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Characterization of a Linked Jumonji Domain of the KDM5/JARID1 Family of Histone H3 Lysine 4 Demethylases*

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The KDM5/JARID1 family of Fe(II)- and α-ketoglutarate-dependent demethylases remove methyl groups from tri- and dimethylated lysine 4 of histone H3. Accumulating evidence from primary tumors and model systems supports a role for KDM5A (JARID1A/RBP2) and KDM5B (JARID1B/PLU1) as oncogenic drivers. The KDM5 family is unique among the Jumonji domain-containing histone demethylases in that there is an atypical insertion of a DNA-binding ARID domain and a histone-binding PHD domain into the Jumonji domain, which separates the catalytic domain into two fragments (JmjN and JmjC). Here we demonstrate that internal deletion of the ARID and PHD1 domains has a negligible effect on in vitro enzymatic kinetics of the KDM5 family of enzymes. We present a crystal structure of the linked JmjN-JmjC domain from KDM5A, which reveals that the linked domain fully reconstitutes the cofactor (metal ion and α-ketoglutarate) binding characteristics of other structurally characterized Jumonji domain demethylases. Docking studies with GSK-J1, a selective inhibitor of the KDM6/KDM5 subfamilies, identify critical residues for binding of the inhibitor to the reconstituted KDM5 Jumonji domain. Further, we found that GSK-J1 inhibited the demethylase activity of KDM5C with 8.5-fold increased potency compared with that of KDM5B at 1 mM KDM5C with 8.5-fold increased potency compared with that of KDM5A. Kinetics of the KDM5 family of enzymes. We present a crystal structure of the linked JmjN-JmjC domain from KDM5A, which reveals that the linked domain fully reconstitutes the cofactor (metal ion and α-ketoglutarate) binding characteristics of other structurally characterized Jumonji domain demethylases. Docking studies with GSK-J1, a selective inhibitor of the KDM6/KDM5 subfamilies, identify critical residues for binding of the inhibitor to the reconstituted KDM5 Jumonji domain. Further, we found that GSK-J1 inhibited the demethylase activity of KDM5C with 8.5-fold increased potency compared with that of KDM5B at 1 mM KDM5B. In contrast, JIB-04 (a pan-inhibitor of the Jumonji demethylase superfamily) had the opposite effect and was ~8-fold more potent against KDM5B than against KDM5C. Interestingly, the relative selectivity of JIB-04 toward KDM5B over KDM5C in vitro translates to a ~10–50-fold greater growth-inhibitory activity against breast cancer cell lines. These data define the minimal requirements for enzymatic activity of the KDM5 family to be the linked JmjN-JmjC domain coupled with the immediate C-terminal helical zinc-binding domain and provide structural characterization of the linked JmjN-JmjC domain for the KDM5 family, which should prove useful in the design of KDM5 demethylase inhibitors with improved potency and selectivity.

It is now well established that cancer arises in part from an altered “epigenome,” with widespread alterations in chromatin modifications (both DNA and histones) contributing to altered gene expression programs and a progressive loss of genome stability (1). Many epigenetic regulators, including DNA methyltransferases (DNMT3A), 5-methylcytosine dioxygenases (TET2), histone methyltransferases (EZH2 and MLL1/2/3), histone demethylases (UTX and KDM5A/B/C), ATP-dependent chromatin remodelers (ATRX and CHD1/4), and even the histone proteins themselves (histone H3.3), undergo somatic mutations and/or are misexpressed in human cancers (2–4). These observations suggest that epigenetic dysregulation is not simply a characteristic of cancer cells but probably plays a direct causal role in the development and progression of the disease. Accordingly, there is a great deal of interest in targeting epigenetic regulators as a therapeutic approach in cancer treatment and prevention with the goal of “reprogramming” the cancer epigenome.

Thus far, the clinical application of epigenetic therapy has been limited to inhibitors of the DNA methyltransferases (5, 6) and the histone deacetylases (7–10). Several histone methyltransferase inhibitors, including pinometostat (EPZ-5676), an inhibitor of the DOT1L histone H3 lysine 79 methyltransferase (11–13), and inhibitors of the EZH2 histone H3 lysine 27 methyltransferase (11, 14–16), are in Phase I trials. Histone lysine demethylases, on the other hand, remain a relatively untapped source of potential “druggable targets.” A few pan-inhibitors of the Jumonji family of demethylases have been identified. GSK-J1, initially identified as a selective inhibitor against KDM6/JMJD3/UTX (17), also inhibits JARID1/KDM5 in vitro (18). JIB-04 was identified using a cell-based screen for epigenetic drivers with improved potency and selectivity.

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The atomic coordinates and structure factors (code 5E6H) have been deposited in the Protein Data Bank (http://wwpdb.org).

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KDM1 (20, 21) that removes methyl groups from mono- and dimethylated lysine 4 of histone H3 (22).

The JARID1 (Jumonji, AT-rich interactive domain) family of histone lysine demethylases represents one such potential target. The JARIDs are multidomain proteins containing a Jumonji domain that catalyzes the removal of methyl marks from histone H3 di- and trimethylated at lysine 4, an ARID DNA binding domain (23, 24), several histone-interacting PHD domains (25, 26), and an uncharacterized but conserved PLU-1 domain (Fig. 1a). Mounting evidence from primary tumors and model systems supports a role for the KDM5 family as onco-genic drivers (reviewed in Ref. 27). KDM5A (also known as JARID1A or RBP2) was originally identified as a retinoblastoma-binding protein (28, 29). Subsequent work showed that the tumor-suppressive activity of retinoblastoma is dependent upon its ability to sequester KDM5A (30). In estrogen receptor-negative breast cancers, KDM5A mediates metastatic spread to the lung (31). Consistent with an oncogenic role, genetic ablation of kdm5a delays tumor onset in retinoblastoma mutant mice (32).

