Crystal Structure and Functional Analysis of JMJD5 Indicate an Alternate Specificity and Function

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JMJD5 is a Jumonji C (JmjC) protein that has been implicated in breast cancer tumorigenesis, circadian rhythm regulation, embryological development, and osteoclastogenesis. Recently, JMJD5 (also called KDM8) has been reported to demethylate dimethylated Lys-36 in histone H3 (H3K36me₂), regulating genes that control cell cycle progression. Here, we report high-resolution crystal structures of the human JMJD5 catalytic domain in complex with the substrate 2-oxoglutarate (2-OG) and the inhibitor *N*-oxalylglycine (NOG). The structures reveal a β -barrel fold that is conserved in the JmjC family and a long shallow cleft that opens into the enzyme's active site. A comparison with other JmjC enzymes illustrates that JMJD5 shares sequence and structural homology with the asparaginyl and histidinyl hydroxylase FIH-1 (factor inhibiting hypoxia-inducible factor 1 [HIF-1]), the lysyl hydroxylase JMJD6, and the RNA hydroxylase TYW5 but displays limited homology to JmjC lysine demethylases (KDMs). Contrary to previous findings, biochemical assays indicate that JMJD5 does not display demethylase activity toward methylated H3K36 nor toward the other methyllysines in the N-terminal tails of histones H3 and H4. Together, these results imply that JMJD5 participates in roles independent of histone demethylation and may function as a protein hydroxylase given its structural homology with FIH-1 and JMJD6.

The Jumonji C (JmjC) enzymes represent a family of nonheme Fe(II)- and 2-oxoglutarate (2-OG)-dependent dioxygenases (5, 12). These enzymes belong to the Cupin protein superfamily whose members share a conserved β -barrel fold and possess a His-X-Glu/Asp-X_n-His triad responsible for Fe(II) coordination (11, 20, 33, 40). Metazoan JmjC proteins comprise several subfamilies that possess distinct substrate specificities and participate in different biological processes, including transcriptional regulation, cell cycle control, chromatin modification, mRNA splicing, and RNA modification (13, 35, 37, 43, 50). Enzymes from several of the JmjC subfamilies have been shown to function as lysine demethylases (KDMs) by hydroxylating the methyl groups of methyllysines in histones and nonhistone proteins. These subfamilies include JHDM1 (KDM2), JHDM2 (KDM3), JMJD2 (KDM4), JARID1, (KDM5), UTX/JMJD3 (KDM6), and PHF8/KIAA1718 (KDM7) (2, 22, 35, 45). In contrast, other JmjC enzymes catalyze stereo- and site-specific hydroxylation of residues in nonhistone proteins. Examples of these enzymes include JMJD6, a lysyl-5S-hydroxylase that oxidizes the splicing factor U2AF65 (32, 50), and FIH-1 (factor inhibiting hypoxia-inducible factor 1 [HIF-1]), an asparaginyl, aspartyl, and histidinyl hydroxylase whose substrates include the HIF and ankyrin repeat-containing proteins (13, 43, 51, 52). Moreover, recent studies have highlighted that JmjC substrates are not restricted to proteins. The tRNA wybutosine-synthesizing enzyme 5 (TYW5) hydroxylates wybutosine to hydroxywybutosine at position 37 in phenylalanine tRNA, illustrating that certain JmjC enzymes can oxidize nucleic acid substrates (28, 37). In summary, the various substrate specificities of the JmjC enzymes underscore their diverse biological functions.

JMJD5 is a metazoan JmjC protein that is conserved from worms to humans. Initial studies identified JMJD5 as a putative tumor suppressor using a retroviral insertional mutagenesis screen in *Blm*^{m3}-deficient mice (46). Correlatively, short hairpin RNA (shRNA)-mediated knockdown of JMJD5 resulted in an increased mutational frequency, implicating it in DNA mismatch repair and maintenance

of genome stability. Subsequent studies reported that JMJD5 is overexpressed in several cancers and promotes breast cancer cell proliferation by demethylating dimethylated Lys-36 in histone H3 (H3K36me₂) in the coding region of the cyclin A1 gene (23). In addition, JMJD5 is essential for mammalian embryological development as a conditional gene knockout results in lethality during mid-gestation in mice (24). This lethality was ascribed to increased H3K36me₂ levels in the *Cdkn1a* locus, inducing the expression of the cyclin-dependent kinase inhibitor p21 and diminishing embryonic cell proliferation. In humans and plants, JMJD5 has also been reported to upregulate dawn-expressed clock genes in circadian rhythm through H3K36 demethylation (25, 26).

