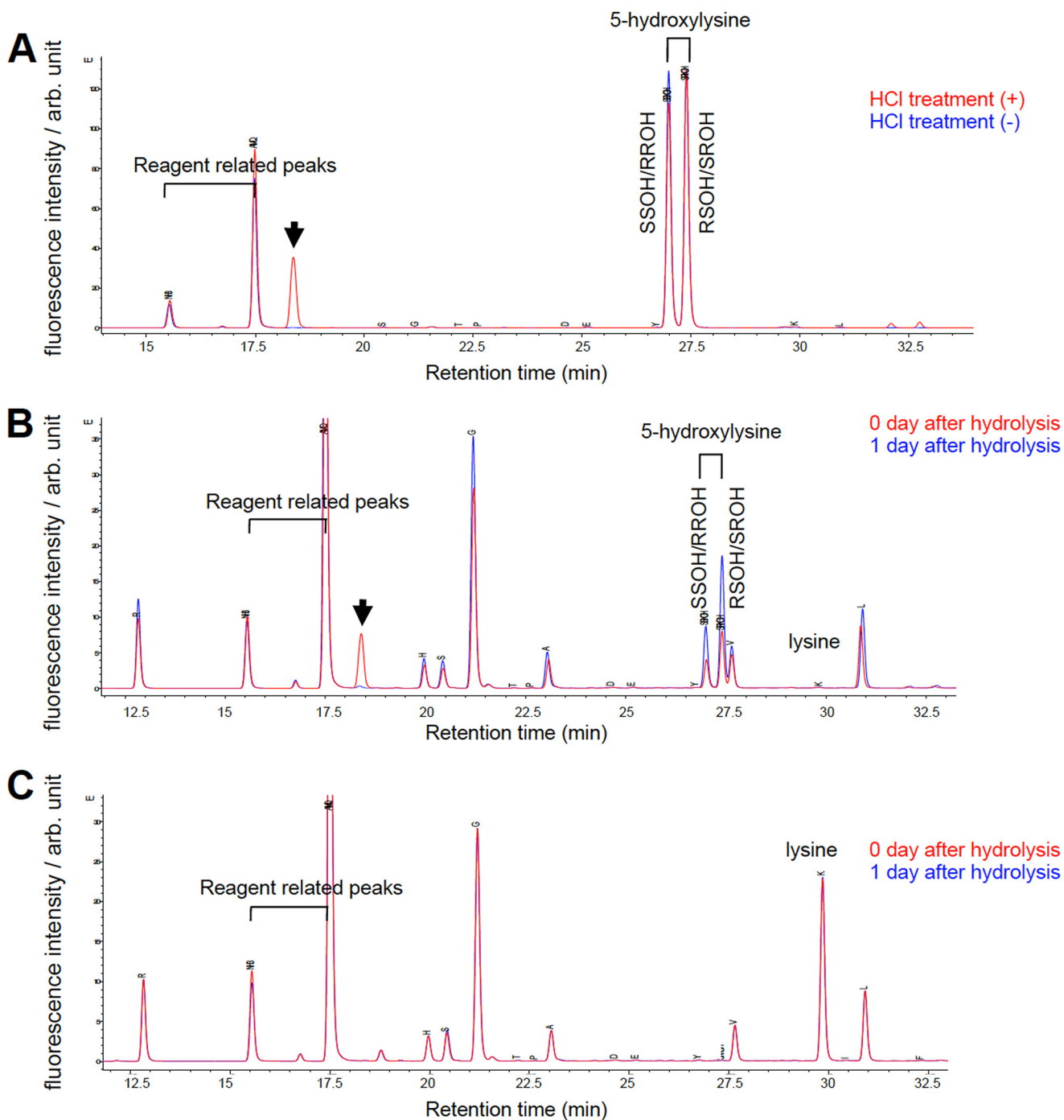


FIGURE 2. **Establishment of amino acid composition analysis for detecting 5-hydroxylysine.** A, analysis of simplicial synthetic SR-hydroxylysine and synthetic racemic mixture (SS/RR/RS/SR) of 5-hydroxylysine either treated with (red) or without (blue) HCl. B and C, analysis of H4<sub>1–20</sub> peptides including synthetic 5-hydroxylysine (B) and unmodified H4<sub>1–20</sub> peptides (C). The peptides were analyzed in the same day of hydrolysis (red) or next day of hydrolysis (blue). The arrow indicates a 5-hydroxylysine derived peak, which possibly corresponds to a lactone derivative, 3-amino-6-(aminomethyl)oxan-2-one. SSOH/RROH, 2S,5S-/2R,5R-hydroxylysine; RSOH/SROH, 2R,5S-/2S5R-hydroxylysine; arb. unit, arbitrary units.

tific, Inc., Waltham, USA), and a fraction including 5-hydroxylysine was derivatized with 6-aminoquinolylcarbonyl. The 6-aminoquinolylcarbonyl amino acids were separated on a C18-reversed phase column (Inertsustain C18HP, 3.0 mm, inner diameter  $\times$  250 mm, 3  $\mu$ m, GL Sciences, Tokyo, Japan) and quantified. Synthetic racemic mixture of DL-5-hydroxylysine (catalog no. H0377, Sigma-Aldrich), and 2S,5R-hydroxylysine (catalog no. 55501, Sigma-Aldrich) were used as standards.

