Characterization of a linked Jumonji domain of the KDM5/JARID1 family of histone H3 lysine 4 demethylases

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Running title: A linked JmjN-JmjC domain of KDM5

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ABSTRACT

The KDM5/JARID1 family of Fe(II)- and α-ketoglutarate-dependent demethylases remove methyl groups from tri- and di-methylated lysine 4 of histone H3. Accumulating evidence from primary tumors and model systems support 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 sub-families, 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 to that of KDM5B at 1 mM α-ketoglutarate. In contrast, JIB-04 (a pan-inhibitor of the Jumonji demethylase superfamily) had the opposite effect and was approximately 8-fold more potent against KDM5B than KDM5C. Interestingly, the relative selectivity of JIB-04 towards 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 Zn-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.

INTRODUCTION

It is now well established that cancer arises in part from an altered ‘epigenome',
with widespread alterations in chromatin modifications (in 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-methylecytosine dioxygenases (TET2), histone methyltransferases (EZH2, MLL1/2/3), histone demethylases (UTX, KDM5A/B/C), ATP-dependent chromatin remodelers (ATRX, 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 likely 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 “re-programming” 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 Jumonji family of histone lysine 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 modulators of a GFP-linked transgene and was found to function as a pan-inhibitor of the Jumonji family of histone demethylases (19). Recent work has also described promising results with an inhibitor of the FAD-dependent demethylase LSD1/KDM1 (20,21) that removes methyl groups from mono- and di-methylated lysine 4 of histone H3 (H3K4me1/2) (22).

The JARID1 (Jumonji, AT-rich interactive domain) family of histone lysine demethylases represents one such potential target. The JARIDs are multi-domain proteins containing a Jumonji domain that catalyzes the removal of methyl marks from histone H3 di- and tri-methylated at lysine 4 (H3K4me2/3), 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 support a role for the KDM5 family as oncogenic drivers [reviewed in (27)]. KDM5A (also known as JARID1A or RBP2) was originally identified as a retinoblastoma (RB)-binding protein (28,29). Subsequent work showed that the tumor-suppressive activity of RB is dependent upon its ability to sequester KDM5A (30). In estrogen receptor (ER) negative breast cancers, KDM5A mediates metastatic spread to the lung (31). Consistent with an oncogenic role, genetic ablation of kdm5a delays tumor onset in Rb 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 MLL-rearranged AML cells, suggesting a crucial role for the H3K4
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’s disease (42). Downregulation of kdm5c in primary neurons reduced the toxicity from mutant Huntingtin expression, restored the level of key neuronal genes, and was neuroprotective in murine and Drosophila Huntington’s 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 insertion of an ARID and PHD1 domain separating it into two subdomains, JmjN and JmjC (46) (Fig. 1a). Previously, we and others have shown that recombinant protein containing the N-terminal halves of KDM5C (residues 1-839), KDM5B (residues 1-769), and KDM5A (residues 1-797) are enzymatically active (43-45). Interestingly, the rice Jumonji demethylase JMJ703 has KDM5-like activity on H3K4me3 but contains no insertion within the catalytic domain (47). We therefore sought to determine the impact of the ARID and PHD domains on the in vitro enzymatic activity of the KDM5 demethylase family. We find that the ARID and PHD1 domains are dispensable for enzymatic activity of KDM5 family members, whereas the immediate C-terminal helical Zn-binding domain is not. Despite the lack of activity, the structure of the linked JmjN-JmjC domain alone of KDM5A demonstrated that the reconstituted domain utilizes the exact same 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 Zn-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 (GeneBank BC110916 for KDM5A, BC054499 for KDM5C, and BC144102 for KDM5D) and KDM5B (a gift of Joyce Taylor-Papadimitriou of King’s College London; GeneBank BC157031 for KDM5B) using VENT or Phusion 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 to PCR 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 E. coli strain BL21(DE3)C+ for protein expression.