KDM5B (also known as JARID1B or PLU-1) expression is normally restricted to the testis and brain but is up-regulated in many tumor types, including breast (33, 34), prostate (35, 36), ovarian, and melanoma (37, 38). KDM5B amplification is particularly prevalent in HER2+ and luminal breast cancers, where it is reported to be a lineage-driving oncogene (39). KDM5B is regulated by HER2 signaling (33), and knockdown of KDM5B slows or inhibits tumor growth in a human tumor xenograft model (40). More recent data indicate that KDM5B negatively regulates leukemogenesis in murine and human mixed lineage leukemia-rearranged acute myeloid leukemia cells, suggesting a crucial role for the histone H3 Lys-4 “methylome” in determining leukemic stem cell fate (41).

KDM5C (JARID1C or SMCX) is located on the X chromosome and has been implicated in the pathogenesis of Huntington disease (42). Down-regulation of kdm5c expression significantly reduces the toxicity from mutant Huntingtonin expression, restored the level of key neuronal genes, and was neuroprotective in murine and Drosophila Huntington disease models. As such, KDM5A, -B, and -C all have potential as drug targets.

The KDM5 family is unique among Jumonji-containing histone demethylases in that the catalytic domain has an atypical binding mode to engage metal and α-ketoglutarate, the two reaction cofactors, as that of other structurally characterized Jumonji demethylases. Our data establish the reconstituted, linked Jumonji domain and the helical zinc-binding domain as the minimal domains required for catalytic activity of the KDM5 family.

**Experimental Procedures**

**Cloning, Expression, and Purification—**N-terminal fragments of human KDM5A, -C, and -D were PCR-amplified from human cDNA clones obtained from the American Type Culture Collection (GenBank™ number BC110916 for KDM5A, BC054499 for KDM5C, and BC144102 for KDM5D) and KDM5B (a gift of Joyce Taylor-Papadimitriou of King’s College London; GenBank™ number BC157031) using Vent or Phenom DNA polymerase and cloned into a pET28 plasmid containing an N-terminal His-SUMO tag sequence (48). The internal deletion constructs, deleting ARID and PHD1 domains (ΔAP), were made by PCR. Primers corresponding to regions flanking the ARID and PHD1 domains plus the linker sequences were used for PCR of the entire parental plasmid except the ARID-PHD1. After DpnI digestion, the PCR fragment was purified, end-labeled by T4 polynucleotide kinase, and ligated by T4 ligase to form the deletion construct used for expression. Recombinant constructs (Fig. 1b) were transformed into Escherichia coli strain BL21(DE3)C+ for protein expression.

Typically 2-liter (for Jumonji domain only) or 12-liter (for Jumonji + helical zinc-binding domain) LB cultures were inoculated with starting culture and grown at 37 °C to an A600 of 0.4 to 0.8, at which point the temperature was reduced to 16 °C. After 1–2 h, 0.1 mM isopropyl-β-D-thiogalactopyranoside was added to induce the expression of recombinant protein over-night. Cells were collected by centrifugation, and the pellets were resuspended in 20 mM HEPES, pH 8.0, 5% glycerol, 300 mM NaCl, and 0.5 mM tris(2-carboxyethyl)phosphine (the KDM5 storage buffer). Cells were broken using a French press and then limited sonication. The cell lysate was cleared by centrifugation at 37,000 × g for ~1 h, and the soluble portion was loaded onto a Ni2+ affinity column and eluted with an imidazole gradient (12–300 mM). The His-SUMO tag was cleaved overnight at 6 °C by Ulp-1 protease (in-house) digestion (48). The protein was further purified by anion exchange (Hitrap Q, GE Healthcare) and gel filtration (Superdex S200, GE Healthcare) chromatography. The final sizing column was equili-brated with the KDM5 storage buffer, and the eluted protein was concentrated using a 10,000 molecular weight cutoff Vivaspin concentrator for crystallization or snap-frozen in liquid nitrogen and stored at ~80 °C for later use.

**Expression and Purification of Formaldehyde Dehydrogenase (FDH)**—Two 1-liter LB cultures of E. coli strain BH249 cells containing a His-tagged Pseudomonas putida FDH construct (a

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4The abbreviations used are: FDH, formaldehyde dehydrogenase; APAD+, 3-acetylpyridine adenine dinucleotide; α-KG, α-ketoglutarate; H3(1–24)K4me3, histone H3(1–24) trimethylated Lys-4 peptide; Gmax, half-maximal growth-inhibitory concentration; PDB, Protein Data Bank; ITC, isothermal titration calorimetry.
A Linked JmjN-JmjC Domain of KDM5

GSK-J1 (Sigma-Aldrich SML0709; 20 mM in 100% DMSO) and APAD was added (0.4 mM) to induce expression over H9262 (0–100 M). Reactions were performed in a 40 M volume at room temperature (~21 °C) for 10 min under the conditions of 0.5 μM [E], 15 μM H3(1–24)K4me3 [S] with variable αKG concentration (0–100 μM) or 1 mM αKG with variable peptide [S] concentration (0–50 μM) in the KDM5 reaction buffer (2 mM ascorbic acid, 50 μM (NH₄)₂Fe(SO₄)₂, 50 mM HEPES (pH 7.0), 7.5 mM NaCl (carried over from the enzyme stock), 0.6 mM APAD⁺, and 10 μM (3 mM tetramer) FDH) (50 mM MES, pH 6.6, for KDM5C). All assay components were preincubated at room temperature for 15 min followed by the addition of αKG or peptide and APAD⁺ to initiate the reaction. A BioTek Synergy™ hybrid microplate reader was used to monitor the reaction using 380/20 excitation and 460/40 emission filters with a 400-nm dichroic mirror. Initial velocity was plotted against αKG or peptide concentration and fitted with the Michaelis-Menten equation.

Inhibition of demethylation activity of KDM5B(1–755)ΔAP and KDM5C(1–789)ΔAP on H3(1–24)K4me3 substrate by GSK-J1 (Sigma-Aldrich SML0709; 20 mM in 100% DMSO) and JIB-04 (20 mM in 100% DMSO) was performed under conditions of 0.5 μM enzyme [E], 15 μM peptide [S], 1 mM αKG, and variable inhibitor [I] in the KDM5 reaction buffer with 10% DMSO. Enzyme was preincubated with αKG and inhibitor at room temperature prior to the addition of the substrate peptide and APAD⁺, and the reactions were carried out for 10 min at room temperature. The demethylation activity was not affected by the addition of less than 10% DMSO (Fig. 2d).