**In Vitro Histone Acetyltransferase (HAT) Assay**—The *in vitro* p300 colorimetric HAT assay was performed according to a protocol from BIOMOL (Plymouth Meeting, PA). In brief, the catalytic domain of human p300 (catalog no. SE-451, BIOMOL) and the indicated amount of control histone H4<sub>1–23</sub> peptides or 5-hydroxylysine containing histone H4<sub>1–23</sub> peptides, in which all lysines were substituted to 5-hydroxylysine (Sigma-Genosys, Hokkaido, Japan), were incubated in 50  $\mu$ l of assay buffer (50 mM HEPES/NaOH (pH 7.9), 0.1 mM EDTA, 50  $\mu$ g/ml BSA) in



## JMJD6 Hydroxylates Histone Lysyl Residues

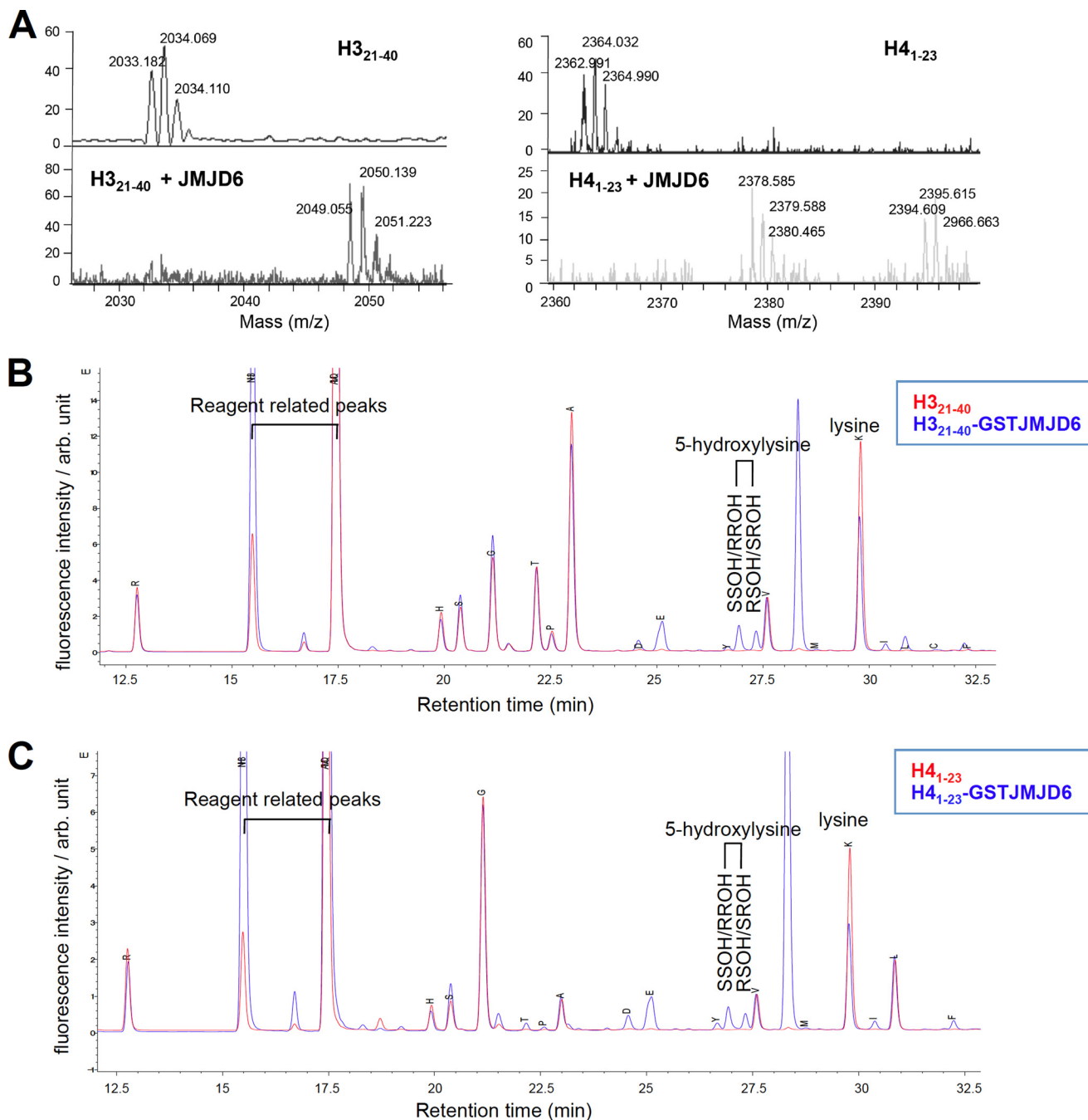


FIGURE 3. **JMJD6 hydroxylates histone H3 and H4 peptides detected by amino acid composition analysis.** A, hydroxylation of H3<sub>21-40</sub> and H4<sub>1-23</sub> peptides by GST-JMJD6 was confirmed by MS analysis. B and C, results of amino acid composition analysis of H3<sub>21-40</sub> (B) and H4<sub>1-23</sub> (C) peptides treated with (blue) or without (red) GST-JMJD6. SSOH/RROH, 2S,5S/2R,5R-hydroxylysine; RSOH/SROH, 2R,5S-/2S,5R-hydroxylysine; arb. unit, arbitrary units.

the presence of acetyl-coenzyme A (CoA, Sigma-Aldrich) at 37 °C. The reaction was stopped by adding 100  $\mu$ l of quenching buffer (3.2 M guanidinium HCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> (pH 6.8)) at the indicated times. Following this, 50  $\mu$ l of 2 mM 5,5'-dithiobis-2-nitrobenzoic acid (Sigma-Aldrich) in 100 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> (pH 6.8) was added, and absorbance at 405 nm was read by a spectrophotometer (ARVO MX/Light 1420 Multilabel/Luminescence Counter, PerkinElmer Life Sciences). The transfer of an acetyl group from an acetyl-CoA to the  $\epsilon$ -amino group of lysine residues was quantified by measurement of the thiol group of CoA. A standard curve was generated using  $\beta$ 2-mercaptoethanol.