In contrast to these findings, recent studies by three separate groups have identified functions of JMJD5 that are independent of histone demethylation. Notably, these studies were unable to detect H3K36 demethylation by JMJD5 *in vivo* (38, 49, 53). In a JMJD5 knockout mouse, loss of JMJD5 upregulated expression of the tumor suppressor p53, resulting in the activation of the p53-target genes encoding p21 and the proapoptotic protein Noxa without an appreciable change in the global H3K36me₂ level (38). Similarly, a proteomic analysis of pleural effusions from patients with lung cancer and benign inflammatory disease identified decreased levels of JMJD5 but found that its overexpression in lung adenocarcinoma cells did not alter the global status of H3K36me₁, H3K36me₂, or H3K36me₃ *in vivo* (49). In a separate study, JMJD5 overexpression failed to yield discernible alterations in mono-, di-,

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and trimethylation of H3K4, H3K9, H3K27, H3K36, and H3K79, as determined by immunoblotting and immunofluorescence analysis (53). Instead, JMJD5 was reported to recognize and hydroxylate the osteoclastogenesis transcription factor NFATc1, mediating its ubiquitination-dependent proteasomal degradation. Collectively, these studies illustrate that JMJD5 participates in diverse nuclear signaling pathways and call into question whether it functions as a bona fide H3K36 demethylase.

To gain insight into the enzymatic activity of JMJD5, we determined high-resolution crystal structures of the catalytic domain of human JMJD5 bound to 2-OG and N-oxalylglycine (NOG), a 2-OG analog-based inhibitor. The structures reveal a catalytic domain whose fold is homologous to the structures of FIH-1, JMJD6, and TYW5 but is more distantly related to the catalytic domains of JmjC KDMs. Phylogenetic analysis of human JmjC proteins illustrates the sequence homology of JMJD5 with other JmjC proteins that are not presently classified as KDMs. Consistent with these findings, biochemical assays of JMJD5 were unable to detect demethylation of H3K36me or other methyllysines in the N-terminal tails of histones H3 and H4. Based on these results and those recently published by other groups (38, 49, 53), it appears that JMJD5 does not possess intrinsic demethylase activity *in vitro* but may function as a protein hydroxylase.

MATERIALS AND METHODS

Cloning, expression, and purification of JMJD5 proteins. Expression vectors for human JMJD5 were generated by PCR amplification of Image clone 5207043 (Open Biosystems), followed by ligation into the Sumo expression plasmid pSMT3 (36). This cloning resulted in the fusion proteins 6×His-Smt3-JMJD5_{183–416} (pDR146) (composed of a six-His tag, Smt3, and residues 183 to 416 of JMJD5) and 6×His-Smt3-JMJD5_{2–416} (pDR150). To minimize metal contamination upon purification, the hexahistidine tag was replaced with a Strep(II) tag (WSHPQFEK) by mutagenesis (10). The resulting constructs generated were Strep(II)-Smt3-JMJD5_{183–416} (pDR157) and Strep(II)-Smt3-JMJD5_{2–416} (pDR158). The JMJD5 fragments from these vectors were excised and subsequently ligated into a modified pET15 vector with an N-terminal Strep(II) affinity tag and tobacco etch virus (TEV) protease cleavage site to produce the constructs Strep(II)-JMJD5_{183–416} (pDR159) and Strep(II)-JMJD5_{2–416} (pDR160). To reproduce a previously reported glutathione S-transferase (GST) fusion of JMJD5 (23), residues 101 to 416 were PCR amplified and ligated into pGST2 (44) to produce GST-JMJD5_{101–416} (pDR163).

For structure determination, the hexahistidine-tagged Smt3-JMJD5_{183–416} was expressed overnight in *Escherichia coli* BL21 Rosetta2 DE3 cells (EMD Millipore) at 20°C, after induction with 0.5 mM isopropyl thiogalactoside. Pellets were resuspended in 50 mM sodium phosphate (pH 7.0), 500 mM NaCl, and 5 mM 2-mercaptoethanol (buffer A) and stored at –80°C. Thawed cells were lysed by sonication in buffer A, and the clarified supernatant was then applied to a Talon Co(II) Superflow column (Clontech) and washed with 2 column volumes of buffer A, followed by a 30-ml gradient into buffer B (buffer A with 500 mM imidazole). Peak fractions containing JMJD5 were pooled and dialyzed into 2 liters of buffer C (50 mM Tris [pH 7.5], 150 mM NaCl, 5.0 mM 2-mercaptoethanol) overnight in the presence of Ulp1 protease that cleaves the Smt3 fusion protein (36). The protein was then concentrated and loaded onto a Superdex 200 gel filtration column (GE Healthcare), and the resulting peak fractions were pooled, concentrated, and flash frozen. Selenomethionine-containing protein was produced for phase determination by expressing the gene product of pDR146 in the *E. coli* methionine auxotrophic B834 strain (EMD Millipore), as described previously (6, 14, 17). Protein concentration was determined by its absorbance at 280 nm. Analysis of the hexahistidine-tagged JMJD5_{183–416} by inductively coupled plasma high-resolution mass spectrometry (ICP-HRMS) (Department of Geology,

University of Michigan) revealed Co(II) bound to the enzyme that was presumably introduced during the Talon affinity chromatography.