Typically 2L (for Jumonji domain only) or 12L (for Jumonji + helical Zn-binding domain) LB cultures were inoculated with starting culture and grown at 37 °C to an OD600 of 0.4 to 0.8, at which point the temperature was reduced to 16 °C. After 1~2 hours, 0.1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce the expression of recombinant protein overnight. Cells were collected by centrifugation and the pellets resuspended in 20 mM HEPES, pH 8.0, 5% glycerol, 300 mM NaCl and 0.5 mM tris (2-carboxyethyl)phosphine (TCEP) (the KDM5 storage buffer). Cells were broken using a French Press and then limited sonication. The cell lysate was cleared by centrifugation at 37,000g for ~1 hr 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 equilibrated with the KDM5 storage buffer and the eluted protein was concentrated using a 10kD MWCO 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 one liter LB cultures of E. coli strain BH249 cells containing a His-tagged pseudomonas putida FDH construct (a gift of Ashok S. Bhagwat, Wayne State University) were grown at 37°C to an OD600 between 0.4 and 0.6 when the temperature of the shaker was set at 16°C. After ~1 hr, IPTG was added (0.4 mM) to induce expression overnight. Except for the requirement of Ulp-1 protease, the purification of FDH was carried out exactly as described above for the KDM5 proteins, including buffers and columns used. After the S200 sizing column, purity of FDH was over 99% based on SDS-PAGE. The final FDH protein was concentrated to about 10 mg/ml using a 30 kDa MWCO Vivaspin concentrator. Purified protein was frozen immediately in liquid nitrogen and stored in 30 µl aliquots at -80 °C.

Formaldehyde dehydrogenase (FDH)-coupled demethylase assay

Purified FDH enzyme and 3-acetylpyridine adenine dinucleotide (APAD+, a more stable analogue of NAD+; Sigma-Aldrich A5251) were utilized in a fluorescence-based formaldehyde release assay (49) (Fig. 2a-b) to determine the kinetic characteristics of KDM5B(1-755)ΔAP or KDM5C(1-789)ΔAP with various concentrations of αKG or H3K4 methylated peptides. Standards of formaldehyde concentrations were made fresh and used to calibrate fluorescence intensities to determine initial velocities (Fig. 2c). The turnover number of FDH is fast enough to ensure that the clearance of formaldehyde from the demethylation catalysis is not delayed by FDH conversion. Reactions were performed in a 40 µl 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 µg (3 µM tetramer) FDH] (50 mM MES pH 6.6 for KDM5C). All assay components were pre-incubated at room temperature for 15 min followed by addition of αKG or peptide and APAD+ to initiate the reaction. A BioTek Synergy™ 4 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) were performed under the 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 pre-incubated with αKG and inhibitor at room temperature prior to the addition of the substrate peptide and APAD+, and the reactions carried out for 10 min at room temperature. The demethylase 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 µM) to pre-incubated 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-hydroxy-cinnamic acid (CHCA) 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 recorded and examined for peptide mass changes in 14, 28, or 42 Daltons before and after the reaction, corresponding to mono-, di-, and tri-demethylation (Fig. 2e). The average number of methyl groups removed per peptide was converted from relative abundance: $n = 3x(me0%) + 2x(me1%) + 1x(me2%)$. The reaction time course was determined by integrating the relative abundance of each methylated K4 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 NOG (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 heat of dilution). Binding constants (K_D) were calculated by fitting the data using the ITC data-analysis module of Origin 7.0 (OriginLab Corporation).

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 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 Rmerge value, which is poorly suited to assess data quality (55), is relatively higher, whereas the CC* and R_pim values are useful indicators for data quality (56) (Table 1).

Sulforhodamine B (SRB) cell growth assay
MCF7 and MDA-MB231 breast cancer cell lines were maintained in Dulbecco’s Modified Eagle 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-Aldrich SML0701) and GSK-J5 (Cayman 12074) were dissolved in 100% DMSO to 20 mM and 40 mM, respectively and stored at -20°C.

For growth inhibition assays, MCF7 and MDA-MB231 cells were seeded in triplicate in a 96 well plate (5000 cells/well), allowed to adhere overnight, and JIB-04, GSK-J4, GSK-J5, or DMSO (vehicle) were added the next day. After 72 hours treatment, cells were fixed on the plate with cold 10% TCA (100 µL/well) for 1 hr at 4°C, washed three times in dH₂O, and air dried. Cells were stained with 0.4% sulforhodamine B in 1% acetic acid (100 µL/well) for 30 minutes, washed three times in 1% acetic acid, and air dried. Sulforhodamine B dye was re-solubilized in 200 µL of 10 mM Tris base (pH 10.5) and the OD₅₁₀ was measured by spectrophotometer. Percent control cell growth was calculated as the OD₅₁₀ 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 the Protein Homology/analogY Recognition Engine (PHYRE2) (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 2OS2) or rice JMJ703 (PDB 4IGP) (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 2EQY), the PHD domain of PHF8 (PDB 3KV4) (59), and the C-terminal helical Zn-binding domain of UTX/KDM6A (PDB 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 3D 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, KDM5C residues 1-769 with and without AP (Fig. 1a-b). We also generated constructs with different C-terminal ends; for example, 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 Zn-binding domain, and are analogous to that of the KDM6/UTX/JMJD3 family (61). A homologous C-terminal helical Zn-binding domain is also present in the rice JMJ703 (47).

