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The Hydroxylation Activity of Jmjd6 Is Required for its Homo-Oligomerization

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ABSTRACT

Jumonji C-terminal (JmjC) domain-containing proteins are protein hydroxylases and histone demethylases that control gene expression. Jumonji domain-containing protein 6 (Jmjd6) is indispensable for embryonic development and has both histone arginine demethylase and lysyl-hydroxylase activities. The protein undergoes post-translational homo-oligomerization, but the underlying mechanism remains unknown. In this study, we examined the enzymatic activity of Jmjd6 and uncovered the mechanism underlying its homo-oligomerization. An in vitro enzymatic assay monitored by matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry indicates that Jmjd6 is unable to remove the methyl group from histone arginine residues but can hydroxylate the histone H4 tail at lysine residues in a 2-oxoglutarate (2-OG)- and Fe (II)-dependent manner. A mutational analysis reveals that the homo-oligomerization of Jmjd6 requires its enzymatic activity and the N- and C-termini. Using an in vitro enzymatic assay, we further demonstrate that Jmjd6 can hydroxylate its N-terminus but not its C-terminus. In summary, we did not detect arginine demethylase activity for Jmjd6, but we did confirm that it could catalyze the lysyl-hydroxylation of histone peptides. In addition, we demonstrated that the homo-oligomerization of Jmjd6 requires its own enzymatic activity and the N- and C-termini. We propose that Jmjd6 forms intermolecular covalent bonds between its N- and C-termini via autohydroxylation. J. Cell. Biochem. 113: 1663–1670, 2012.

KEY WORDS: Jmjd6; ARGININE DEMETHYLATION; HYDROXYLATION; HOMO-OLIGOMERIZATION

Histone methylation plays an important role in the epigenetic control of gene expression [Strahl and Allis, 2000; Bhaumik et al., 2007; Kouzarides, 2007]. Histones can be methylated at both lysine residues with 1, 2, or 3 methyl groups and arginine residues with 1 or 2 methyl groups in a symmetric or asymmetric manner [Bannister and Kouzarides, 2005; Bedford and Richard, 2005]. Histone lysine methylation is dynamically regulated by methyl transferases and histone demethylases [Martin and Zhang, 2005; Shi, 2007]. Two classes of histone lysine demethylases have been identified: the lysine-specific demethylase 1 (LSD1) family, which can only demethylate mono- or dimethylated lysines, and the Jumonji C-terminal (JmjC) domain-containing protein family, which can demethylate mono-, di-, and trimethylated lysines [Mosammaparast and Shi, 2010]. The demethylation reaction catalyzed by the former requires flavin adenine dinucleotide (FAD) as a cofactor and releases formaldehyde as a byproduct [Shi et al., 2004]. The latter catalyzes the hydroxylation of the methyl group coupled to the oxidative decarboxylation of 2-oxoglutarate (2-OG), forming an unstable hydroxymethyl ammonium intermediate, which is released as formaldehyde [Klose et al., 2006]. However, whether these mechanisms also take place on arginine residues has long been of interest.

Abbreviations used: JmjC domain, Jumonji C-terminal domain; Jmjd6, Jumonji domain-containing protein 6; MALDI-TOF MS, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry; 2-OG, 2-oxoglutarate; LSD1, lysine-specific demethylase 1; FAD, flavin adenine dinucleotide; U2AF65, splicing factor U2 small nuclear ribonucleoprotein auxiliary factor 65-kDa subunit; Flt1, VEGF-receptor 1; N-OG, *N*-oxalylglycine; FACS, fluorescence-activated cell sorting; RP-HPLC, reversed phase high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; LOX, lysyl oxidase; TG, transglutaminase; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Jumonji domain-containing protein 6 (Jmjd6) was originally identified as a membrane protein and putative receptor for phosphatidylserine, which plays a role in phagocytosis [Fadok et al., 2000]. Studies in knockout mice indicated that Jmjd6 is indispensable for embryonic development because Jmjd6-deficient mice die perinatally [Li et al., 2003; Bose et al., 2004; Kunisaki et al., 2004]. However, contradictory findings regarding the localization and function of this protein have since been published. Several groups reported that Jmjd6 is actually localized to the nucleus instead of the plasma membrane [Cikala et al., 2004; Cui et al., 2004; Hahn et al., 2010]. In addition, knockout mice did not show impaired phagocytosis [Bose et al., 2004]. Moreover, in 2007, Jmjd6 was reported to possess a histone arginine demethylase activity [Chang et al., 2007]. However, the histone arginine demethylase activity could not be reproduced in another study, which instead revealed that Jmjd6 has a hydroxylase activity that adds a hydroxyl group at the 5-C of a lysine side chain of the splicing factor U2 small nuclear ribonucleoprotein auxiliary factor 65-kDa subunit (U2AF65) [Webby et al., 2009]. Silencing of Jmjd6 impairs angiogenic functions in endothelial cells by altering the splicing of VEGF-receptor 1 (Flt1) [Boeckel et al., 2011]. These results indicate that Jmjd6 is a nuclear protein and plays an essential role in embryonic development. However, the biochemical function of the protein requires further investigation.

The intermolecular cross-linking of collagens, which determines the mechanical strength of collagen fibrils, was discovered many years ago [Prockop and Kivirikko, 1995; Myllyharju and Kivirikko, 2004]. However, the cross-linking of nuclear proteins has rarely been studied. Although Jmjd6 undergoes oligomerization [Tibrewal et al., 2007; Hahn et al., 2010], the molecular mechanism remains unknown.