For demethylase assays, JMJD5 constructs were purified with Strep(II) or GST affinity tags to minimize inhibition by contaminating transition metal ions, as previously reported (29). Strep(II)-Smt3-JMJD5_{2–416} and Strep(II)-Smt3-JMJD5_{183–416} fusion proteins were expressed as outlined above and lysed in 50 mM sodium phosphate (pH 7.5), 250 mM NaCl, and 5.0 mM 2-mercaptoethanol (buffer D). Clarified supernatants were applied to a StrepTactin Superflow Plus (Qiagen) column and eluted with buffer D containing 2.5 mM desthiobiotin. Peak fractions were collected and pooled, followed by dialysis against 2.0 liters of 25 mM Tris (pH 8.5), 150 mM NaCl, and 5.0 mM 2-mercaptoethanol in the presence of Ulp1 protease that cleaves the Strep(II)-Smt3 fusion protein. The cleaved JMJD5 constructs were further purified on a Superdex 200 gel filtration column (GE Healthcare) in the same buffer. The GST-JMJD5_{101–416} construct was purified in a similar manner as described above, with the exception that the fusion protein was purified on a glutathione-Sepharose 4 Fast Flow (GE Healthcare) column in Tris (pH 8.5), 300 mM NaCl buffer and eluted with 15 mM glutathione using the same buffer. The GST fusion protein was left intact to permit a direct comparison of its enzymatic activity to that previously reported for a GST-JMJD5_{101–416} fusion (53). Strep(II)-tagged JMJD2A was purified as previously described (29). To examine the content of contaminating transition metals that may inhibit JMJD5, ICP-HRMS was used to analyze three purifications of the StrepTactin-purified JMJD5_{183–416} and revealed a total percent metal content (Ni, Cu, Co, Zn, and Mn) of 20% ± 0.4%, which is defined as the molar ratio of the metal/enzyme concentration. These data indicate that the majority of the enzyme was devoid of inhibitory transition metals.

Crystallization and structure determination. Crystals of the selenomethionyl-JMJD5_{183–416} bound to NOG were obtained by hanging-drop vapor diffusion at 20°C. Protein was prepared at 12 mg/ml with 15 mM bis-Tris (pH 7.2), 25 mM NaCl, 1.0 mM dithiothreitol, 1.0 mM NOG, and 0.5 mM CoCl₂. This cocktail was then mixed 1:1 with the mother liquor: 4.5% (wt/vol) polyethylene glycol 3000, 0.1 M bis-Tris (pH 5.5), and 50 mM MgCl₂. Crystals of the JMJD5_{183–416}–2-OG complex were obtained by mixing protein cocktail (12 mg/ml JMJD5 in 15 mM Tris [pH 8.5], 25 mM NaCl, 1.5 mM 2-OG, 1.5 mM dithiothreitol, 4 mM H3K36me2-L peptide [see Table S1 in the supplemental material]) with an equal volume mother liquor (9.5% [wt/vol] polyethylene glycol 3000, 0.1 M bis-Tris [pH 6.2], 50 mM MgCl₂). X-ray diffraction data were collected at Life Sciences Collaborative Access Team ([LS-CAT] beamline 21-ID-G) at the Advanced Photon Source, Argonne National Laboratory, using a MarMosaic 300 charge-coupled-device (CCD) detector (Rayonix). Data were processed and scaled using HKL2000 (39). Phases were determined by selenium single anomalous diffraction (SAD) phasing, and the resulting model was refined with individual anisotropic B-factor refinement using PHENIX (1). The JMJD5–2-OG complex was solved by molecular replacement and refinement in PHENIX using the JMJD5–NOG complex as the search model. Although the H3K36me2-L peptide was included in the crystallization condition of this complex, electron density corresponding to the peptide was not observed in the final refined maps. After model building and refinement, structures were validated by MolProbity (8). Secondary structure elements were defined by DSSP (27), molecular graphics were rendered using PyMOL (Schrodinger, LLC), and the electrostatic surface model was generated using the APBS plug-in for PyMOL (4).

Bioinformatics analyses. JmjC-containing proteins were identified using NCBI BLAST (3) using the JMJD5 catalytic domain as the query. The JmjC domains of these proteins, as defined by UniProtKB (48), were used for the structural alignment in Clustal W, version 2.0 (31). The boot-strapped parsimony tree was generated using the PHYLIP bioinformatics package (19) and was plotted using NJPlot (41). Structural homologs of JMJD5 were identified and analyzed using the Dali server (21).