*In Vitro Histone Methyltransferase Assay*—The *in vitro* histone methyltransferase assay was performed as described previously (10), except for slight modifications. In brief, a fixed amount of purified baculovirus-produced recombinant SMYD3 (1  $\mu$ M) was incubated with indicated histone peptides, which were also used for the *in vitro* HAT assay, and 1  $\mu$ Ci of *S*-adenosyl-L-methionine (AdoMet; GE Healthcare) as the methyl donor in a mixture of 60  $\mu$ l of methylase activity buffer (50 mM Tris-HCl (pH 8.5), 100 mM NaCl, 10 mM dithiothreitol) at 30 °C. The incorporated <sup>3</sup>H-labeled methyl groups in the substrates were measured by a scintillation counter after filter binding (units, cpm). The concentration

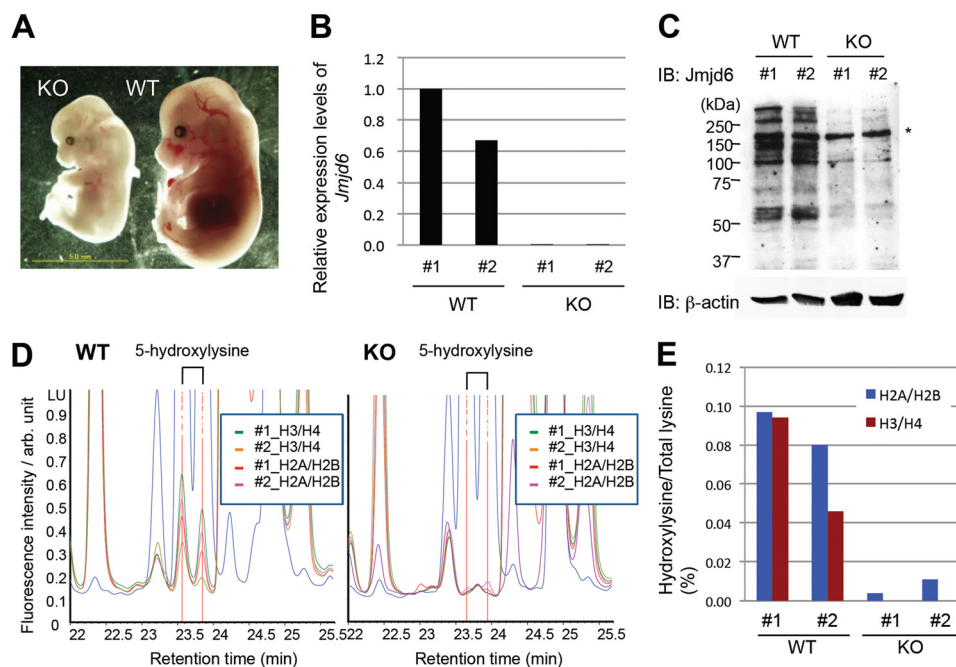


FIGURE 4. JMJD6 hydroxylates histones H2A/H2B and H3/H4 in mouse embryos. A, a representative image of JMJD6 knock-out and wild-type E14.5 embryos. B, JMJD6 knock-out was confirmed by qRT-PCR. GAPDH was used as an internal control. C, JMJD6 knock-out was confirmed by Western blotting. The asterisk indicates a nonspecific band.  $\beta$ -Actin was used as a loading control. D, result of amino acid composition analysis of histones derived from two Jmjd6 wild-type (left) and knock-out (right) E14.5 embryos. E, % of 5-hydroxylysine in total lysine of histones H2A/H2B (blue) and H3/H4 (red) was calculated from the HPLC data (D). IB, immunoblot; arb. unit, arbitrary units.

(nM) of the methylated substrate was calculated based on the basis of radioactivity.