Here, by examining the catalytic activity of Jmjd6, we demonstrate that this protein can hydroxylate the histone H4 tail in a 2-OG- and Fe (II)-dependent manner but that it fails to demethylate dimethylated H4R3 in vitro. Importantly, we demonstrate that the enzymatic activity of Jmjd6 is required for its homo-oligomerization, which represents a new biochemical function of the JmjC domain.

MATERIALS AND METHODS

REAGENTS

The peptides used were biotin-conjugated H4 1–21 (Upstate 12-405), H3 1–21 (Upstate 12-403), H3 21–44 (Upstate 12-404), H2A 1–22 (Upstate 12-406), H2B 1–21 (Upstate 12-407), and H2B 21–41 (Upstate 12-408). The symmetric or asymmetric dimethyl histone H4R3 peptides are gifts from Dr. Yang Shi (Harvard Medical School, USA). Unmodified H4 1–22, Jmjd6 1–23, 371–391, and 392–414 peptides were purchased from Abmart (Shanghai, China).

The Jmjd6 rabbit polyclonal antibody was purchased from Abcam (Ab10526), the Flag antibody was obtained from Sigma (F3165), the H4R3me2s antibody was purchased from Abcam (Ab5823), and the H3R2me2s antibody was obtained from Upstate (07-585).

The chemicals used are α-ketoglutaric acid disodium salt dehydrate (Sigma Cat. #75892), ascorbic acid (Sigma Cat. #A2218), ammonium iron (II) sulfate hexahydrate (Sigma Cat. #F1543), and Ni-NTA agarose (Qiagen Cat. #30210).

CLONING PROCEDURE

The open reading frame of human Jmjd6 was PCR amplified and cloned into an *Nhe*I/*Hin*dIII-digested pET28(a) vector (Novagen) for His-tag recombinant protein expression and purification. For cellular expression, Flag-tag human Jmjd6 was PCR amplified and cloned into an *Nhe*I/*Bgl*II-digested pIRES2-EGFP vector (Clontech). Mutations and deletions were generated by PCR and cloned into either the pET28(a) or pIRES2-EGFP vector. Please refer to Supplementary Table I for detailed information.

RECOMBINANT PROTEIN PURIFICATION

Rosetta *E. coli* transformed with the His-Jmjd6 plasmid was cultured at 37°C and 250 rpm. Protein expression was induced with 0.1 mM IPTG overnight at 16°C when the OD reached 0.6. The cells were collected, re-suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0), and sonicated 25 times at 200 W (sonicated for 2 s and paused for 12 s). The supernatant was loaded onto a lysis buffer-equilibrated Ni-NTA column and washed with buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8.0), buffer 2 (50 mM NaH_2PO_4, 300 mM NaCl, and 40 mM imidazole, pH 8.0), buffer 3 (50 mM NaH_2PO_4, 300 mM NaCl, and 60 mM imidazole, pH 8.0), and buffer 4 (50 mM NaH_2PO_4, 300 mM NaCl, and 100 mM imidazole, pH 8.0). The recombinant proteins were eluted with buffer 5 (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 8.0) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) analysis to determine the purity and concentration.

IN VITRO ENZYMATIC ACTIVITY ASSAY

The purified Jmjd6 recombinant protein was incubated with 200 ng of each synthetic peptide in reaction buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 50 μM $[NH_4]_2Fe[SO_4]_2$, 1 mM 2-OG, and 2 mM ascorbic acid] for 3 h at 37°C. The reaction mixtures were desalted using a Zip-tip and then subjected to assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis.

CELL TRANSFECTION

HEK293T cells were transfected with the pIRES2-EGFP-Jmjd6 plasmid using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. The cells were collected 48 h after the transfection and lysed with 1× SDS loading buffer for Western blot analysis.

RESULTS

Jmjd6 IS A 2-OG- AND Fe (II)-DEPENDENT LYSYL-HYDROXYLASE

Jmjd6 has both histone arginine demethylase and protein hydroxylase activities [Chang et al., 2007; Webby et al., 2009]. To examine its arginine demethylase activity, we expressed a His-tagged full-length human Jmjd6 protein in bacteria and purified to near homogeneity (Fig. 1A). The purified protein was