FIG 1 Structure of JMJD5. (A) Active site of the SeMet-JMJD5₁₈₃₋₄₁₆-NOG complex, showing selected residues from SeMet-JMJD5₁₈₃₋₄₁₆ (gray carbon atoms) in complex with Co(II) (magenta), NOG (green carbon atoms), and a coordinated water molecule (red sphere). The 2.0 σ electron density map shown was calculated from the experimental phases after density modification in PHENIX. (B) Overall structure of the JMJD5₁₈₃₋₄₁₆-2-OG complex. Residues belonging to the JmJC domain are shown in yellow, and Co(II) (magenta), 2-OG (green carbon atoms), and a coordinated water molecule (red sphere) are shown. Secondary structure elements were assigned by DSSP. (C) Active site of the JMJD5-2-OG complex with residues colored as in panel B. (D) Electrostatic surface of the JMJD5-2-OG complex. Electrostatic potential is contoured from $+5k_B T e^{-1}$ (blue) to $-5k_B T e^{-1}$ (red), where k_B is the Boltzmann constant, T is temperature (K), and e^{-1} is the charge of an electron. The 2-OG, Co(II) ion, and water molecule are visible in the active site.

Demethylase assays. Mass spectrometry-based demethylase assays were performed using 10 μM enzyme, 50 μM peptide, 50 mM HEPES (pH 7.5), 50 mM NaCl, 50 μM (NH₄)₂Fe(SO₄)₂, 1.0 mM L-ascorbic acid, 1.0 mM NAD⁺, 0.1 μM formaldehyde dehydrogenase (FDH), and 0.5 mM 2-OG. Peptides used in the assays are detailed in Table S1 in the supplemental material. Reaction mixtures were incubated at 37°C for 4 h, quenched with an equal volume of 1% trifluoroacetic acid, and stored at -20°C. Thawed samples were desalted using a ZipTip (Millipore) and mixed 1:1 with matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid, dissolved in a equal mixture of acetonitrile and ethanol) and spotted onto target plate. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was performed using either a Waters TofSpec-2E in reflectron mode with delayed extraction (Department of Chemistry, University of Michigan) or a Shimadzu Biotech Axima CFR in reflectron mode (Texas A&M University).

FDH-coupled fluorescent demethylase assays were performed as described previously (29). Briefly, the assay was conducted using 5.0 μM enzyme purified by StrepTactin or glutathione affinity chromatography in a buffer containing 50 mM HEPES (pH 7.5), 50 mM NaCl, 50 μM (NH₄)₂Fe(SO₄)₂, 1.0 mM L-ascorbic acid, 1.0 mM NAD⁺, and 0.1 μM recombinant FDH (29). Reactions were initiated by the addition of 0.5 mM 2-OG and 1.0 mM peptide H3K36me2-L (see Table S1 in the supplemental material). NADH production was measured at 37°C in 30-s intervals by fluorescence (excitation wavelength, 340 nm; emission wavelength, 490 nm) using a Sapphire2 fluorescence microplate reader (Tecan Group Ltd.).

Protein structure accession numbers. The atomic coordinates and structure factors for the JMJD5 crystal structures, under accession codes 4GJY and 4GJZ, have been deposited in the Protein Data Bank (PDB), Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

RESULTS

Crystal structure of JMJD5. The crystal structure of the C-terminal catalytic domain of human JMJD5 (residues 183 to 416) bound to NOG was determined by selenomethionine SAD phasing to 1.25-Å resolution (Fig. 1A and Table 1). This structure was subsequently used as a molecular replacement model to solve the structure of a JMJD5-2-OG binary complex to 1.05-Å resolution. The overall quality of the electron density maps permitted the entire structure to be assigned for the JMJD5-2-OG and NOG complexes, with the exception of residues 241 to 248 in the NOG complex (residues 241 to 247 in the 2-OG complex) that represent a flexible loop linking the β3 and β4 strands. In addition, density corresponding to an H3K36me2 peptide was absent in the electron density maps of the JMJD5-2-OG complex despite the peptide's inclusion during crystallization. In both structures, Co(II) was modeled in the Fe(II) coordination site because Co(II) was included during crystallization and was detected in the enzyme purified by immobilized Co(II) metal affinity chromatography using ICP-HRMS (data not shown), as described for the JMJD2 KDMs (29).