## RESULTS

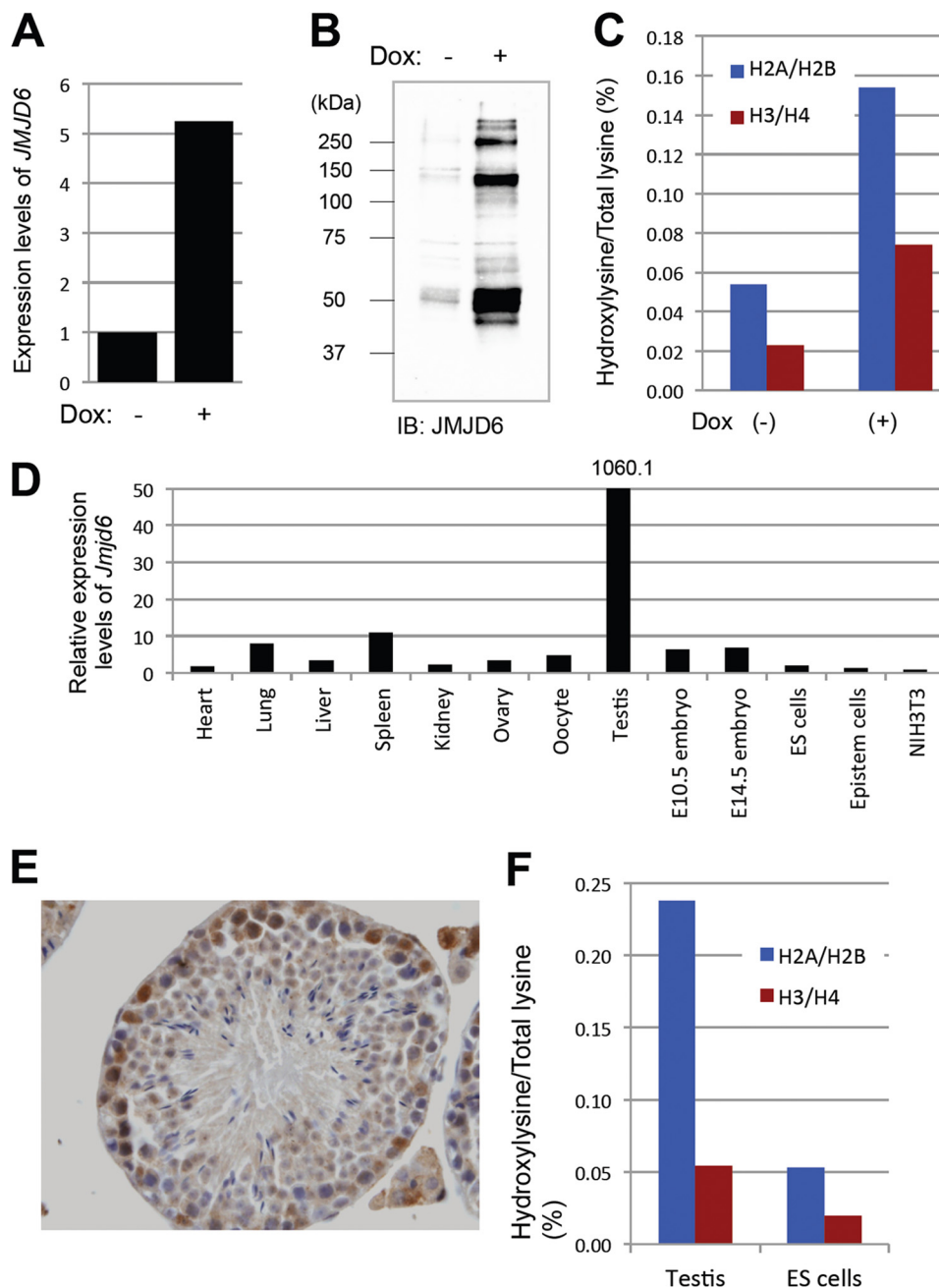
**JMJD6 Effectively Hydroxylates Histone Lysyl Residues *In Vitro***—During screening of UHRF1-interacting proteins, we identified JMJD6 as a novel binding partner of UHRF1 (data not shown). Because UHRF1 recognizes hemimethylated DNA and histone modifications, we assumed that JMJD6 might be recruited by UHRF1 to nucleosomes and modify histone lysyl residues. *In vitro* experiments showed that recombinant GST-JMJD6 possessed the ability to bind to histone H3<sub>1–20</sub> tail and histone H4 (Fig. 1, A and B) and hydroxylate multiple lysyl residues in the N-terminal tails of histone H3<sub>1–20</sub> and H4<sub>1–30</sub>, which was detected as of 16, 32, or 48 Da shifts by MS analysis (Fig. 1, C and D); subsequent MS/MS analysis revealed that JMJD6 mediates monohydroxylation of lysyl residues. As indicated by Webby *et al.* (1), JMJD6 preferentially hydroxylated lysyl residues in the basic peptides, and no apparent sequence preference was observed *in vitro* (data not shown).

Next, we established a sensitive hydroxylysine detection method based on amino acid composition analysis as an alternative to the MS-based method. For amino acid composition analysis, we briefly hydrolyzed peptides or proteins with HCl and separated each amino acid residue by reversed phase HPLC to detect 5-hydroxylysine. To evaluate this method, we first performed reversed phase HPLC using simplicial synthetic 2*S*,5*R*-hydroxylysine and synthetic racemic mixture of 5-hydroxylysine composed of 2*S*,5*S* (SS)-, 2*R*,5*R* (RR)-, 2*R*,5*S* (RS)-, and 2*S*,5*R* (SR)-stereoisomers (Fig. 2A). We detected two peaks corresponding to SS/RR- and RS/SR-hydroxylysine by analyzing these synthetic 5-hydroxylysines without HCl treatment

(Fig. 2A). After HCl treatment of these synthetic 5-hydroxylysines, another peak was appeared (Fig. 2A, arrow). This peak possibly corresponds to a lactone derivative, 3-amino-6-(aminomethyl)oxan-2-one, generated by dehydration condensation between C5 hydroxyl group and carboxyl group, which is described in a previous report (11). Next, we evaluated the method using unmodified H4<sub>1–23</sub> peptides and 5-hydroxylysine containing H4<sub>1–23</sub> peptides in which all the lysines at positions 5, 8, 12, and 20 were substituted with 5-hydroxylysines. After hydrolysis of these peptides, we detected two peaks corresponding to SS/RR- and RS/SR-hydroxylysine only in the 5-hydroxylysine containing peptides but not in the unmodified peptides (Fig. 2, B and C). We also detected the peak of the possible lactone derivative in the 5-hydroxylysine containing peptides by reversed phase HPLC performed in the same day of hydrolysis, but the peak disappeared in the next day of hydrolysis, indicating that the derivative is unstable. Because quantification of the derivative is technically difficult, we only quantified SS/RR- and RS/SR-hydroxylysine.

Using this method, we analyzed H3<sub>21–40</sub> and H4<sub>1–23</sub> peptides treated with or without recombinant GST-JMJD6. First, we confirmed hydroxylation of the peptides by GST-JMJD6 by MS analysis (Fig. 3A). Then, the peptides were separated from the enzyme reaction mixture, by reversed phase HPLC. The separated peptides were treated with HCl, and each amino acid residue was separated by reversed phase HPLC (Fig. 3, B and C). Comparison of the chromatograph between amino acids derived from the JMJD6-treated and -untreated peptides identified two additional peaks in the peptides treated with JMJD6, which are matched with the standard synthetic 5-hydroxylysine (Fig. 3, B and C).