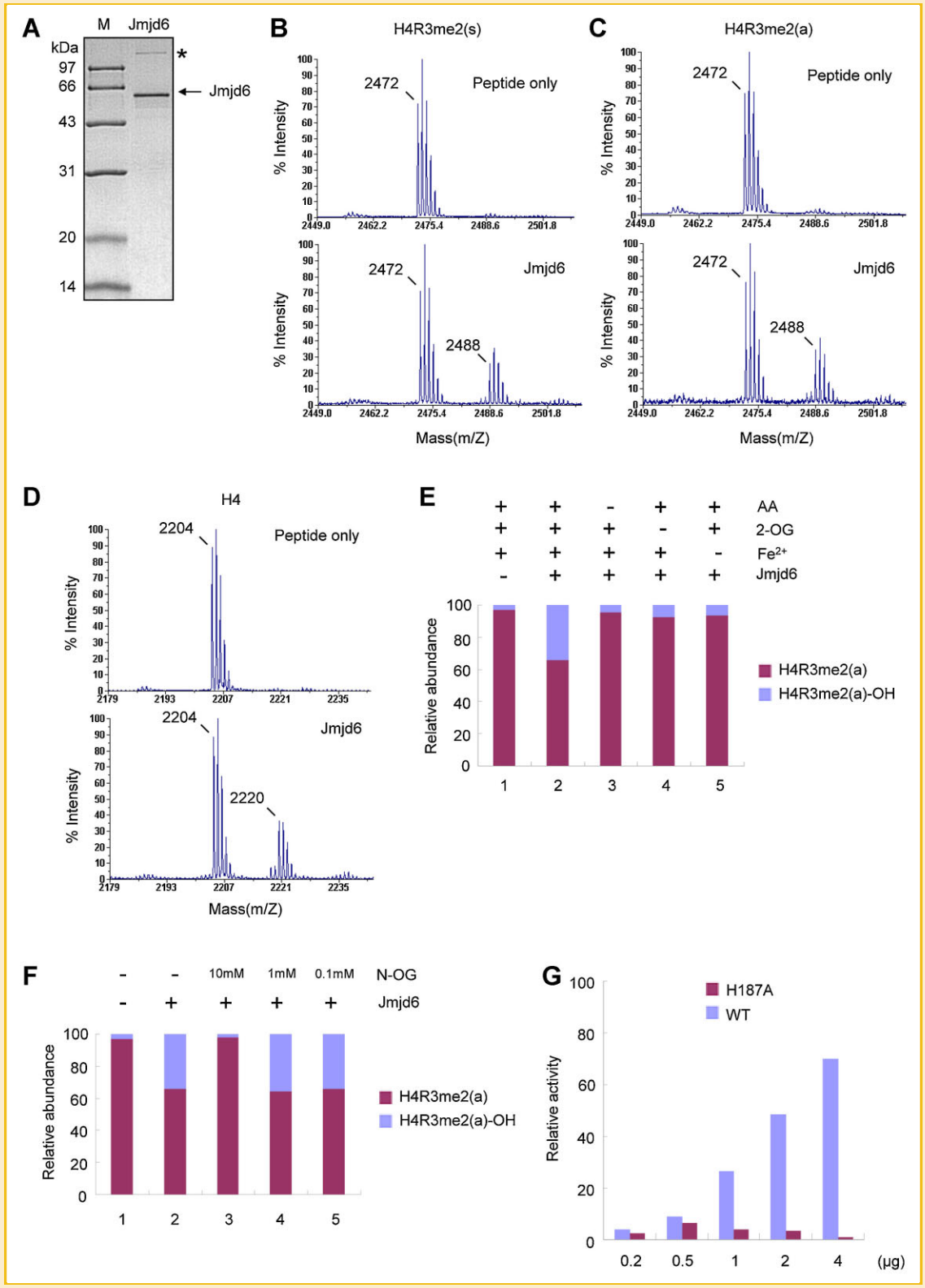

Fig. 1. Jmjd6 catalyzes histone H4 hydroxylation. A: Purified His-tagged recombinant human Jmjd6 was separated by SDS–PAGE and stained with Coomassie blue. An asterisk indicates a possible Jmjd6 dimer. B,C: A total of 200 ng of symmetrically (B) or asymmetrically (C) dimethylated H4R3 peptide was incubated with (lower panel) or without (upper panel) 3 μg of the recombinant Jmjd6 protein in the presence of 2 mM of ascorbic acid, 1 mM of 2-OG, and 50 μM of Fe (II), and then subjected to MALDI-TOF MS analysis. D: A total of 200 ng of unmodified H4 1–22 peptide was incubated with (lower panel) or without (upper panel) 3 μg of Jmjd6 and subjected to MALDI-TOF MS analysis. E: A total of 200 ng of asymmetrically dimethylated H4R3 peptide was incubated with (lanes 2–5) or without (lane 1) 3 μg of Jmjd6 in the presence of the indicated cofactors and subjected to MALDI-TOF MS analysis. The blue and red bars represent the relative abundances of the product and substrate, respectively. F: A total of 200 ng of asymmetrically dimethylated H4R3 peptide was incubated with (lanes 2–5) or without (lane 1) 3 μg of Jmjd6 in the presence of all cofactors without (lane 2) or with decreased amounts of N-OG (lanes 3–5) and subjected to MALDI-TOF MS analysis. The blue and red bars represent the relative abundances of the product and substrate, respectively. G: A total of 200 ng of unmodified H4 peptide was incubated with increased amounts of recombinant WT or H187A Jmjd6 protein and then subjected to MALDI-TOF MS analysis. Relative activity was defined as the percentage of the hydroxylated product over the total amount of the substrate. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

incubated with a symmetrically or an asymmetrically dimethylated H4R3-containing peptide in the presence of 2-OG, Fe (II), and ascorbic acid as cofactors. The products were then analyzed by MALDI-TOF MS. Consistent with a previous study [Webby et al., 2009], we did not observe a demethylated product with a reduced molecular weight (MW) of 14 Da. Instead, a product with a shift of MW of +16 Da was detected when symmetrically and asymmetrically dimethylated H4R3-containing peptides were used as the substrates (Fig. 1B,C). These results suggest that Jmjd6 has a hydroxylase activity using histone H4 as the substrate, consistent with previously reported results [Chang et al., 2007; Webby et al., 2009]. To test whether the hydoxylase activity requires the methylation of the substrate, we used an unmodified histone H4 peptide as the substrate in an in vitro assay and found that the unmodified H4 peptide was also hydroxylated (Fig. 1D), suggesting that the substrate does not need to be methylated for the hydroxylation reaction.

Because the demethylase activity of JmjC domain-containing proteins requires 2-OG and Fe (II) as cofactors [Tsukada et al., 2006], we examined whether the same set of cofactors is required for the hydroxylase activity of Jmjd6. Indeed, Jmjd6 had a strong hydroxylase activity on histone H4 in the presence of 2-OG, Fe (II), and ascorbic acid (Fig. 1E, lane 2); the omission of any of the cofactors in the reaction diminished the hydoxylase activity (Fig. 1E, lanes 3–5). In addition, the hydroxylase activity was inhibited by *N*-oxalylglycine (N-OG), a 2-OG competitive inhibitor, in a dose-dependent manner (Fig. 1F). Furthermore, the hydroxylase activity was abolished when the conserved histidine 187 that is required for Fe (II) binding in the JmjC domain was mutated to alanine (Fig. 1G). These results indicate that the hydroxylase activity of Jmjd6 requires the same set of cofactors as the demethylase activity of other JmjC domain-containing proteins.