The structure of the JMJD5 catalytic core comprises a JmJC domain (residues 271 to 416) preceded by an N-terminal extension (residues 183 to 270) composed of mixed helical and β-sheet topology (Fig. 1B). The JmJC β-barrel is composed of two antiparallel β-sheets consisting of β7-β12-β9-β10 and β5-β6-β13-β8-β11. Interleaved between these β-strands are several α- and 3₁₀-helices that surround the exterior of the β-barrel. The N-terminal extension wraps around the β-barrel and appears to stabilize its

TABLE 1 Crystal data collection and refinement statistics

Parameter	SeMet-JMJD5-NOG	JMJD5-2-OG
Data collection		
Beamline	21-ID-G	21-ID-G
Wavelength (Å)	0.9786	0.9786
Space group	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions (<i>a</i> , <i>b</i> , <i>c</i> [Å])	49.82, 64.17, 76.87	49.70, 64.06, 76.80
Resolution (Å)	30.4–1.25	25.4–1.05
Highest-resolution shell (Å)	1.29–1.25	1.09–1.05
R_{merge} (%) ^a	5.9 (35.1)	8.9 (45.3)
$I/\sigma I$ ^a	49.6 (3.78)	16.17 (2.28)
No. of reflections	68,062	110,949
Completeness (%) ^a	98.8 (98.1)	96.5 (76.2)
Redundancy ^a	13.1 (7.8)	12.6 (3.6)
Phasing		
Figure of merit	0.47	
Refinement and validation ^b		
No. of reflections	67,099	107,259
No. of atoms	4,242	4,593
Protein atoms	3,874	4,065
Ligand atoms	13	24
Metals and other atoms	1	1
Water	354	503
$R_{\text{work}}/R_{\text{free}}$ ^c	14.0/16.1	14.7/16.3
Avg B-factors (Å ²)		
Overall	14.3	15.1
Protein	13.4	13.3
Ligands	7.19	13.1
Metals	14.0	11.0
Waters	24.1	29.5
RMSD		
Bond angles (°)	1.25	1.31
Bond lengths (Å)	0.008	0.008
MolProbity scores		
Clashscore (all atoms) ^d	2.73 (97th percentile, <i>n</i> = 285)	2.2 (96th percentile, <i>n</i> = 176)
MolProbity score	1.06 (98th percentile, <i>n</i> = 1,486)	1.0 (98th percentile, <i>n</i> = 686)
Resolution range (Å)	1.249 ± 0.25	1.048 ± 0.25
Ramachandran statistics		
Favored (%)	99	98
Allowed (%)	1	2
Outliers (%)	0	0

^a Values in parentheses correspond to the highest-resolution shell.

^b Structures were refined in PHENIX using anisotropic temperature-factor refinement.

^c $R_{\text{work}} = \sum ||F_o| - |F_c|| / \sum |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively; $R_{\text{free}} = 5\%$ of the total reflections.

^d Clashscore is the number of serious steric overlaps (>0.4 Å) per 1,000 atoms, where *n* is the number of structures included in this comparison.

structure. Specifically, the $\beta 1$ – $\beta 2$ and $\beta 3$ – $\beta 4$ sheets in the N-terminal motif extend the β -barrel by forming β -interactions with $\beta 11$ and $\beta 5$, respectively, anchoring the N-terminal extension to the JmjC domain. The structures of the 2-OG and NOG complexes share a homologous tertiary fold with unpublished structures recently deposited into the Protein Data Bank of a JMJD5–2-OG complex (3UYJ; 2.35-Å resolution) and JMJD5–NOG complex (4AAP; 2.60-Å resolution), with root mean square deviation (RMSD) values for aligned C α atoms of 0.3 Å and 0.4 Å, respectively.

The active site of JMJD5 is located within the β -barrel and harbors the Fe(II) and 2-OG binding pockets. Co(II) occupies the Fe(II) binding site in each structure and is coordinated to the side chains of His-321, Asp-323, and His-400 in JMJD5 (Fig. 1C). A water molecule and 2-OG complete the octahedral coordination

of Co(II), with the water occupying the O₂ binding site. 2-OG is bound through its coordination to Co(II), hydrogen bonding to Tyr-272, Ser-318, Asn-327, and Lys-336, and van der Waals interactions with Trp-310, Leu-329, Val-402, and Trp-414. In the structure of the JMJD5–NOG binary complex, the inhibitor adopts a conformation homologous to that of 2-OG and engages in interactions essentially identical to those described for the co-substrate.

An electrostatic potential mapped onto the surface of JMJD5 yields insights into its substrate specificity. A striking feature of the surface is a long acidic channel that runs the length of the catalytic domain and opens into its active site (Fig. 1D). This channel is approximately ~ 10 Å wide with a maximum depth of ~ 11 Å and is defined by two lobes that flank the β -barrel. One lobe encompasses the $3_{10}3$ and $3_{10}4$ helices from the JmjC domain, whereas the second is composed of the $\alpha 3$ and $\alpha 4$ helices and the $\beta 3$ – $\beta 4$ sheet from the N-terminal extension, as well as the $\alpha 5$ helix from the JmjC domain. The electrostatic surface illustrates that the entire channel is acidic, with the greatest density of net negative charge at the bottom of the channel that adjoins the active-site entrance. The overall dimensions and charge of the cleft are suited to recognizing a positively charged substrate, such as a histone N-terminal tail or a basic loop in a protein substrate, suggesting possible modes for substrate recognition by JMJD5.