## JMJD6 Hydroxylates Histone Lysyl Residues

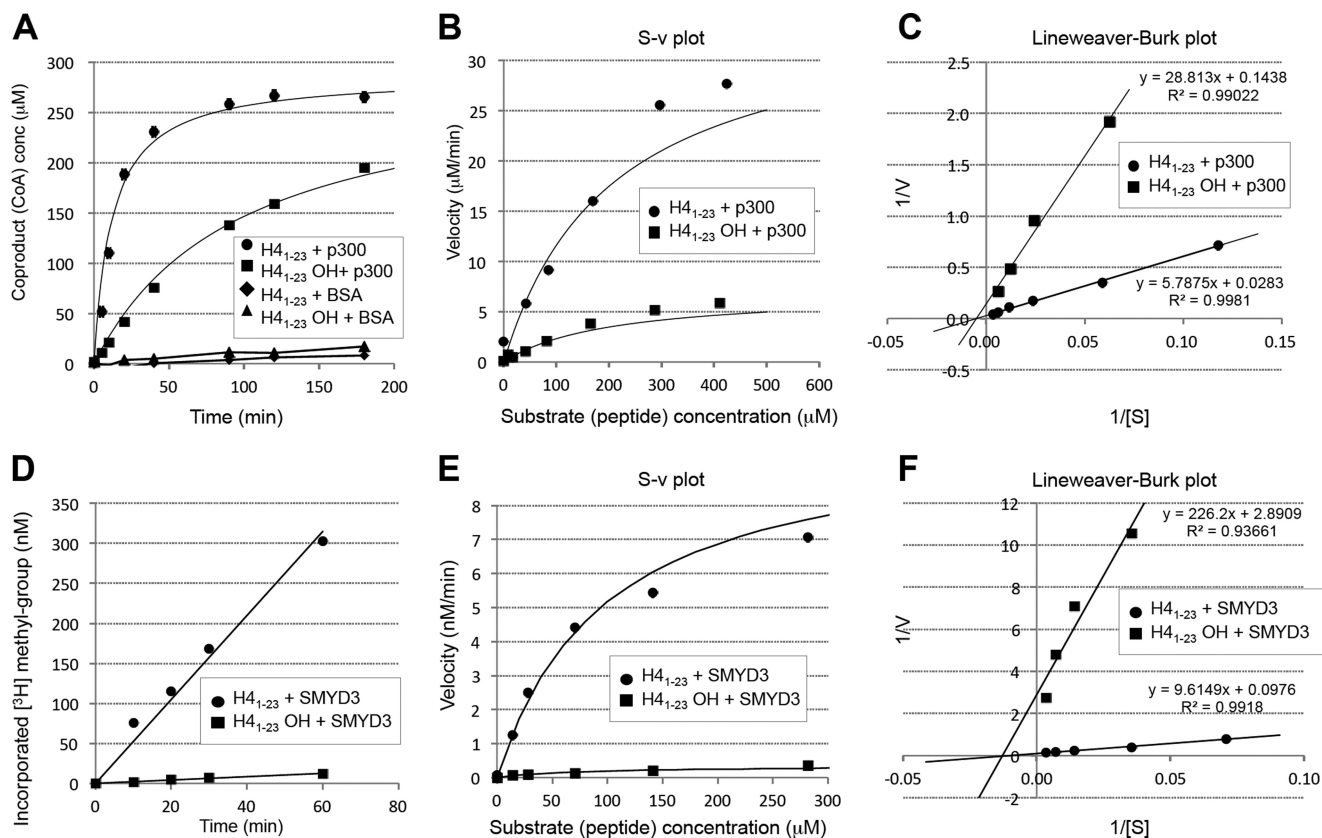


**FIGURE 5. Amount of 5-hydroxylysine in JMJD6 overexpressed HEK 293 cells, mouse testis, and ES cells.** *A*, expression levels of *JMJD6* in Dox-inducible *JMJD6* stable cells were examined by qRT-PCR before and after 48-h Dox induction. *B*, induction of *JMJD6* by Dox in the cells was confirmed by Western blotting. *C*, amino acid composition analysis of histones derived from Dox-inducible *JMJD6* stable cell lines. The blue and red bars indicate % of 5-hydroxylysine in the total lysine of histone H2A/H2B and in the H3/H4, respectively. *D*, relative expression levels of *Jmjd6* in various mouse tissues and cells were examined by qRT-PCR. *E*, expression of *Jmjd6* in a 6-month-old mouse testis was examined by immunohistochemistry. *F*, amino acid composition analysis of histones derived from 6-month-old mouse testis and J1 ES cells. The blue and red bars indicate % of 5-hydroxylysine in the total lysine of histone H2A/H2B and in the H3/H4, respectively.

*JMJD6 Hydroxylates Histone Lysyl Residues in Vivo*—To investigate histone lysyl hydroxylation *in vivo*, we performed the amino acid composition analysis for analyzing a mixture of histone H2A/H2B and a mixture of histone H3/H4 proteins isolated from two *JMJD6* wild-type and two *JMJD6* knock-out whole embryos at E14.5 (Fig. 4*A*). *JMJD6* knock-out was confirmed by qRT-PCR and Western blotting (Fig. 4*B* and *C*). The results showed that 0.097 and 0.080% of total lysyl residues in histone H2A/H2B and 0.094 and 0.046% of those in histone H3/H4 were 5-hydroxylated in each of the two *JMJD6* wild-type

mice (Fig. 4, *D* and *E*), whereas 0.004 and 0.011% of total lysyl residues in histone H2A/H2B and 0.000 and 0.000% of those in histone H3/H4 were 5-hydroxylated in each of the two *JMJD6* knock-out mice (Fig. 4, *D* and *E*), indicating that *JMJD6* hydroxylates histone lysyl residues *in vivo*.