To identify the hydroxylation site, we performed MS/MS experiments after the in vitro reaction and compared matched y-ions from the parental H4 peptide and its hydroxylated product following MS fragmentation. Both of the peptides generated the same set of y-ions, from y4 to y14, but different sets of y-ions after y14 (Fig. 2A). Whereas y15–y19 exactly matched the expected fragment ions generated from the parental H4 peptide, each of those generated from the hydroxylated product had a shift in spectrum, with an addition of 16 Da. Because y15 corresponds to lysine 8 of histone H4 (Fig. 2B), these results indicate that lysine 8 is the hydroxylation site.

To determine whether other histone tails can be hydroxylated by Jmjd6, we performed an in vitro assay using other histone peptides. Although Jmjd6 could hydroxylate a peptide containing residues 1–21 of histone H4 (H4 1–21; Fig. 3A), it could not hydroxylate the other histone peptides examined, including residues 1–21 of H3 (H3 1–21), residues 21–44 of H3 (H3 21–44), residues 1–22 of H2A (H2A 1–22), and residues 1–21 of H2B (H2B 1–21; Fig. 3B–E). These results indicate that Jmjd6 specifically hydroxylates the histone tail of H4.

THE ENZYMATIC ACTIVITY IS REQUIRED FOR THE HOMO-OLIGOMERIZATION OF Jmjd6

Previous studies indicate that Jmjd6 could undergo post-translational homo-oligomerization [Tibrewal et al., 2007; Hahn et al., 2010]; however, the molecular mechanism was not elucidated. To begin to delineate the underlying molecular mechanism, we first confirmed the homo-oligomerization. When Flag-tagged Jmjd6 was

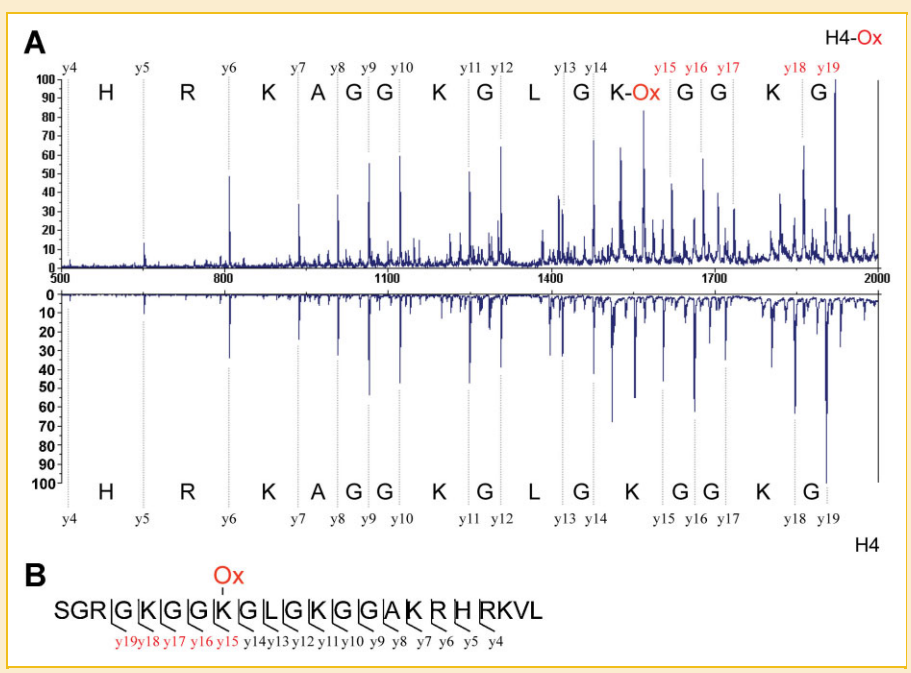

Fig. 2. Jmjd6 hydroxylates H4 at lysine 8. A: Unmodified or mono-hydroxylated H4 peptide was subjected to MS/MS analysis. B: Sequence of the histone H4 tail. All of the y-ions are marked, and those y-ions with a mass shift of +16 Da are indicated in red. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