Mass Spectrometry-based Demethylation Assays—Activity assays with histone peptides were measured by MALDI-TOF mass spectrometry using a Bruker Ultra FlexII TOF/TOF instrument (Biochemistry Department). Reactions were initiated by adding histone peptide H3(1–24)K4me3 ([S] = 15 mM) to preincubated KDM5B(1–755)ΔAP or KDM5C(1–789)ΔAP ([E] = 0.75 μM) with 1 mM αKG at room temperature in the KDM5 reaction buffer (without FDH and APAD⁺) (50 mM MES, pH 6.6, for KDM5C). At different time points, 2 μl of sample was withdrawn and mixed with 5 μl of 0.1% trifluoroacetic acid (TFA) to stop the reaction. These mixtures representing reaction progression at various time points were mixed with α-cyano-4-hydroxycinnamic acid as a MALDI matrix in 70% acetonitrile, 30% H₂O (1:1) and spotted onto a stainless steel mass spectrometry sample plate. After the sample dried out, the sample plate was mounted in the MALDI-TOF instrument, and the sample mass spectra were recorded and examined for peptide mass changes at 14, 28, or 42 daltons before and after the reaction, corresponding to mono-, di-, and trimethylation (Fig. 2e). The average number of methyl groups removed per peptide was converted from relative abundance: $n = 3 \times (\text{me0\%}) + 2 \times (\text{me1\%}) + 1 \times (\text{me2\%})$. The reaction time course was determined by integrating the relative abundance of each methylated Lys-4 species. The Dynafit program (BioKin Ltd.) was used for global fitting of the demethylation kinetic data.

Isothermal Titration Calorimetry (ITC)—The ITC experiments were carried out at an enzyme concentration of 0.2–0.4 mM with 4–8 mM αKG (Sigma-Aldrich K2010-25G) or N-oxalylglycine (the cofactor analog; Alexis Biochemicals) in the KDM5 storage buffer with 2 mM MnCl₂. A MicroCal Auto-iTC200 was used to perform 25 4-μl injections of compounds into protein or buffer (to measure the heat of dilution). Binding constants ($K_d$) were calculated by fitting the data using the ITC data analysis module of Origin version 7.0 (OriginLab Corp.).

Crystallography—KDM5A(1–588)ΔAP was mixed with MnCl₂ and α-ketoglutarate (in the KDM5 storage buffer) at a molar ratio of 1:5, concentrated to 53 mg/ml, and then diluted with concentration filtrate to 40, 20, and 5 mg/ml for crystallization trials. Crystals of this tertiary complex were grown using sitting drop vapor diffusion at 16 °C by mixing 0.2 μl of the complex with an equal volume of a well solution containing trial conditions. KDM5A(1–588)ΔAP crystals grew in clusters with the well condition 1.5 M ammonium sulfate, 12% glycerol, and 0.1 M Tris-HCl, pH 8.5. The best crystals appeared in low concentration samples (5 mg/ml). The crystal cluster was broken, and the best visual crystals were mounted into nylon cryoloops (Hampton Research, Inc.). Crystals were frozen in liquid nitrogen after the addition of ~15% more glycerol to the mother liquor as a cryoprotectant.

X-ray diffraction data were collected on the SER-CAT beamline ID-22 at the Advanced Photon Source at Argonne National Laboratory at 100 K and were processed and merged with HKL2000 (50). The structure was determined by molecular replacement using the PHyre2-generated homology model (Fig. 1c) as an initial search model. Repeated rounds of manual refitting and crystallographic refinement were performed using COOT (51, 52) and PHENIX (53, 54) (Table 1). We note that the traditional $R_{merge}$ value, which is poorly suited to assess data quality (55), is relatively higher, whereas the $CC_{diff}$ and $R_{merge}$ values are useful indicators for data quality (56) (Table 1).

Sulfuricodemine B Cell Growth Assay—MCF7 and MDA-MB231 breast cancer cell lines were maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and 2 mM glutamine and cultured in a humidified incubator at 37 °C and 5% CO₂. JIB-04 (Sigma-Aldrich SML0808), GSK-J4 (Sigma-
TABLE 1

Statistics of x-ray data collection and refinement

<table>
<thead>
<tr>
<th></th>
<th>KDM5A(1–588)ΔAP</th>
</tr>
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<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
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<tr>
<td>Space group</td>
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<tr>
<td>Cell dimensions</td>
<td></td>
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<td>α = γ, β (degrees)</td>
<td>90, 92.6</td>
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<tr>
<td>a, b, c (Å)</td>
<td>115.4, 61.6, 46.6</td>
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<td>Beamline (SERCAT)</td>
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<tr>
<td>Resolution (Å)</td>
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<tr>
<td>Rmerge-b</td>
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<tr>
<td>Rmerge</td>
<td>0.068 (0.262)</td>
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<tr>
<td>CC1-c</td>
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<tr>
<td>CC1</td>
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<tr>
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<td>13.3 (3.7)</td>
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<tr>
<td>Completeness (%)</td>
<td>99.5 (97.0)</td>
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<tr>
<td>Redundancy</td>
<td>11.7 (4.5)</td>
</tr>
<tr>
<td>Observed reflections</td>
<td>183,848</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>15,751          (1538)</td>
</tr>
</tbody>
</table>

| **Refinement**           |                 |
| Resolution (Å)           | 2.24            |
| No. of reflections       | 15,751          |
| Rwork/Rmerge             | 0.179/0.220     |
| No. of atoms             | 2529            |
| Protein                  | 2406            |
| Mn(II), aKG              | 11              |
| Solvent                  | 38.5            |
| Mn (II), aKG             | 28.6            |
| Solvent                  | 38.5            |
| Root mean square deviations |                 |
| Bond lengths (Å)         | 0.004           |
| Bond angles (degrees)    | 0.7             |

* Values in parenthesis correspond to the highest resolution shell.