We also generated Dox-inducible *JMJD6* stable HEK293 cells. *JMJD6* induction by Dox was confirmed by qRT-PCR and Western blotting (Fig. 5, *A* and *B*) and increased 5-hydroxylation levels of histone lysyl residues (Fig. 5*C*). In addition, we purified histones from a 6-month-old *JMJD6* wild-type mouse



**FIGURE 6. 5-Hydroxylation of lysyl residue impairs *N*-acetylation and *N*-methylation *in vitro*.** A–C, the *in vitro* colorimetric HAT assay was performed using a fixed amount of p300 (0.44  $\mu\text{M}$ ) and control H4<sub>1-23</sub> peptides (●) or 5-hydroxylysine-containing peptides (H4<sub>1-23</sub> OH, ■). BSA was used as a negative control (◆, ▲). After the reactions, absorbance (405 nm) of the coproduct (CoA) was measured. A, reactions were terminated at the indicated time points, and the concentration of CoA was calculated on the basis of a standard curve that was generated from  $\beta$ -2-mercaptoethanol. B and E, substrate concentration-velocity (s-v) plot. C and F, Lineweaver-Burk plot. The vertical axis is 1/velocity [v], and the horizontal axis is 1/substrate concentration [S]. D–F, the *in vitro* histone methyltransferase assay was performed using a fixed amount of SMYD3 (1  $\mu\text{M}$ ) and control H4<sub>1-23</sub> peptides (●) or 5-hydroxylysine-containing peptides (H4<sub>1-23</sub> OH, ■). AdoMet was used as a methyl donor. After the reactions, radioactivity (cpm) of the <sup>3</sup>H-methylated substrates was measured. The concentration of incorporated <sup>3</sup>H-methyl groups (nm) was calculated based on the basis of radioactivity (1 cpm was 0.02563 nm in the reaction). D, reactions were performed with fixed amounts of the peptides (141  $\mu\text{M}$ ) and terminated at the indicated time points.

testis, which expressed JMJD6 at the highest level among various tissues and cells (Fig. 5, D and E). In the testis, 0.238 and 0.054% of total lysyl residues in histone H2A/H2B and H3/H4, respectively, were 5-hydroxylated (Fig. 5F). In the mouse J1 ES cells, 0.053 and 0.020% of total lysyl residues in histone H2A/H2B and H3/H4, respectively, were 5-hydroxylated (Fig. 5F).

**5-Hydroxylation Prevents *N*-Acetylation and *N*-Methylation of Histone Lysyl Residues *in Vitro***—Because lysyl residues in histone tails are often subjected to *N*-acetylation and *N*-methylation, we examined whether 5-hydroxylation of lysyl residues affects modifications at the  $\epsilon$ -amino groups. First, we examined the effect of lysyl 5-hydroxylation on histone H4 *N*-acetylation by p300, which catalyzes *N*-acetylation of the  $\epsilon$ -amino group of lysyl residues, including histone H4K5 and H4K8, through its HAT domain (12). Kinetic analysis using the unmodified and the 5-hydroxylysine containing H4<sub>1-23</sub> peptides in which all the lysines were substituted with 5-hydroxylysines as substrates revealed that 5-hydroxylation largely interfered with the HAT activity of p300 *in vitro* (Fig. 6, A–C). Lineweaver-Burk plot analysis was performed to calculate the maximum velocity ( $V_{max}$ ) and Michaelis constant ( $K_m$ ) values (Table 1; Fig. 6C,  $R^2 = 0.9981$  and 0.9902).  $V_{max}$  of the reactions in which p300 acetylated the 5-hydroxylysine-containing peptides (H4<sub>1-23</sub>OH) was 5-fold

**TABLE 1**

**Effect of 5-hydroxylation on *N*-acetylation of  $\epsilon$ -amino group of lysyl residues**

Lineweaver-Burk plots were used for estimation of the kinetic constants,  $V_{max}$ , and  $K_m$ .  $R^2$  is the determination coefficient (see Fig. 6C).

	$V_{max}$	$K_m$
	$\mu\text{M}/\text{min}$	$\mu\text{M}$
H4 <sub>1-23</sub> + p300	35.34 ± 1.65 ( $R^2 = 0.9981$ )	204.51 ± 10.12
H4 <sub>1-23</sub> OH + p300	6.95 ± 0.45 ( $R^2 = 0.9902$ )	200.37 ± 14.32

**TABLE 2**

**Effect of 5-hydroxylation on *N*-methylation of  $\epsilon$ -amino group of lysyl residues**

Lineweaver-Burk plots were used for estimation of the kinetic constants,  $V_{max}$ , and  $K_m$ .  $R^2$  is the determination coefficient (see Fig. 6F).

	$V_{max}$	$K_m$
	nm/min	$\mu\text{M}$
H4 <sub>1-23</sub> + SMYD3	10.90 ± 0.92 ( $R^2 = 0.9918$ )	80.63 ± 16.26
H4 <sub>1-23</sub> OH + SMYD3	0.48 ± 0.26 ( $R^2 = 0.9366$ )	75.26 ± 9.60

less than that of the control peptides (6.95 ± 0.45 and 35.34 ± 1.65  $\mu\text{M}/\text{min}$ , respectively), whereas  $K_m$  of the two reactions was quite similar (204.51 ± 10.12 and 200.37 ± 14.32  $\mu\text{M}$ , respectively), indicating that 5-hydroxylation does not inhibit binding of lysyl residues to p300 but reduces the catalytic efficiency.