Biochemical analysis of demethylase activity. Previous biochemical studies reported that a JMJD5 truncation mutant lacking its first 100 residues fused to GST (GST-JMJD5_{101–416}) demethylated H3K36me₂, whereas as the full-length enzyme lacked this activity based on a MALDI mass spectrometry-based assay using an H3K36me₂ peptide (23). To corroborate these findings, we repeated the mass spectrometry assay by testing the activity of full-length JMJD5 (JMJD5_{2–416}), GST-JMJD5_{101–416}, and the JMJD5_{183–416} construct used in structure determination with methylated H3K36 peptides of identical lengths (see Table S1 in the supplemental material) to those previously reported (23). Assays were performed with a relatively high ratio of enzyme to peptide substrate concentration (1:5) over a 4-h period to ensure detection of the demethylated product(s). Surprisingly, none of the JMJD5 constructs tested demethylated H3K36me₁, H3K36me₂, or H3K36me₃ peptides under these conditions (Fig. 2A). In contrast, JMJD2A, a dual-specificity H3K9me₃/H3K36me₃ KDM, catalyzed essentially complete demethylation of an H3K9me₃ peptide to H3K9me₂ and, to a lesser extent, H3K9me₁ under identical reaction conditions (Fig. 2B). To further substantiate these findings, we attempted to measure demethylation of a longer H3K36me₂ peptide (see Table S1) by JMJD5_{2–416}, GST-JMJD5_{101–416}, and JMJD5_{183–416} using a complementary assay that detects formaldehyde formation via an enzyme-coupled reaction (29) (Fig. 2C). The JMJD5 constructs exhibited negligible activity toward this substrate in comparison to a JMJD2A S288A mutant that efficiently demethylates both di- and trimethyllysines (9, 15).

Our finding that JMJD5 is inactive against H3K36me peptides prompted us to examine whether it can demethylate other methyllysines in the N-terminal tails of histones H3 and H4. Mass spectrometry-based assays revealed that JMJD5_{2–416}, GST-JMJD5_{101–416}, and JMJD5_{183–416} are inactive toward mono-, di-, or trimethylated forms of H3K4, H3K9, H3K27, and H4K20 (see Fig. S1 in the supplemental material), in agreement with previous studies (23, 53). Collectively, these results indicate that the various JMJD5 constructs analyzed, including the previously reported GST-JMJD5_{101–416}, lack

FIG 2 Histone demethylase assays of JMJD5. (A) The results from the MALDI mass spectrometry-based demethylase assay using H3K36me peptides are shown. The construct of JMJD5 used in each reaction mixture is indicated on the left, and the peptide assayed is indicated above. The spectra shown in the first row correspond to control reactions in which JMJD5 was omitted from the assays. (B) MALDI mass spectrometry assay of JMJD2A. The upper panel shows a control assay of the H3K9me3 peptide in the absence of enzyme, whereas the bottom panel represents the H3K9me peptide products formed in the presence of JMJD2A. (C) FDH-coupled fluorescent demethylase assays of the JMJD5 constructs tested in panel A and a JMJD2A S288A mutant. Each enzyme was assayed with an H3K36me2-L peptide substrate (see Table S1 in the supplemental material). Activity is reported relative to the JMJD2A S288A mutant, and error bars represent standard deviations from three replicates. The negative values observed for the activities of the JMJD5 constructs resulted from a slight background drift that was due to the high protein and peptide concentrations used in the demethylase assay.

intrinsic demethylase activity toward H3K36 or other methyllysines in histone H3 or H4 N-terminal tails *in vitro*.

Comparison with other JmjC enzymes. Based on JMJD5's apparent lack of KDM activity, we employed a bioinformatics approach to identify homologs of JMJD5 that might offer insight into its enzymatic activity and biological functions. Strikingly,

phylogenetic analysis of human JmjC proteins illustrates that JMJD5 segregates into a clade containing TYW5, JMJD8, and PLA2G4B that is distinct from clades containing the JmjC KDM subfamilies (Fig. 3). Consistent with the phylogenetic data, a search for structural homologs in the Protein Data Bank using the Dali server (21) identified several JmjC proteins as sharing high