**References**

1. Aldrich SML0701 and GSK-J5 (Cayman 12074) were dissolved in 100% DMSO to 20 and 40 mm, respectively, and stored at −20 °C.

2. For growth inhibition assays, MCF7 and MDA-MB231 cells were seeded in triplicate in a 96-well plate (5000 cells/well) and allowed to adhere overnight, and JLB-04, GSK-J4, GSK-J5, or DMSO (vehicle) was added the next day. After 72-h treatment, cells were fixed on the plate with cold 10% TCA (100 µl/well) for 1 h at 4 °C, washed three times in distilled H2O, and air-dried. Cells were stained with 0.4% sulforhodamine B in 1% acetic acid (100 µl/well) for 30 min, washed three times in 1% acetic acid, and air-dried. Solubrhamine B dye was resolubilized in 200 µl of 10 mm Tris base (pH 10.5), and the A510 was measured by a spectrophotometer. The percentage of control cell growth was calculated as the A510 at day 3 minus that at day 0 among treated cells relative to that of an untreated control.

**Results**

A Hypothetical Model of the N-terminal Half of KDM5—To accurately define the domain boundaries between JmJN and ARID and between PHD1 and JmJC (Fig. 1a), we generated a hypothetical structural model for the N-terminal half of the KDM5 family, which is enzymatically active in vitro (43, 44), using PHYRE2 (Protein Homology/analogy Recognition Engine) (57). With PHYRE2, we generated individual domain models based on known structural information. For KDM5B, the JmJN (residues 26–95) and JmJC (residues 363–604) were modeled as an integral domain based on JMJD2A (PDB entry 2OS2) or rce JM703 (PDB entry 4I6P) (47, 58). The ARID (residues 96–188), PHD1 (residues 301–362), and C-terminal (residues 605–751) domains were modeled based on the structures of the ARID domain of mouse jarid1b (PDB entry 2EQY), the PHD domain of PHF8 (PDB entry 3KV4) (59), and the C-terminal helical zinc-binding domain of UTX/KDM6A (PDB entry 3AVS) (60), respectively. No homology model was generated for residues 189–300 between the ARID and PHD1 domains.

The domain structures were juxtaposed manually and assembled to finalize a hypothetical three-dimensional model (Fig. 1c). With this model, we observed that the connections between JmJN and ARID and between PHD1 and JmJC are immediately adjacent to each other, possibly via two antiparallel β strands (Fig. 1d). We hypothesized that an extended loop could replace the ARID and PHD1 domains and connect the two strands together, maintaining the Jumonji catalytic domain structure. Similar models for KDM5A and -5C were generated (not shown).

Generating Constructs for Directly Linked JmJN-JmJC of KDM5A, -B, and -C—Based on the PHYRE2 model, we generated two constructs for KDM5B: residues 1–755 and residues 1–755ΔAP (deleting ARID and PHD1 (AP) domains by connecting residues 100 and 363). Corresponding constructs were also made for KDM5A residues 1–739 with and without AP and for KDM5C residues 1–769 with and without AP (Fig. 1, a and b). We also generated constructs with different C-terminal ends (e.g. at residues 769, 789, and 839 of KDM5C). These ΔAP constructs represent the domain arrangement of the conventional Jumonji domain followed by a C-terminal helical zinc-binding domain and are analogous to that of the KDM6B/UTX/JMJD3 family (61). A homologous C-terminal helical zinc-binding domain is also present in the rice JM703 (47).

In KDM6A/UTX, the C-terminal zinc-binding domain is involved in recognizing a portion of the histone H3 peptide (residues 17–23) N-terminal to the methylated target lysine (H3 Lys-27) (60), thereby excluding the histone H3 Lys-9 with identical immediate neighboring residues (ARKS) as a substrate. In a separate structural study, a short H3 peptide (residues 24–34) was used in complex with mouse KDM6B/JMJD3, and only the Jumonji domain was involved in peptide binding (17). Similarly, the isolated Jumonji domain of the rice JM703 was observed to interact with three of the 10 residues in the trimethylated histone H3 Lys-4 peptide (1–10) used in crystallization (47). We thus generated constructs corresponding to the approximate size of the Jumonji domain, KDM5A(1–588)ΔAP, KDM5B(1–604)ΔAP, and KDM5C(1–618)ΔAP, by removing the C-terminal helical zinc-binding domain.

All constructs were expressed in E. coli and exhibited variable expression levels and solubility (Fig. 1, b and c). In general, constructs with longer C-terminal ends that included the helical zinc-binding domain were much less soluble than those without. For example, the shorter constructs, KDM5A(1–588)ΔAP, KDM5B(1–604)ΔAP, and KDM5C(1–618)ΔAP, generated 20–30 mg of soluble protein from a 1-liter culture.
The longer constructs resulted in either insoluble products (KDM5A) or 1–3 mg of purified enzyme/liter of culture (KDM5B and KDM5C) (Fig. 1b).

Deletion of ΔAP Has No Effect on Kinetics of KDM5C—To investigate the effect of deleting ARID and PHD1 domains (ΔAP), we compared the kinetic parameters of KDM5C(1–789)ΔAP with those of KDM5C(1–839), which we had previously characterized (43). Under the optimum conditions established for KDM5C(1–839) (Fig. 2f), we obtained kinetic parameters for KDM5C(1–789)ΔAP showing nearly identical characteristics between the two enzyme constructs: K_m values for αKG (6 ± 1 versus 5.4 ± 0.5 μM), K_m value for histone H3(1–24)K4me3 (3.3 ± 0.2 versus 3.3 ± 0.4 μM), and turnover rate k_cat (2.0–2.7 versus 2.5–3.1 min⁻¹) (Fig. 3, a and b). We thus concluded that deletion of AP and further shortening the C-terminal residues from 839 to 789 have negligible effects on the in vitro enzymatic activity of KDM5C using peptide substrates.