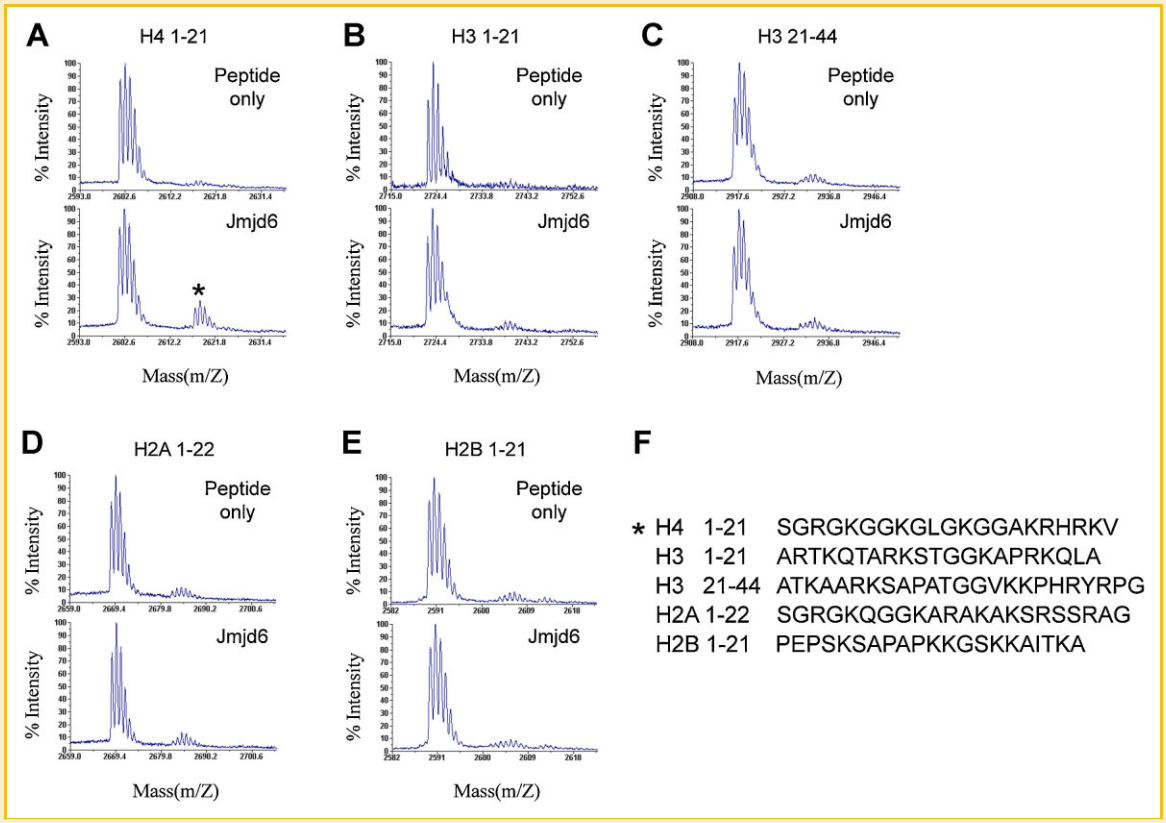

Fig. 3. Jmjd6 specifically hydroxylates the histone H4 tail. A–E: A total of 200 ng of various histone tail peptides were incubated with 3 μg of the recombinant Jmjd6 protein in the presence of all of the cofactors and then subjected to MALDI-TOF MS analysis. F: The amino acid sequences of histone tail peptides are shown. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

overexpressed in HEK293T cells and the lysates were subjected to Western blot analysis, we detected bands with MW of approximately 55, 110, 165, and 220 kDa using an antibody against the Flag epitope (Fig. 4A, upper panel, lane 3). The bands correspond to oligomerized products of 1, 2, 3, and 4 molecules of overexpressed Flag-Jmjd6. However, these bands were not detected when Jmjd6 without Flag was overexpressed (Fig. 4A, upper panel, lane 2), indicating the specificity of the anti-Flag antibody. When an antibody against Jmjd6 was used in the immunoblotting, we detected bands with MW of approximately 55, 110, 165, and 220 kDa in lysates from both Jmjd6 and Flag-tagged Jmjd6-overexpressing cells (Fig. 4A, lower panel, lanes 2 and 3). These results indicate that the exogenous Jmjd6 could oligomerize. More importantly, the similar pattern of bands with MW of approximately 55, 110, 165, and 220 kDa were also detected in the mock-transfected cells by the Jmjd6 antibody, and the intensity of each band was comparable (Fig. 4A, lower panel, lane 1). These results indicate that the endogenous Jmjd6 also oligomerizes in cells. In addition to the monomer and oligomerized products, we observed an additional band with a lower MW than the monomer of Jmjd6, which was previously suggested to be a cleavage product of Jmjd6 [Tibrewal et al., 2007; Hahn et al., 2010].

To examine whether hydroxylase activity is required for the formation of the oligomerized products with high MW, we mutated histidine 187 to alanine (H187A), a substitution that abolishes hydroxylase activity. The mutated Jmjd6 was overexpressed in HEK293T cells, and Western blot analysis was performed. Surprisingly, we only detected the monomer in the Jmjd6- or Flag-tagged Jmjd6-overexpressing cells when anti-Jmjd6 or anti-Flag antibodies were used; we did not detect the oligomerized products with high MW (Fig. 4A, lanes 4 and 5). These results suggest that hydoxylase activity is required for the homo-oligomerization of the protein.

The N- and C-termini of the Jmjd6 protein contain many lysine and arginine residues, similar to the histone tail that is the substrate for Jmjd6. To determine whether oligomerization takes place in the N- or C-terminus, we deleted either the N- or C-terminus of Jmjd6. As expected, the overexpression of the wild-type protein gave rise to the high MW products (Fig. 4C, lane 1), but the overexpression of the catalytically inactive mutant, H187A, did not (Fig. 4C, lane 2). The overexpression of either the N- or the C-terminus-deleted mutant also abolished the oligomerization (Fig. 4C, lanes 3 and 4). To rule out the possibility that the deletion of the N- or the C-terminus impairs the hydroxylase activity of Jmjd6, the N- and the C-termini-deleted Jmjd6 variants were co-overexpressed in HEK293T cells. Western blot analysis shows that they form a dimer with a MW of 100, which corresponds to the dimerized product of the N and the C-terminus-deleted proteins, indicating that deletion of the N- or the C-terminus does not affect Jmjd6 activity. Together with the observation that 1, 2, 3, and 4 monomers of the wild-type protein can oligomerize (Fig. 4C, lane 1), these results indicate that the