In KDM6A/UTX, the C-terminal Zn-binding domain is involved in recognizing a
portion of the histone H3 peptide (residues 17-23) N-terminal to the methylated target lysine (H3K27) (60), thereby excluding the histone H3K9 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 JMJ703 was observed to interact with three of the ten residues in the H3K4me3 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 Zn-binding domain. All constructs were expressed in E. coli and exhibited variable expression levels and solubility (Fig. 1b and 1e). In general, constructs with longer C-terminal ends that included the helical Zn-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 soluble protein from a liter culture. The longer constructs resulted in either insoluble products (KDM5A) or 1-3 mg purified enzyme per liter 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 to that 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 vs. 5.4±0.5 \( \mu \)M), \( K_M \) value for histone H3 peptide (1-24)K4me3 (3.3±0.2 vs. 3.3±0.4 \( \mu \)M), and turnover rate \( k_{cat} \) (2.0-2.7 vs. 2.5-3.1 min\(^{-1}\)) (Fig. 3a-b). We thus concluded that deletion of AP and further shortening the C-terminal residues from 839 to 789 has 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 Zn-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 Zn-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 Zn-binding domain was shown to be essential for enzymatic activity by binding a portion of the histone H3 peptide, and (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. 2e, 2g, and 2i). Under these conditions, we determined the \( K_M \) value for αKG to be ~8 \( \mu \)M and the \( K_M \) for peptide substrate to be ~4 \( \mu \)M (Fig. 3c-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 H3K4me3 peptide substrate. We further found that KDM5B(1-755)ΔAP has no activity on the corresponding H3K9me3- or H3K27me3-containing peptides (Fig. 2j).

Using tri-methylated H3(1-24)K4me3 peptide as the initial substrate, we applied quantitative mass spectrometry to monitor the
kinetics of product formation (Fig. 3e-f). For the KDM5B(1-755)ΔAP and KDM5C(1-789)ΔAP enzymes, we observed a rapid disappearance of H3K4me3 in the first 5-10 min of the reaction with a corresponding appearance of a transient peak of H3K4me2 (up to 50-60%), followed by conversion to H3K4me1. We determined the kinetic rate constants for each of the three demethylation steps using nonlinear least-squares regression analysis. The first conversion from me3 to me2 is faster (k1 ≈ 47 h⁻¹ for KDM5B and 32 h⁻¹ for KDM5C) than the second conversion from me2 to me1. The two enzymes showed a larger difference (approximately 7-fold) in the second conversion rate with k2 ≈ 22 h⁻¹ for KDM5B and 3 h⁻¹ for KDM5C. The last conversion from me1 to me0 is the slowest with the negligible k3 ≈ 0.1-0.2 h⁻¹, resulting in prolonged accumulation of me1. Fig. 3g summarizes apparent kcat 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 H3K4me1 despite occupancy by KDM5B/C (62-65).