We next compared the activities of two KDM5C constructs, KDM5C(1–789)ΔAP and KDM5C(1–618)ΔAP, representing the linked Jumonji domain with and without the C-terminal helical zinc-binding domain (Fig. 2h). Using the histone peptide H3(1–24)K4me3, the shorter construct without the C-terminal domain was inactive (Fig. 2h). The lack of activity in the absence of the zinc-binding domain may stem from the inability to properly engage the H3 substrate, consistent with our previous observation that the activity of KDM5C requires the substrate H3 peptide to be at least 12 amino acids long (residues 1–12) (43). KDM5 enzymes might be similar to KDM6A in this regard, where the C-terminal helical zinc-binding domain was shown to be essential for enzymatic activity by binding a portion of the histone H3 peptide (60).

KDM5BΔAP and KDM5CΔAP Have Comparable Demethylation Activity—In parallel, we analyzed the demethylase activity of KDM5B(1–755)ΔAP, using the optimal conditions of room temperature, pH 7.0, and low ionic strength (Fig. 2, e, g, and i). Under these conditions, we determined the K_m value for αKG to be ~8 μM and the K_m for peptide substrate to be ~4 μM (Fig. 3, c and d). The turnover rate (k_cat) is ~2 min⁻¹. These results indicate that KDM5B(1–755)ΔAP and KDM5C(1–789)ΔAP have comparable demethylase activity on the same trimethylated histone H3 Lys-4 peptide substrate. We further found that KDM5B(1–755)ΔAP has no activity on the corresponding trimethylated histone H3 Lys-9– or H3 Lys-27-containing peptides (Fig. 2j).

Using H3(1–24)K4me3 as the initial substrate, we applied quantitative mass spectrometry to monitor the kinetics of product formation (Fig. 3, e and f). For the KDM5B(1–755)ΔAP and KDM5C(1–789)ΔAP enzymes, we observed a rapid disappearance of trimethylated histone H3 Lys-4 in the first 5–10 min of the reaction with a corresponding appearance of a transient peak of dimethylated histone H3 Lys-4 (up to 50–60%), followed by conversion to monomethylated H3 Lys-4. We determined the kinetic rate constants for each of the three demethylation steps using nonlinear least-squares regression analysis. The first conversion from trimethylated to dimethylated is faster (k_1 ~ 47 h⁻¹ for KDM5B and 32 h⁻¹ for KDM5C) than the second conversion from dimethylated to monomethy-
A Linked JmjN-JmjC Domain of KDM5

(a) me0
K4—N—H
| H
(b) O=CH₂
(Formaldehyde)

(b) (Formaldehyde dehydrogenase)

(c) γ = 0.0008x
R² = 0.99222

(d) V (μM/min)

(e) Room Temperature

(f) # of CH₃ removed per Peptide

(g) # of CH₃ removed per Peptide

(h) KDM5C(1-789)ΔAP

(i) KDM5C(1-41B)ΔAP

(j) H3(1-15)K9me3

(k) H3(21-45)K27me3

[|E|=0.325 μM
[S]=5 mM peptide
[t]=120 min]
FIGURE 2. Overview of demethylation reactions catalyzed by KDM5 family. a, for protein lysine demethylation, the Fe(II)- and α-ketoglutarate-dependent Jumonji dioxygenases generate a hydroxymethyl intermediate (-N-CH$_2$OH) for each reaction that subsequently decomposes to release a formaldehyde spontaneously (without additional enzymatic activities) and the demethylated lysine (with one methyl group removed). b, the reaction product formaldehyde can be converted by FDH to formate. This process is coupled with the reduction of NAD$^+$ to NADH, and the fluorescence generated by NADH can be monitored to reflect the rate of the coupled reactions, by converting fluorescence intensities to formaldehyde concentrations using the calibration plot shown in c. c, maximum fluorescence intensities obtained after saturation of the fluorophore were plotted against the corresponding formaldehyde standard solutions of known concentrations to convert fluorescence intensity into formaldehyde concentration. d, low concentrations of DMSO (≤ 10%) have no effect on activity of KDM5C(1–789)AP. The velocity of reactions was determined with $[S] = 15$ μM H3K4(1–24)me3 and $[E] = 0.5$ μM JARID1C(1–789)AP. The DMSO concentrations tested were 2-fold dilutions of 10% to 5, 2.5, 1.25, and 0.63%. e, traces of MALDI-TOF mass spectrometry of sample reactions of KDM5B(1–755)AP at room temperature (left) and 37 °C (right). f, optimal pH for the demethylase activities of KDM5C(1–789)AP activity (g), which is the same as our previously determined optimal conditions established for KDM5C(1–789)AP (43), and KDM5B(1–755)AP (h), which has an optimum pH of 7.0. The reactions were carried out under the KDM5 reaction buffer conditions with varying pH as indicated (50 mM MES for KDM5C or 50 mM HEPES for KDM5B). h, FDH-coupled assays of demethylase activities of KDM5C(1–789)AP and KDM5C(1–618)AP under the conditions of $[E] = 0.5$ μM and $[S] = 50$ μM H3(1–24)K4me3. An assay with no enzyme was used to determine background fluorescence. i, KDM5B(1–755)AP has no activity on trimethylated Lys-9- and trimethylated Lys-27-containing peptides.

FIGURE 3. Kinetic parameters of KDM5C(1–789)AP and KDM5B(1–755)AP. a–d, Michaelis-Menten kinetic plots of KDM5C(1–789) (a and b) and KDM5B(1–755) (c and d) for the cofactors αKG (a and c) and substrate H3 peptide (b and d), measured by an FDH-coupled demethylase assay. Initial velocities were plotted against αKG or peptide concentrations and fit with the Michaelis-Menten equation using GraphPad Prism version 5.0. Error bars, S.E. from two independent experiments. e and f, kinetic progression of demethylation reaction catalyzed by KDM5C(1–789) (e) and KDM5B(1–755) (f), measured by MALDI-TOF mass spectrometry. g, summary of the kinetic constants for each demethylation step derived from the global fitting of the experimental data. The apparent $k_{cat}$ value for each step was derived from the data in e and f using the formula $k_{cat} = k_i \times [S]/[E]$, where $i = 1, 2$, and 3, and $[S]/[E] = 20$. H3K4me0, -me1, -me2, and -me3, un-, mono-, bi-, and trimethylated histone H3 Lys-4, respectively.