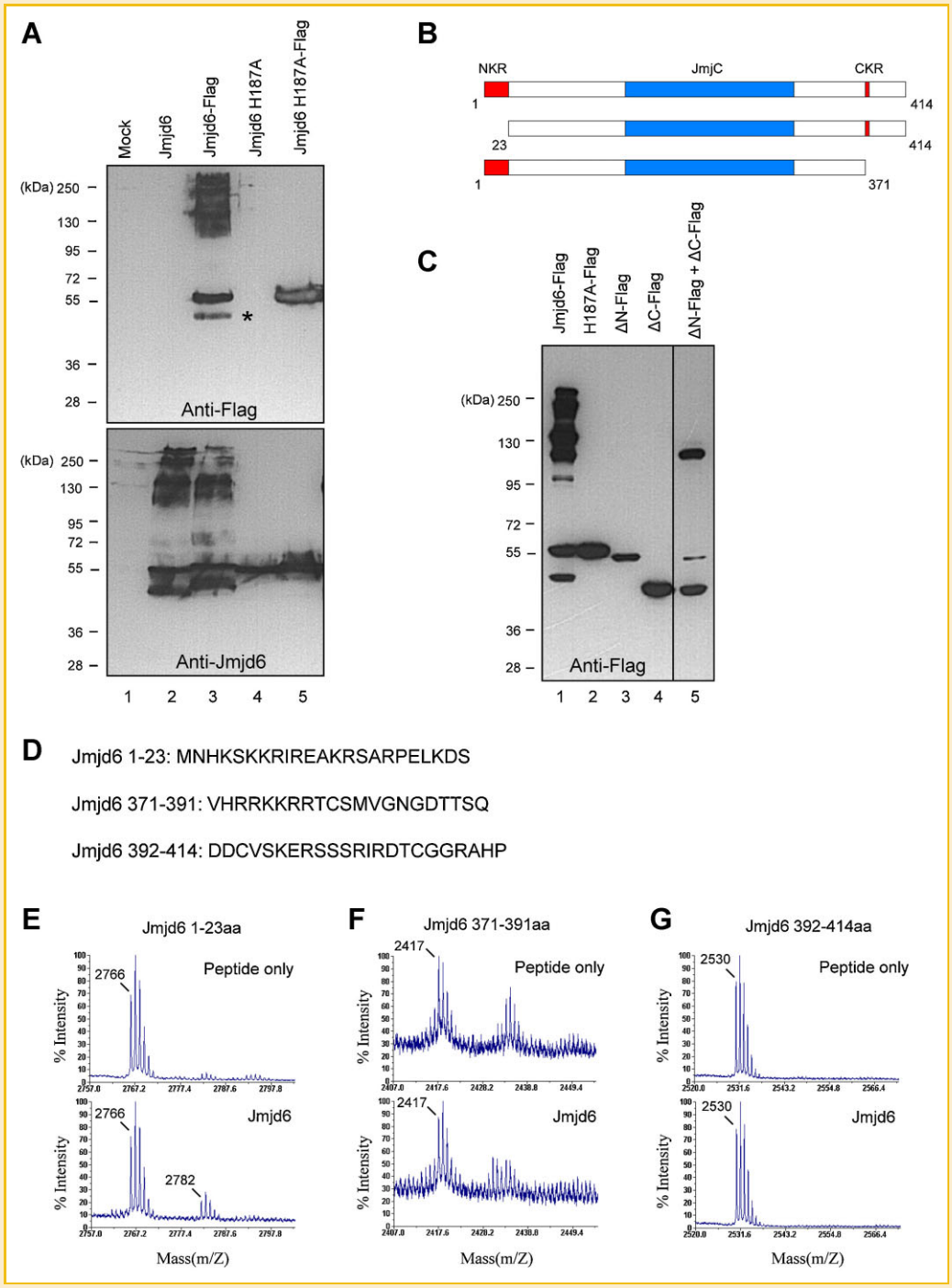

Fig. 4. The enzymatic activity of Jmjd6 is required for its homo-oligomerization. A: Un-tagged (lanes 2 and 4) or Flag-tagged (lanes 3 and 5) wild-type (lanes 2 and 3) or H187A (lanes 4 and 5) variants of Jmjd6 were overexpressed in HEK293T cells and then assayed by immunoblotting using an anti-Flag (upper panel) or anti-Jmjd6 (lower panel) antibody. An asterisk indicates a possible cleaved product of Jmjd6. B: The schematic representation of Jmjd6 and its terminal truncations are shown. C: Flag-tagged WT (lane 1), H187A mutant (lane 2), or N- or C-terminal-deleted Jmjd6 was individually expressed (lanes 3 and 4) or co-overexpressed (lane 5) in HEK293T cells and then assayed by immunoblotting using an anti-Flag antibody. D: The amino acid sequences of the Jmjd6 1–23 aa, 371–391 aa, and 392–414 aa peptides are shown. E–G: A total of 200 ng of Jmjd6 1–23 aa (E), 371–391 aa (F), or 392–414 aa (G) peptide was incubated with 3 μg of Jmjd6 protein in the presence of all of the cofactors and then subjected to MS analysis. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

oligomerization occurs between the N- and C-termini of Jmjd6 and not between the N- and N- or the C- and C-termini of the monomers.

Because the hydroxylase activity and the N- and C-termini of Jmjd6 are required for the observed oligomerization, we examined whether Jmjd6 can hydroxylate its own N- or C-terminus. One N-terminal peptide with residues 1–23 (Jmjd6 1–23 aa) and 2 C-terminal peptides with residues 371–391 (Jmjd6 371–391 aa), and 392–414 (Jmjd6 392–414 aa) were subjected to an in vitro

hydroxylation reaction with the purified wild-type Jmjd6 protein. Indeed, the product with a mass shift of +16 Da was detected when Jmjd6 1–23 aa was used, suggesting that Jmjd6 can hydroxylate its own N-terminus. The hydroxylation is specific because no MW shift was observed when the peptide Jmjd6 371–391 aa or Jmjd6 392–414 aa was used as the substrate.