FIG 3 Phylogenetic tree of human JmjC proteins. A parsimony tree derived from protein sequences of selected JmjC domains is depicted. The sequences used in this analysis include (UniProtKB accession number and amino acid residues): JHDM1A (Q9Y2K7; 148 to 316), JHDM1B (Q8NHM5; 178 to 346), JMJD1A (Q9Y4C1; 1058 to 1281), JMJD1B (Q7LBC6; 1498 to 1721), JMJD1C (Q15652; 2274 to 2498), JMJD2A (O75164; 142 to 308), JMJD2B (O94953; 146 to 309), JMJD2C (Q9H3R0; 144 to 310), JMJD2D (Q6B0I6; 146 to 312), JARID1A (P29375; 437 to 603), JARID1B (Q9UGL1; 453 to 619), JARID1C (P41229; 468 to 634), JARID1D (Q9BY66; 458 to 624), UTX (O15550; 1095 to 1258), JMJD3 (O15054; 1339 to 1502), PHF8 (Q9UPP1; 231 to 387), K1AA1718 (Q6ZMT4; 230 to 386), TYW5 (A2RUC4; 102 to 267), FIH-1 (Q9NWT6; 142 to 312), PHF2 (O75151; 197 to 353), UTY (O14607; 1042 to 1205), JMJD4 (Q9H9V9; 188 to 347), JMJD5 (Q8N371; 271 to 416), JMJD6 (Q6NYC1; 141 to 305), JMJD7 (P0C870; 128 to 307), JMJD8 (Q96S16; 201 to 334), JARID2 (Q92833; 884 to 1048), PLA2G4B (P0C869; 1 to 97), HSPB1 (P04792; 1 to 205), Hairless (O43593; 946 to 1157), MINA53 (Q8IUF8; 139 to 271), and NO66 (Q9H6W3; 294 to 439).

degrees of structural conservation with JMJD5. The top three scores from the Dali search were TYW5 (Z-score, 26.2; RMSD, 1.9 Å), FIH-1 (Z-score, 24.3; RMSD, 2.2 Å), and JMJD6 (Z-score, 21.3; RMSD, 2.8 Å). The remaining proteins identified by Dali share weaker structural conservation with JMJD5 and include several JmjC KDMs, such as PHF8, K1AA1718, and JMJD2 KDMs, in addition to Fe(II)-dependent dioxygenases belonging to the Cupin superfamily.

To further examine the structural homology identified through the Dali search, we generated structural alignments of the catalytic domains of TYW5 and FIH-1 with JMJD5. Superimposition of JMJD5 with the crystal structure of TYW5 (28) illustrates striking structural homology between their tertiary structures as well as conservation in certain active-site residues (Fig. 4A). In addition to sharing an Fe(II)-coordinating His-X-Asp-X_n-His triad, the residues Tyr-272, Asn-327, and Lys-336, which engage in hydrogen bonding with 2-OG in JMJD5, are conserved in TYW5 (Fig. 1C and 4A). In contrast, the residues that presumably form the respective binding pockets of the hydroxyl acceptor substrates diverge considerably between these enzymes. Notably, Trp-310 in JMJD5 is replaced by Arg-149 in TYW5, which is potentially positioned to interact with the phosphate backbone of the RNA substrate (28). Further, Ser-318 and Trp-414 in JMJD5 align with Leu-157 and Phe-249 in TYW5. These variations between the JMJD5 and TYW5 active sites imply that they hydroxylate different substrates.

An alignment of JMJD5 with the structure of an FIH-1–2-OG–HIF-1 peptide complex (18) also reveals homology between these JmjC enzymes. In addition to the overall structural similarity of their β -barrel folds, the Fe(II)-coordinating His-X-Asp-X_n-His triad and 2-OG-binding residues are highly conserved between JMJD5 and FIH-1 (Fig. 4B). In particular, residues Tyr-272, Ser-318, Asn-327 (data not shown), and Lys-336, which form hydrogen bonds with 2-OG in JMJD5, are strictly conserved in FIH-1, with the exception of Ser-318 that aligns with Thr-196 in FIH-1. Conversely, Arg-238 and Gln-239 in FIH-1, which hydrogen bond to Asn-803 in the HIF-1 substrate peptide and position its C β atom for hydroxylation, are replaced by Asn-359 and Thr-360 in JMJD5, respectively. These differences in their hydroxyl acceptor recognition sites suggest that FIH-1 and JMJD5 have evolved to recognize distinct substrates.