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lated. The two enzymes showed a larger difference (~7-fold) in the second conversion rate, with \( k_2 \approx 22 \text{ h}^{-1} \) for KDM5B and 3 \text{ h}^{-1} \) for KDM5C. The last conversion from monomethylated to unmethylated is the slowest, with the negligible \( k_3 \approx 0.1–0.2 \text{ h}^{-1} \), resulting in prolonged accumulation of monomethylated form. Fig. 3 summarizes the apparent \( k_{\text{cat}} \) values for each of the three demethylation steps. Our in vitro observations are in agreement with the observation that distal gene regulatory elements, such as enhancers, are typically enriched for monomethylated H3 Lys-4 despite occupancy by KDM5B/C (62–65).

**Inhibition of KDM5B\(1–755\)AP and KDM5C\(1–789\)AP by GSK-J1 and JIB-04—**Two small molecule inhibitors, GSK-J1 and JIB-04, have been reported to inhibit KDM5 family members, in addition to other Jumonji-containing histone demethylases (18, 19). We found that GSK-J1 inhibited the demethylase activity of KDM5B\(1–755\)AP with an \( IC_{50} \approx 94 \mu \text{M} \) (Fig. 4a) under the assay conditions of 1 mM αKG, whereas it had ~8.5-fold higher potency against KDM5C\(1–789\)AP (\( IC_{50} \approx 11 \mu \text{M} \)) (Fig. 4b). On the other hand, JIB-04 had the opposite effect. JIB-04 was an ~8-fold more potent inhibitor of KDM5B\(1–755\)AP activity than it was of KDM5C\(1–789\)AP (\( IC_{50} \approx 5 \mu \text{M} \) versus 42 \( \mu \text{M} \)) (Fig. 4c, compare c and d). These observations seem to suggest that selective inhibition within the KDM5 family members is attainable. In a separate study, GSK-J1 was shown to have greater in vitro inhibitory activity against KDM5B and KDM5C than against KDM5A, by factors of ~40 and 12, respectively (18). (We note that different fragments of KDM5A, -B, and -C and different assay conditions and detection methods were used in this study (Table 2)).

To understand how in vitro differences in the specificity of the KDM inhibitors influence cellular activity, we determined the impact of JIB-04 and GSK-J4 on the growth of two breast cancer cell lines, MDA-MB231 and MCF7. GSK-J4 is a cell-permeable prodrug that is hydrolyzed by esterases within the cell to generate GSK-J1. GSK-J5 is a less active isomer of GSK-J4 (17) and was used as a negative control. Overall, the breast cancer cells were ~10–50-fold more sensitive to JIB-04 treatment than to GSK-J4, with half-maximal growth-inhibitory concentrations (\( GI_{50} \)) in the 20–300 nM range for JIB-04 versus...
1–3 μM for GSK-J4 after 72 h of treatment (Fig. 4, compare e and f). Additionally, JIB-04 exhibited differential activity among the breast cancer cell lines. MCF7 cells were 14-fold more sensitive to the antiproliferative effects of JIB-04 than were MDA-MB231 cells, with Gl50 ~ 22 nM (range = 16–25 nM, n = 3) versus 300 nM (range 270–330 nM, n = 3) (Fig. 4f). In contrast, GSK-J4-induced growth inhibition demonstrated relatively little cell type specificity, comparing MCF7 cells (GI50 ~ 0.9–1.0 μM, n = 3) versus MDA-MB231 cells (GI50 ~ 2.9 μM, range = 1.3–5.2 μM, n = 4) (Fig. 4e). GSK-J5 showed little growth-inhibitory activity against either cell line (GI50 > 40 μM). Thus, the relative selectivity of JIB-04 toward KDM5B versus KDM5C above correlated with an increased cellular potency overall and a propensity for cell type specificity not observed with GSK-J4 (at least between these two cell lines) and may reflect dissimilarities in the underlying biology of the cell lines. For example, breast cancer cells may be more dependent on the KDM5 family versus KDM6, the preferred target of GSK-J1/4 in vitro (17, 18). Cell type-specific differences in the levels or activity of KDM4 may also contribute because JIB-04 has nearly equivalent activity against KDM4 family enzymes in vitro and against cellular histone H3 trimethylated Lys-9 demethylase activity (19). Differential effects on cytoplasmic targets of the KDM enzymes may also play a role because JIB-04 has been recently shown to inhibit translation and to sensitize cells to the growth-inhibitory effects of mechanistic target of rapamycin (mTOR) inhibitors, possibly through its effects on KDM4A or KDM5A, both of which were found to associate with polyamines (66).

Structure of the KDM5A(1–588)ΔAP-linked JmjN-JmjC Domain—We set up crystallization trials of KDM5A/B/C in various configurations. Our first success came with KDM5A(1–588)ΔAP in complex with α-ketoglutarate and Mn(II). The crystal diffracted x-rays to a resolution of 2.24 Å (Table 1). The structure was determined by molecular replacement using the PHYRE2-generated homology model (described above) as the initial search model. Previously, we had used a PHYRE homology model in structure determination of the ankyrin repeat domain of G9a histone methyltransferases in complex with a histone peptide (67).

The electron density map is of excellent quality, and much of the backbone of the structure was visible with the exception of three regions: the N-terminal 11 residues, the 12-residue linker between JmjN and JmjC, and a 10-residue loop (amino acids 457–466) prior to strand β7. The disordered loop is right next to the C-terminal residue (after strand β14 in Fig. 5a), which would lead to the C-terminal helical zinc-binding domain in the full-length protein, and the flexibility may suggest that the loop would adopt a more stable conformation upon interaction with the C-terminal domain. Although the crystallized fragment, KDM5A(1–588)ΔAP, is inactive in the absence of the immediate C-terminal helical domain (Fig. 2h), deletion of the AP alone from the active KDM5C (e.g. KDM5C(1–789)ΔAP) does not affect activity. We thus infer that our current structure should shed light on the active site geometry and the interactions with the cofactors.