DISCUSSION

The dynamic regulation of histone arginine methylation is an area of interest, and the existence of arginine demethylase has been debated for a long time [Cuthbert et al., 2004; Wang et al., 2004; Chang et al., 2007; Webby et al., 2009]. Jmjd6 was characterized as the first histone arginine demethylase specific for H3R2 and H4R3 [Chang et al., 2007]. Recently, Webby et al. [2009] demonstrated that Jmjd6 possesses a lysine hydroxylase activity, but its arginine demethylase activity was not reproduced in that study. Our in vitro study also indicates that Jmjd6 does not have arginine demethylase activity: we did not observe any arginine demethylase activity in vivo by immunofluorescence staining (Fig. S1). The different results observed among various groups may arise from different experimental procedures. In an in vitro assay, Chang et al. enriched their demethylated product by immunoprecipitation using an antibody against the product before MS analysis. Conversely, in Webby et al. and our studies, the products were analyzed by MS directly after the in vitro reaction without enrichment. However, when we examined the global change of histone arginine methylation upon the overexpression of Jmjd6 at the cellular level, we adopted the same procedure as Chang et al., but we failed to detect any changes of the H3R2me2 and H4R3me2 levels in the Jmjd6-transfected and non-transfected control cells. The arginine demethylase activity of Jmjd6 (which is likely very weak if it does exist) will continue to be a subject of debate, and its biological significance should be evaluated in vivo.

In contrast to the lack of histone arginine demethylase activity, we observed a strong hydroxylase activity of Jmjd6 on the histone H4 tail in our in vitro system. Lysine hydroxylase activity on the H3 and H4 tails was also reported in studies by the Bruick [Chang et al., 2007] and Bottger [Webby et al., 2009] groups. To determine the substrate specificity, we screened all of the core histone tails and demonstrated that Jmjd6 has a strong hydroxylase activity on the H4 tail, but it had a very weak activity on the H3 tail, indicating that the H4 tail is the preferred substrate of Jmjd6. Lysine hydroxylation is independent of H4R3 methylation because the unmodified H4 tail can also be hydroxylated. Occasionally, we observed mono-, di-, and tri-hydroxylated products, with mass shifts of +16, +32, and +48 Da, respectively, when highly active enzymes were used. Hydroxylation of the H4 tail by Jmjd6 at multiple lysine sites was also observed in the study by the Bruick group [Chang et al., 2007]. The mono-hydroxylated product is not a unique isoform, as analyzed by MS/MS. Based on our data, we estimate that lysine 8 on the H4 tail is the preferred hydroxylation site, with a relative abundance of more than 60% among all of the hydroxylation sites.

To demonstrate its histone hydroxylase activity in vivo, we overexpressed Jmjd6 in HEK293T cells by transiently transfecting with a Jmjd6-IRES-EGFP plasmid. GFP-positive cells were sorted by fluorescence-activated cell sorting (FACS), and the histones were extracted using an acid extraction method. The histones were further separated into H2A, H2B, H3, and H4 fractions by reversed phase high-performance liquid chromatography (RP-HPLC). After digestion with chymotrypsin, the digested peptides were analyzed by liquid chromatography-mass spectrometry (LC-MS)/MS. Whereas the known modifications, such as the acetylation of lysines 5, 8, 12, 16, and the methylation of lysine 20 on H4, were observed, we did not detect any hydroxylation of H4, even though the expression level of exogenous Jmjd6 is at least 10 times higher than the endogenous Jmjd6 expressed in 293T cells (unpublished data). These results suggest that histone H4 may not be an in vivo substrate of Jmjd6.

Instead of identifying histone H4 as the hydroxylation substrate of Jmjd6 in vivo, we discovered that the hydroxylase activity of Jmjd6 is required for its homo-oligomerization in vivo. Jmjd6 was previously found to homo-oligomerize [Tibrewal et al., 2007; Hahn et al., 2010], but the underlying mechanism was not described. Our study demonstrates that Jmjd6 indeed can homo-oligomerize and that the oligomerization requires the N- and C-termini. The homo-oligomerization also requires its hydroxylase activity because the mutation of a conserved residue that is required for the binding of the cofactor abolishes the oligomerization. Furthermore, we found that Jmjd6 can hydroxylate a peptide comprising its N-terminus in vitro. Thus, we propose that Jmjd6 homo-oligomerizes via autohydroxylation in vivo. The identification of the hydroxylation-mediated homo-oligomerization of Jmjd6 adds another player to the family of cross-linking enzymes. This family of enzymes includes lysyl oxidase (LOX) and transglutaminase (TG). LOX is an amine oxidase that catalyzes the lysine residues of collagen and elastin to form aldehydes that spontaneously react with other aldehyde residues or unmodified lysine residues, resulting in the cross-linking of collagen and elastin [Lucero and Kagan, 2006]. TG catalyzes the cross-linking between a free amine group and the gamma-carboxamide group of polypeptides [Lorand and Graham, 2003]. Therefore, our study reveals a catalytic role for Jmjd6 in its homo-oligomerization and provides a new biochemical function for the JmjC domain.

Apart from the observations in frequently used cell lines, the multimerization of Jmjd6 was also observed in MEF cells [Hahn et al., 2010]. However, the function of multimerization has not been studied, and the revelation of this effect may help to understand the biological function of this protein. Our study indicates that the C- and N-termini are essential for multimerization, a finding that can be used in the future to dissect the function of Jmjd6 multimerization without affecting its catalytic activity. An understanding of the regulation of Jmjd6 will help to determine how Jmjd6 functions in tissue development.