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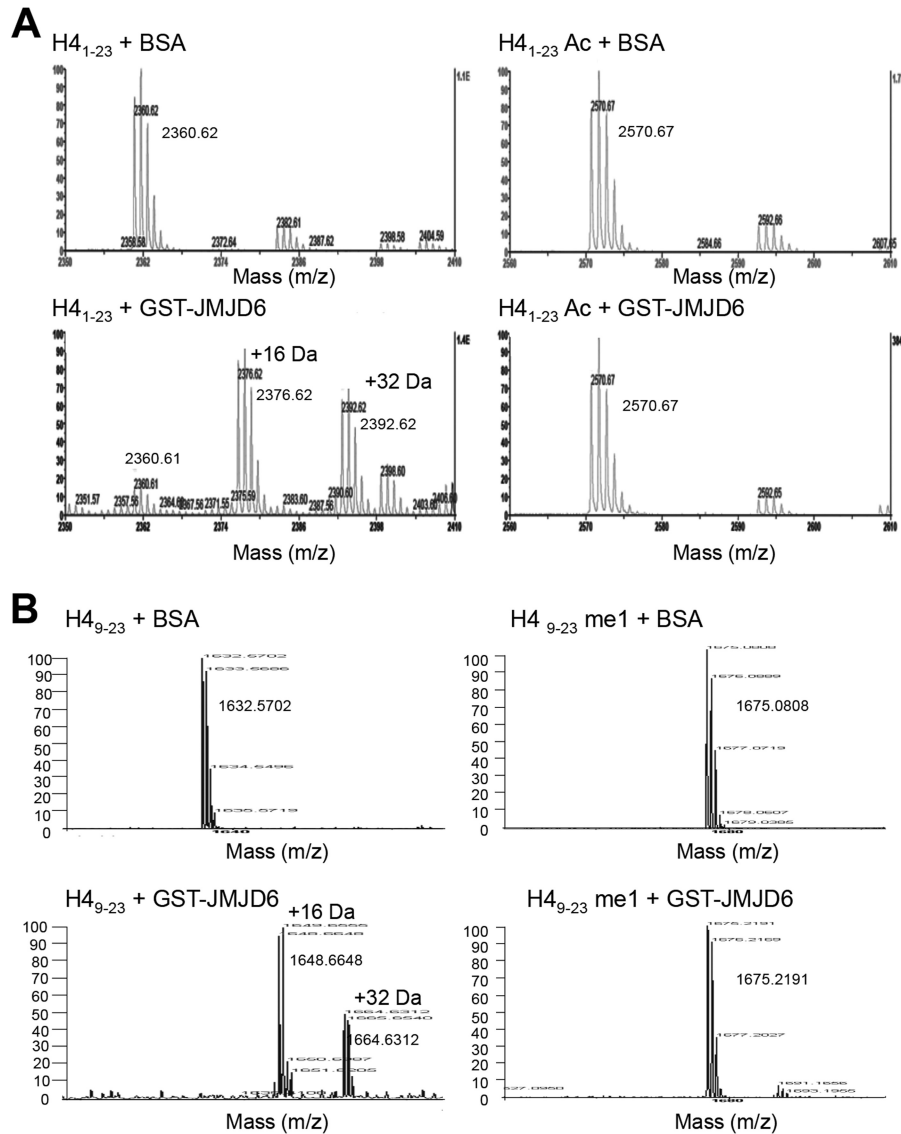


FIGURE 7. **N**-Acetylation and **N**-methylation of lysyl residues impairs 5-hydroxylation by JMJD6 *in vitro*. **A**, the *in vitro* hydroxylation assay was performed using GST-JMJD6 (10  $\mu$ M) and 85  $\mu$ M of control H4<sub>1-23</sub> peptides or *N*-acetyl-lysine-containing peptides. **B**, the *in vitro* hydroxylation assay was performed using GST-JMJD6 (10  $\mu$ M) and 85  $\mu$ M control H4<sub>1-23</sub> peptides or *N*-monomethyl-lysine-containing peptides. BSA was used as a negative control. 5-Hydroxylation by JMJD6 was detected by MS analysis.

We also examined the effect of lysyl 5-hydroxylation on the histone methyltransferase activity of SMYD3, which catalyzes lysyl *N*-methylation of histone H3 (13) and also H4 (data not shown) through its SET (su(var) 3-9 enhancer-of-zeste trithorax) domain by the histone methyltransferase assay. The results showed that 5-hydroxylation at lysyl residues almost completely inhibited *N*-methylation catalyzed by SMYD3 (Table 2 and Fig. 6, *D–F*);  $V_{\max}$  values of the reactions with the control peptides (H4<sub>1-23</sub>) and the 5-hydroxylysine-containing peptides (H4<sub>1-23</sub>OH) as substrates were  $10.90 \pm 0.92$  and  $0.48 \pm 0.26$  nm/min, respectively. Similar to the HAT assay,  $K_m$  values of the two reactions were  $\sim 80.63 \pm 16.26$  and  $75.26 \pm 9.60$   $\mu$ M, respectively.

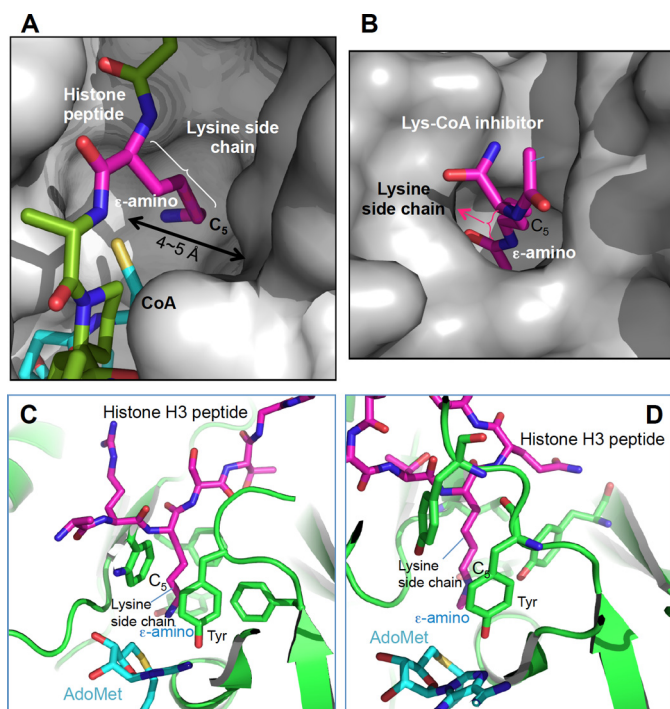
Subsequently, we performed reciprocal experiments using H4<sub>1-23</sub> or H4<sub>9-23</sub> peptides, in which all the lysines are either unmodified, *N*-acetylated, or *N*-monomethylated. JMJD6 effectively hydroxylated the control peptides (Fig. 7, *A* and *B*, left

panels); however, *N*-acetylation and *N*-monomethylation at the  $\epsilon$ -amino group of the lysines completely blocked 5-hydroxylation by JMJD6 (Fig. 7, *A* and *B*, right panels).