Inhibition of KDM5BΔAP and KDM5CΔAP 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)ΔAP with an IC50 (half-maximal inhibitory concentration) of approximately 94 µM (Fig. 4a) under the assay conditions of 1 mM αKG, whereas it had ~8.5-fold higher potency against KDM5C(1-789)ΔAP (IC50 ≈ 11 µM) (Fig. 4b). On the other hand, JIB-04 had the opposite effect. JIB-04 was a ~8-fold more potent inhibitor of KDM5B(1-755)ΔAP activity than it was of KDM5C(1-789)ΔAP (IC50 ≈ 5 µM vs. 42 µM) (comparing Fig. 4c and 4d). 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 approximately 40 and 12, respectively (18) [We note that different fragments of KDM5A, B, and C, different assay conditions and detection method 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 (GI50) in the 20-300 nM range for JIB-04 versus 1-3 µM range for GSK-J4 after 72 hours of treatment (comparing Fig. 4e and 4f). Additionally, JIB-04 exhibited differential activity among the breast cancer cell lines. MCF7 cells were ~10-50-fold more sensitive to the antiproliferative effects of JIB-04 than were MDA-MB231 cells, with GI50 ≈ 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 towards 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 as JIB-04 has nearly equivalent activity against KDM4 family enzymes in vitro and against cellular H3K9me3 demethylase activity (19). Differential effects on cytoplasmic targets of the KDMs may also play a role as JIB-04 has been recently shown to inhibit translation and to sensitize cells to the growth inhibitory effects of mTOR inhibitors, possibly through its effects on KDM4A or KDM5A, both of which were found to associate with polysomes (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 Zn-binding domain in the full-length protein, and the flexibility may suggest 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 (eg. 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. 5b-c-d). Two twisted β-sheets (a four-stranded minor sheet in cyan and an eight-stranded major sheet in dark blue and grey) pack together with helices on the outer surfaces of the major and minor sheets (Fig. 5a). The JmjN residues (dark grey) 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. Pro41, located in the middle of the kinked helix αB, is conserved among the four KDM5 family members (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 stand 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 $\beta_8$ of the minor sheet to $\beta_9$ of the major sheet and strand $\beta_{13}$ provide invariant residues for binding of the metal ion (H483, E485, and H571), forming the ferrous binding motif, HxE/D...H (68). The $\alpha$-ketoglutarate and its non-reactive analog N-oxalylglycine (NOG) are bound with the dissociation constant $K_D$ of approximately 80 $\mu$M and 47 $\mu$M respectively, measured by isothermal titration calorimetry (Fig. 6a-b). The cofactor $\alpha$KG is in extensive polar (Y409, S491, N493, K501) and hydrophobic interactions with the protein (Y472, F480, W503 and A583) (Fig. 5f-g). The importance of these interactions is underscored by the fact that $\alpha$KG-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 $\alpha$KG binding are conserved in KDM6A (Fig. 6a), except for F480 of KDM5A, which is replaced by S1143 in KDM6A in the primary sequence. Although K501 of KDM5A and K1137 of KDM6A do not align in the primary sequence, and project from different sides of the catalytic site, their terminal $\varepsilon$-amino groups occupy the same location in space, both in close contact with $\alpha$KG (Fig. 6a).

**Comparison with KDM6**

The high structural similarity of KDM5A with other structurally characterized catalytic Jumonji domains (such as KDM6A: root-mean-squared 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. Superimposition of KDM6A in complex with histone H3 peptide (PDB 3AVR) (60) placed a trimethylated lysine residue in the active site of KDM5A, surrounded on four sides by W470, Y472, N585 and the metal-ligand water molecule (Fig. 6b). The aromatic indole ring of W470 is in parallel with the hydrophobic portion of the target lysine. The side chains of Y472 and N585 (both of which are conserved in KDM6A: Y1135 and N1240) 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 $O_2$ 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 4ASK) (17) (rmsd = 2.5 Å across 208 Cα atoms). As noted by Kruidenier et al. (17), GSK-J1 partially overlaps with $\alpha$KG via the propanoic acid moiety and the pyridyl-pyrimidine biaryl of GSK-J1 makes a bidentate interaction with the metal ion. Thus the interactions we observed with $\alpha$KG-Mn(II)-KDM5A would likely be maintained with the propanoic portion of GSK-J1 (Fig. 6c). Furthermore, the polar residues of KDM6B (R1246, Q1248 and N1331) that surround the tetrahydrobenzazepine moiety of GSK-J1 are also conserved in KDM5A (R73, Q75 and D412), supporting the observed cross reactivity of GSK-J1 against the KDM5 family members (18) (Fig. 4a and 4b). One notable exception is that C481 of KDM5A (an invariant residue among KDM5 family; Fig. 7) replaces P1388 of KDM6B, which packs directly against the aromatic ring of tetrahydrobenzazepine (Fig. 6d). Exploring the interaction between the noncatalytic C481 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
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 non-catalytic 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 biologic 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 Zn-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 towards KDM5 over KDM6. For example, the use of cysteine-reactive cyanoacrylamine electrophile (71) might provide a strategy to target the unique noncatalytic cysteine residue near the KDM5 active site (C481 in KDM5A) for enhanced selectivity and duration of target engagement in vitro and in vivo.