Like other structurally characterized αKG-dependent dioxygenases (68), the linked JmjN-JmjC of KDM5A has a core double-stranded β-helix fold that binds Fe(II) and αKG (Fig. 5, b–d). Two twisted β-sheets (a four-stranded minor sheet in cyan and an eight-stranded major sheet in dark blue and gray) pack together with helices on the outer surfaces of the major and minor sheets (Fig. 5a). The JmjN residues (dark gray) appear to be critical to the structural integrity of the molecule; the segment surrounds nearly the entire molecule in the orientation shown (Fig. 5a), providing three strands (β1, β2, and β4) on the edges of the major β sheet and two helices (αA and αB) supporting the sheet. Pro-41, located in the middle of the kinked helix αB, is conserved among the four KDM5 family members (see Fig. 7). Interestingly, a kinked helix is also observed in the Naegleria Tet-like dioxygenase, performing the same function of supporting the corresponding major β sheet (69).

A third, two-stranded antiparallel β sheet is formed by strand β3 of JmjN and strand β5 of JmjC (Fig. 5a, bottom). These are the two corresponding strands initially suggested by the PHYRE2 model; one leads to the ARID domain, and the other returns from the PHD1. At the C-terminal end of JmjN (which is also the beginning of ARID), we observed a small one-turn helix αC (magenta); the same helix was observed in the NMR structure of the ARID domain of KDM5C/JARID1C (70) (Fig. 5e).

The unequal number of strands of the two sheets creates an active site located asymmetrically on the side of the molecule where the mouth of the two sheets opens up. The loop interconnecting strand β8 of the minor sheet to β9 of the major
sheet and strand β13 provide invariant residues for binding of the metal ion (His-483, Glu-485, and His-571), forming the ferrous binding motif, HX(E/D) . . . H (68). The α-ketoglutarate and its non-reactive analog N-oxalylglycine are bound with a dissociation constant (K_D) of 80 and 47 µM, respectively, measured by isothermal titration calorimetry (Fig. 5, f and g). The cofactor αKG is in extensive polar (Tyr-409, Ser-491, Asn-493, and Lys-501) and hydrophobic interactions with the protein (Tyr-472, Phe-480, Trp-503, and Ala-583) (Fig. 5, b and c). The importance of these interactions is underscored by the fact that αKG-interacting residues are invariant among the KDM5 family members and highly conserved among the other Jumonji family of histone demethylases. For example, seven of eight residues of KDM5A involved in αKG binding are conserved in KDM6A (Fig. 6a), except for Phe-480 of KDM5A, which is replaced by Ser-1143 in KDM6A in the primary sequence. Although Lys-501 of KDM5A and Lys-1137 of KDM6A do not align in the primary sequence and project from different sides of the catalytic site, their terminal ε-amino groups occupy the same location in space, both in close contact with αKG (Fig. 6a).

Comparison with KDM6 — The high structural similarity of KDM5A with other structurally characterized catalytic Jumonji domains (such as KDM6A; root mean square deviation = 2.4 Å across 207 aligned Cα pairs) allows us to model a methyl-lysine substrate and known inhibitors into the active site of KDM5A.

FIGURE 5. Structure of linked JmjN-JmjC of KDM5A(1–588),ΔAP and αKG binding. a, the KDM5A catalytic domain has two twisted β-sheets (a four-stranded minor sheet in cyan and an eight-stranded major sheet in dark blue and gray) with helices packed on the outer surfaces of the major and minor sheets. The yellow circle indicates the bound metal Mn(II). b and c, two views of Mn(II)-αKG (in yellow) binding in the active site of KDM5A. Note the octahedral coordination of Mn(II) by the His-483, Glu-485, His-571, αKG (two ligands), and a water molecule. d, omit electron densities, contoured at 5 and 3 above the mean, are shown for Mn(II) (magenta mesh) and αKG (green mesh), respectively. e, the corresponding helix C (magenta) in the independently determined structure of KDM5C ARID domain (PDB entry 2JRZ). f and g, ITC measurements of binding of αKG (f) or N-oxalylglycine (g) to KDM5A(1–588),ΔAP and binding of αKG to KDM5B(1–604),ΔAP (h) or KDM5B(1–755),ΔAP (i). The dissociation constant (K_D) and the one-site binding model (n = 1) were calculated by fitting the ITC data.
tide (PDB entry 3AVR) (60) placed a trimethylated lysine residue in the active site of KDM5A, surrounded on four sides by Trp-470, Tyr-472, Asn-585, and the metal-ligand water molecule (Fig. 6b). The aromatic indole ring of Trp-470 is in parallel with the hydrophobic portion of the target lysine. The side chains of Tyr-472 and Asn-585 (both of which are conserved in KDM6A: Tyr-1135 and Asn-1240) each coordinate one methyl group, whereas the third methyl group is in close proximity to a metal ligand-coordinated water molecule. During the catalytic cycle, this site would be occupied by a dioxygen \( \text{O}_2 \) molecule that initiates the reaction by abstracting a hydrogen atom from the substrate.