## DISCUSSION

We found a novel histone modification, 5-hydroxylation, by JMJD6. JMJD6 reportedly hydroxylates a splicing factor, U2AF65 (1). That study and another report (1, 14) stated that evidence of histone lysyl hydroxylation was not found by MS-based analysis *in vivo*. In the present study, we developed an alternative method, amino acid composition analysis, to detect 5-hydroxylation of histone lysyl residues. As reported previously, we have not detected clear evidence of 5-hydroxylation of histone lysyl residues by MS-based analysis. We think that there are several causes for this. 1) The amount of 5-hydroxylysine is too small to detect by MS-based analysis. 2) Artificial methionine oxidation during preparation of samples for MS analysis





**FIGURE 8. Structure around the active site of HAT domains and SET domains, a lysine side chain, and an S-adenosyl methionine (AdoMet).** *A*, spatial localization among the HAT domain of GCN5 (gray), a lysine side chain (magenta), and CoA (cyan) (Protein Data Bank code 1QSN). The 5-hydroxyl group may locate close to acetyl-CoA, indicating that this may work as a steric barrier and prevent effective *N*-acetylation by the catalytic domain. *B*, structure of the HAT domain of p300 (gray) in complex with inhibitor, Lys-CoA (magenta) (Protein Data Bank code 3BLY). The 5-hydroxyl group may restrict the conformation of lysine side chain in the catalytic pocket of p300. The figures were prepared by using program PyMOL. *C*, spatial localization among the SET domain of *N. crassa* Dim-5 (green), a histone H3 peptide (magenta), and AdoMet (cyan) (Protein Data Bank code 1PEG). *D*, spatial association among the SET domain of human SETD7/9 (green), a histone H3 peptide containing monomethylated Lys-4 (magenta), and AdoMet (cyan) (Protein Data Bank code 1O9S). A side chain of a lysine residue locates in tightly hydrophobic pocket of the SET domains (*C* and *D*). Hydroxylation at position C<sub>5</sub> of the chain may prevent a lysine side chain to locate in the pocket, causing inhibition of *N*-methylation by SET domains.

makes detection of 5-hydroxylysine difficult. 3) 5-Hydroxylysine could be an intermediate form as it is in collagen, and a further unknown modification(s) such as glycosylation could be added; the final product of collagen is glucosylgalactosyl hydroxylysine (4). The collagen hydroxylase, PLOD3, possesses galactosyltransferase and glucosyltransferase activities. Unlike PLOD3, JMJD6 does not appear to possess any other enzymatic activities by domain search; therefore, it is difficult to assume possible further modification(s) by its protein structure. We may have been able to detect 5-hydroxylation in histone lysyl residues by amino acid composition analysis but not by the MS-based analysis because many modifications such as glycosylation or galactosylation could be removed during the hydrolysis process of amino acid composition analysis. By this analysis, we detected both *SS/RR*- and *SR/RS*-hydroxylysine in JMJD6-treated histone peptides and also in JMJD6 wild-type E14.5 embryos, ES cells, and the Dox-inducible JMJD6 stable HEK293 cells. Because the relationship between *RR* and *RS* and also between *SS* and *RS* is a diastereomer, we were able to distinguish them. However, because a relationship between *SS* and *RR*, and also between *RS* and *SR* is an enantiomer, we were not

able to separate them by this method. Despite this, these two peaks are most likely *SS*- and *RS*-hydroxylysine because JMJD6 is reported to generate *SS*-hydroxylysine (11). The *RS*-hydroxylysine could be generated from *SS*-hydroxylysine through the lactone derivative, 3-amino-6-(aminomethyl)oxan-2-one, which is unstable and difficult to be quantified. Because of this difficulty, we only quantified *SS/RR*- and *RS/SR*-hydroxylysine in this report. Therefore, actual quantity of 5-hydroxylysine in the samples examined here could be a little higher.

Because we detected 5-hydroxylysines in the UHRF1 KO ES cells (data not shown), UHRF1 is not required for 5-hydroxylation of histone lysyl residues by JMJD6. Therefore, biological significance of the interaction between UHRF1 and JMJD6 remains unclear. Further analysis is also required to determine the biological significance of 5-hydroxylation of histone lysyl residues. *In vitro* experiments suggest that 5-hydroxylation can inhibit *N*-acetylation and *N*-methylation by p300 and SMYD3. The active site structure of the p300 and general control of amino acid synthesis 5 (GCN5) HAT domains showed that the side chain of the 5-hydroxylysine can invade the catalytic pocket; however, the 5-hydroxyl group may disturb active form formation of the substrate (Fig. 8, *A* and *B*). The catalytic site of SET domains of *Neurospora crassa* Dim-5 and human SETD7, which are structurally similar to SMYD3 (15), suggested that the side chain of 5-hydroxylysine can invade the catalytic pocket; however, the 5-hydroxyl group may disturb an active form formation of the substrate (16, 17) similar to that in HAT domains (Fig. 8, *C* and *D*). Therefore, 5-hydroxylation could be important in the context of the histone code. It is known that histones H2A and H2B move more dynamically between the nucleosome and nucleoplasm. 5-Hydroxylation of these histones may have some effects for the movement because the modification was detected more in histones H2A/H2B than in histones H3/H4. The expression pattern of JMJD6 is also interesting. JMJD6 may play important role(s) in the testis, such as a role in histone-protamine exchange. We believe that our present finding provides a novel insight into epigenetic regulations of gene transcription and/or chromosomal rearrangement.

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