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REFERENCES

Bannister AJ, Kouzarides T. 2005. Reversing histone methylation. Nature 436:1103–1106.

Bedford MT, Richard S. 2005. Arginine methylation an emerging regulator of protein function. Mol Cell 18:263–272.

Bhaumik SR, Smith E, Shilatifard A. 2007. Covalent modifications of histones during development and disease pathogenesis. Nat Struct Mol Biol 14:1008–1016.

Boeckel JN, Guarani V, Koyanagi M, Roexe T, Lengeling A, Schermuly RT, Gellert P, Braun T, Zeiher A, Dimmeler S. 2011. Jumonji domain-containing protein 6 (Jmjd6) is required for angiogenic sprouting and regulates splicing of VEGF-receptor 1. Proc Natl Acad Sci USA 108:3276–3281.

Bose J, Gruber AD, Helming L, Schiebe S, Wegener I, Hafner M, Beales M, Kontgen F, Lengeling A. 2004. The phosphatidylserine receptor has essential functions during embryogenesis but not in apoptotic cell removal. J Biol 3:15.

Chang B, Chen Y, Zhao Y, Bruick RK. 2007. JMJD6 is a histone arginine demethylase. Science 318:444–4447.

Cikala M, Alexandrova O, David CN, Proschel M, Stiening B, Cramer P, Bottger A. 2004. The phosphatidylserine receptor from Hydra is a nuclear protein with potential Fe(II) dependent oxygenase activity. BMC Cell Biol 5:26.

Cui P, Qin B, Liu N, Pan G, Pei D. 2004. Nuclear localization of the phosphatidylserine receptor protein via multiple nuclear localization signals. Exp Cell Res 293:154–163.

Cuthbert GL, Daujat S, Snowden AW, Erdjument-Bromage H, Hagiwara T, Yamada M, Schneider R, Gregory PD, Tempst P, Bannister AJ, Kouzarides T. 2004. Histone deimination antagonizes arginine methylation. Cell 118:545–553.

Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM. 2000. A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature 405:85–90.

Hahn P, Wegener I, Burrells A, Bose J, Wolf A, Erck C, Butler D, Schofield CJ, Bottger A, Lengeling A. 2010. Analysis of Jmjd6 cellular localization and testing for its involvement in histone demethylation. PLoS ONE 5:e13769.

Klose RJ, Kallin EM, Zhang Y. 2006. JmjC-domain-containing proteins and histone demethylation. Nat Rev Genet 7:715–727.

Kouzarides T. 2007. Chromatin modifications and their function. Cell 128:693–705.

Kunisaki Y, Masuko S, Noda M, Ilnayoshi A, Sanui T, Harada M, Sasazuki T, Fukui Y. 2004. Defective fetal liver erythropoiesis and T lymphopoiesis in mice lacking the phosphatidylserine receptor. Blood 103:3362–3364.

Li MO, Sarkisian MR, Mehal WZ, Rakic P, Flavell RA. 2003. Phosphatidylserine receptor is required for clearance of apoptotic cells. Science 302:1560–1563.

Lorand L, Graham RM. 2003. Transglutaminases: Crosslinking enzymes with pleiotropic functions. Nat Rev Mol Cell Biol 4:140–156.

Lucero HA, Kagan HM. 2006. Lysyl oxidase: An oxidative enzyme and effector of cell function. Cell Mol Life Sci 63:2304–2316.

Martin C, Zhang Y. 2005. The diverse functions of histone lysine methylation. Nat Rev Mol Cell Biol 6:838–849.

Mosammaparast N, Shi Y. 2010. Reversal of histone methylation: Biochemical and molecular mechanisms of histone demethylases. Annu Rev Biochem 79:155–179.

Myllyharju J, Kivirikko KI. 2004. Collagens, modifying enzymes and their mutations in humans, flies and worms. Trends Genet 20:33–43.

Prockop DJ, Kivirikko KI. 1995. Collagens: Molecular biology, diseases, and potentials for therapy. Annu Rev Biochem 64:403–434.

Shi Y. 2007. Histone lysine demethylases: Emerging roles in development, physiology and disease. Nat Rev Genet 8:829–833.

Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA. 2004. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119:941–953.

Strahl BD, Allis CD. 2000. The language of covalent histone modifications. Nature 403:41–45.

Tibrewal N, Liu T, Li H, Birge RB. 2007. Characterization of the biochemical and biophysical properties of the phosphatidylserine receptor (PS-R) gene product. Mol Cell Biochem 304:119–125.

Tsukada Y, Fang J, Erdjument-Bromage H, Warren ME, Borchers CH, Tempst P, Zhang Y. 2006. Histone demethylation by a family of JmjC domain-containing proteins. Nature 439:811–816.

Wang Y, Wysocka J, Sayegh J, Lee YH, Perlin JR, Leonelli L, Sonbuchner LS, McDonald CH, Cook RG, Dou Y, Roeder RG, Clarke S, Stallcup MR, Allis CD, Coonrod SA. 2004. Human PAD4 regulates histone arginine methylation levels via demethylimination. Science 306:279–283.

Webby CJ, Wolf A, Gromak N, Dreger M, Kramer H, Kessler B, Nielsen ML, Schmitz C, Butler DS, Yates JR III, Delahunty CM, Hahn P, Lengeling A, Mann M, Proudfoot NJ, Schofield CJ, Bottger A. 2009. Jmjd6 catalyses lysyl-hydroxylation of U2AF65, a protein associated with RNA splicing. Science 325:90–93.