We also superimposed the structure of KDM5A with that of KDM6B/JMJD3 in complex with GSK-J1 (PDB entry 4ASK) (17). As noted by Kruidenier et al. (17), GSK-J1 partially overlaps with \( \alpha \text{KG} \) via the propanoic acid moiety, and the pyridyl-pyrimidine biaryl of GSK-J1 makes a bidentate interaction with the metal ion (silver ball). Thus, the interactions we observed with \( \alpha \text{KG} \)-Mn(II)-KDM5A would probably be maintained with the propa-
FIGURE 7. Sequence alignment of KDM5 family members. White-on-black residues are invariant between the four sequences examined, whereas gray-highlighted positions are conserved (R and K; E and D; T and S; F and Y, V, I, L, and M). Positions highlighted with red arrows indicate junction points or termination sites in the various constructs indicated in Fig. 1, a and b. The Rice Jmj703 (another trimethylated histone H3 Lys-4 demethylase) was included for comparison (47).
noic portion of GSK-J1 (Fig. 6c). Furthermore, the polar residues of KDM6B (Arg-1246, Gln-1248, and Asn-1331) that surround the tetrahydrobenzazepine moiety of GSK-J1 are also conserved in KDM5A (Arg-73, Gln-75, and Asp-412), supporting the observed cross-reactivity of GSK-J1 against the KDM5 family members (18) (Fig. 4, a and b). One notable exception is that Cys-481 of KDM5A (an invariant residue among the KDM5 family; Fig. 7) replaces Pro-1388 of KDM6B, which packs directly against the aromatic ring of tetrahydrobenzazepine (Fig. 6d). Exploring the interaction between the noncatalytic Cys-481 and GSK-J1 analogs might provide an avenue for improved potency, selectivity, and prolonged on-target residence times of inhibitors of the KDM5 family. This approach of using reversible covalent inhibitors that target noncatalytic cysteine residues to achieve prolonged and tunable residence time has recently been demonstrated with protein kinases (71).

Discussion

The ascribed physiologic functions of the KDM5 histone demethylase family members stem largely from studies in which the KDM5 genes have been deleted or suppressed, making it difficult to discern the relative contributions of the enzymatic activity from those of the noncatalytic domains (DNA-binding ARID, histone-binding PHDs, and uncharacterized PLU-1) within the same polypeptide or as a structural component of other multiprotein complexes. Thus, in addition to providing an opportunity for the development of novel therapeutics, the discovery of small molecule inhibitors that specifically target the catalytic domain of the KDM5 family while leaving its “reader” domains and scaffolding properties intact will provide important information about the biological functions of this class of histone modifying enzymes. Here we have expressed, purified, and characterized the minimal catalytic domain of the KDM5 family members (A, B, and C) and determined the structure of a linked KDM5A JmjN-JmjC. Our results indicate that the ARID and PHD1 domains have little impact on the catalytic activity of the KDM5 family and that the linked JmjN-JmjC together with the immediate C-terminal helical zinc-binding domain are necessary for catalytic activity in vitro. We further show that the two reaction cofactors (metal ion and α-ketoglutarate) bind in the active site of a linked JmjN-JmjC domain in a manner similar to that of other families of structurally characterized demethylases with contiguous Jumonji domains. The observed differences in the active sites of KDM5 and KDM6 family members (Fig. 6d) might provide new avenues for designing and improving the potency and selectivity of GSK-J1-based derivatives toward KDM5 over KDM6. For example, the use of cysteine-reactive cyanoacrylamidine electrophile (71) might provide a strategy to target the unique noncatalytic cysteine residue near the KDM5 active site (Cys-481 in KDM5A) for enhanced selectivity and duration of target engagement in vitro and in vivo.

KDM5A and KDM5B exhibit frequent gain of function alterations across a broad range of primary human cancers. In particular, breast (19%), prostate (12%), and ovarian cancers (20%) show a high frequency of KDM5A/B gene amplification, whereas melanoma, lung adenocarcinomas, and cervical cancers show a similar rate of alteration though a mix of point mutations, gene amplification, and overexpression (14–19%) (Cancer Genome Atlas; cBioportal). Interestingly, high KDM5A/B expression marks a small subpopulation of slowly cycling, tumor-initiating cells that are intrinsically resistant to a wide variety of cancer therapeutics, including both cytotoxic (e.g. cis-platinum) and targeted agents (tyrosine kinase inhibitors, bortezomib, B-raf inhibitors) (37, 38, 72). Thus, in addition to primary therapy, inhibition of JARID1A/B might prove useful in combination with conventional therapies to combat drug resistance. KDM5C, on the other hand, exhibits loss-of-function alterations in ~7% of clear cell renal cell carcinomas (73), where it plays a tumor-suppressive role (74), and inherited mutations in KDM5C are associated with X-linked mental retardation.

As noted above, KDM5C overexpression has been linked to Huntington disease (42), and reducing the kdm5c level was neuroprotective in mouse and Drosophila HD models. Thus, selective inhibition of KDM5 family members in different contextual applications will be desirable. Although the active site residues are invariant among the KDM5 family members, we and others have observed differences in the ability of GSK-J1 (18) and JIB-04 to inhibit the enzymatic activity of the various family members in vitro (Fig. 4, a–d). These differences might be attributed to drug effects on regions outside of the catalytic domain influencing protein dynamics, entropic characteristics, or other factors not obvious from examination of the crystal structures. For example, αKG binds 3-fold more tightly to the larger catalytically active KDM5B protein that includes the zinc-binding domain than to the linked JmjN-JmjC domain alone (Fig. 5, h and i). Disrupting the interface between the catalytic Jumonji domain (where the disordered residues 457–466 are located) and the C-terminal helical zinc-binding domain (where the sequences are more variable within the KDM5 family) might be an alternative approach to selective inhibition of KDM5 function. Iterative cycles of crystallography, synthesis, and bioactivity assays will ultimately aid successful design of selective and potent epigenetic inhibitors of KDM5 di-/trimethylated histone H3 Lys-4 demethylases.

Author Contributions—J. R. H. developed the PHYRE2 model, designed constructs, performed purification and crystallographic experiments, and performed KDM5B kinetics and inhibition; A. E. performed KDM5C kinetics and inhibition; X. L. generated some constructs and performed activity assays of KDM5A and KDM5C; J. R. S. performed routine protein purifications; E. L. Z and P. M. V. designed constructs, performed purification and crystallographic experiments, and performed KDM5B kinetics and inhibition; X. Z. participated in discussion throughout; M. A. J. and H. F. participated in organization and discussion of the study; X. C. organized and designed the scope of the study; and all authors were involved in analyzing data and preparing the manuscript.

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