In contrast to its structural similarities to TYW5 and FIH-1, JMJD5 displays weaker homology with JmjC KDMs. Superimpositions of JMJD5 with structures of the JMJD2A-NOG-H3K36me3 and PHF8-NOG-H3K9me2 peptide complexes reveal divergence between the tertiary structures of JMJD5 and the KDMs (Fig. 4C and D). In particular, JMJD2A and PHF8 possess narrow histone substrate binding clefts, contrasting with the wide, unobstructed substrate binding channel observed in JMJD5 (see Fig. S2 in the supplemental material). The alignment of PHF8 and JMJD5 illustrates that the residues in PHF8 composing its H3K9me2 binding pocket, Ile-191, Leu-236, Tyr-234, and Asn-333, are occupied by Gln-275, Trp-310, and Trp-414 in JMJD5, which physically occlude dimethyllysine binding (Fig. 4C). Similarly, the trimethyllysine binding pocket of JMJD2A is occupied by the indole side chain of Trp-414, precluding trimethyllysine recognition (Fig. 4D). In addition, Tyr-177 in JMJD2A, whose hydroxyl group engages in methyl carbon-oxygen hydrogen bonding with di- and trimethyllysine substrates (15), is replaced by Trp-310 in JMJD5, abolishing these interactions. Based on the comparisons with PHF8 and JMJD2A, the active site of JMJD5 appears to be incompatible with recognizing and hydroxylating methyllysine residues. These observations are consistent with our biochemical data indicating that JMJD5 lacks KDM activity *in vitro* (Fig. 2; see also Fig. S1 in the supplemental material) and with previous studies that were unable to detect lysine demethylase activity *in vivo* (38, 49, 53).

DISCUSSION

JMJD5 has been classified as an H3K36me2-specific demethylase that regulates the expression of genes that govern cell cycle progression. However, our biochemical data indicate that JMJD5 is incapable of demethylating H3K36me as well as other methyllysines in histones H3 and H4 *in vitro*. These findings agree with those recently reported by three independent groups that were unable to detect H3K36me2 demethylation by JMJD5 *in vivo* (38, 49, 53). Further, Youn et al. presented immunoblot and immunofluorescence data illustrating that overexpression of JMJD5 does not appreciably alter histone H3 methyllysine levels *in vivo* (53). One potential explanation for these discrepancies is that JMJD5 may assemble into a multimeric complex that endows the enzyme with H3K36me2 activity *in vivo*, as observed in certain histone acetyltransferase and methyltransferase complexes that require additional subunits for optimal activity (7, 16, 30, 34, 42). Further, a JMJD5-containing complex may serve to recruit the enzyme to specific loci and would potentially explain why H3K36me2 dem-

FIG 4 Structural superimposition of JMJD5 with other JmjC enzymes. In each panel, the JMJD5–2-OG complex is shown as yellow carbon atoms, with a pink Co(II) atom and light gray 2-OG bound. The JmjC proteins aligned with JMJD5 include the following: TYW5 (PDB: 3AL6) shown in light blue with a bound Ni(II) atom in teal (A); FIH-1 (PDB: 2ILM) shown in purple in complex with 2-OG (green), Fe(II) (gray), and HIF-1 peptide (pink) (B); PHF8 (PDB: 3KV4) depicted in cyan bound to NOG (green), Fe(II) (gray), and an H3K4me3-K9me2 peptide (pink) (C); and JMJD2A (PDB: 2Q8E) illustrated in green in complex with NOG (dark green), Ni(II) (cyan), and an H3K36me3 peptide (pink) (D).

ethylation has been reported in certain target genes, whereas its overexpression does not result in global H3K36 demethylation (49, 53). Alternatively, JMJD5 may masquerade as a KDM *in vivo* by upregulating the expression of an H3K36me2-specific demethylase, such as JHDM1A or JHDM1B (47).

Despite these possibilities, the lack of intrinsic KDM activity points toward JMJD5 functions that are independent of histone demethylation. Sequence and structural analyses of JMJD5 reveal that it shares a high degree of homology with JmjC enzymes that hydroxylate proteins and nucleic acids, such as TWY5, FIH-1, and JMJD6, in comparison to JmjC KDMs (Fig. 3 and 4). Although the catalytic domains of TWY5 and JMJD5 are highly homologous, it is unlikely that JMJD5 functions as a DNA or RNA hydroxylase due to differences in their respective active sites and, in particular, the acidic substrate binding cleft of JMJD5 that is incompatible with nucleic acid recognition (Fig. 1D and 4A; see also Fig. S2 in the supplemental material). The properties and dimensions of the substrate binding cleft and active site of JMJD5 appear to be conducive to protein hydroxylation based in part on its structural homology with FIH-1 (Fig. 4B). While completing these studies, Youn et al. reported that JMJD5 hydroxylates the transcription factor NFATc1, stimulating its ubiquitin-mediated proteasomal degradation (53). Although the hydroxylated residue(s) in NFATc1 has yet to be identified, the structure and dimensions of the active site of JMJD5 suggest that its substrate specificity may differ from that of FIH-1 as the residues responsible for asparaginyl and histidinyl substrate recognition in FIH-1 are not conserved in JMJD5 (Fig. 4B). Future studies are required to address the enzymatic activity, substrate specificity, and biological functions of JMJD5 as a putative protein hydroxylase.

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