KDM5A and 5B 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%) (The Cancer Genome Atlas; cBioportal). Interestingly, high KDM5A/B expression marks a small subpopulation of slow-cycling, tumor initiating cells that are intrinsically resistant to a wide variety of cancer therapeutics, including both cytotoxic (eg. 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’s disease (42), and reducing 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. 3a-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 Zn-binding domain than to the linked JmjN-JmjC domain alone (Fig. 5h-i). Disrupting the interface between the catalytic Jumonji domain (where the disordered residues 457-466 are located) and the C-terminal helical Zn-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 H3K4me3/2 demethylases.

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Author Contributions - J.R.H. developed PHYRE2 model, designed constructs, performed purification and crystallographic experiments, and 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 and performed cell growth assays; 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 were involved in analyzing data and preparing the manuscript.

The atomic coordinates and structure factors of KDM5A (codes 5E6H) have been deposited in the Protein Data Bank.

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Footnotes
The abbreviations used are: α-KG, α-ketoglutarate; FDH, Formaldehyde dehydrogenase;

Figure legends

Figure 1. The KDM5/JARID1 family.
(a) Schematic representation of KDM5 family members. JmjN and JmjC represent the two parts of the Jumonji domain; ARID (AT-rich interactive domain) is a DNA binding domain, shown to binds to CCGCCC and GCAC(A/C sequences for KDM5A and KDM5B, respectively (23,24). In addition, KDM5 family members contain two or three PHD (plant homeodomain) domains some of which have been shown to bind to H3K4me0 (PHD1 in KDM5A (26,45)), H3K4me3/me2 (PHD3 in KDM5A (26)), and H3K9me3 (PHD1 in KDM5C (25)). (b) KDM5 family expression constructs made in this study. (c) A manually assembled hypothetical 3D model of the N-terminal half of KDM5B, based on PHYRE2 generated homology models. (d) A closer view showing that the two joints (JmjN-ARID and PHD1-JmjC) are adjacent to each other. (e) A 12% SDS-PAGE showing examples of the purified proteins used in this study. Lane 1, KDM5A(1-588)ΔAP, Lane 2, KDM5B(1-755)ΔAP, Lane 3, KDM5B(1-604)ΔAP, Lane 4, KDM5C(1-839), Lane 5, KDM5C(1-789)ΔAP, and Lane 6, KDM5C(1-618)ΔAP.

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-CH2OH) 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 formaldehyde dehydrogenase (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 panel c. (e) 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%) has no effect on activity of KDM5C(1-789)ΔAP. Velocity of reactions was determined with [S]=15 µM of H3K4(1-24)me3 and [E]=0.5 µM JARID1C(1-789)ΔAP. 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)ΔAP at room temperature (left panel) and 37 °C (right panel). (f-g) The optimal pH for the demethylase activities of KDM5C(1-789)ΔAP activity (f), which is the same as our previously determined optimal conditions established for KDM5C(1-789) without the internal deletion ΔAP (43), and KDM5B(1-755)ΔAP (g), 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 HEPES for KDM5B). (h) FDH-coupled assays of demethylase activities of KDM5C(1-789)ΔAP and KDM5C(1-618)ΔAP under the conditions of [E]=0.5 µM and [S]= 50 µM of H3(1-24)K4me3 peptide. Assay with no enzyme was used to determine background fluorescence. (i) The demethylase activity of KDM5B(1-755)ΔAP is more active at low ionic strength. (j) KDM5B(1-755)ΔAP has no activity on K9me3- and K27me3-containing peptides.

Figure 3. Kinetic parameters of KDM5C(1-789)ΔAP and KDM5B(1-755)ΔAP.
(a-d) Michaelis–Menten kinetic plots of KDM5C(1-789) (a-b) and KDM5B(1-755)ΔAP (c-d) for the cofactors αKG (a, c) and substrate H3 peptide (b, d), measured by FDH-coupled demethylase assay. Initial velocities were plotted against αKG or peptide concentrations and fit with the Michaelis–Menten equation using GraphPad Prism5.0. Error bars indicate standard error of the mean value from two independent experiments. (e-f) Kinetic progression of demethylation reaction catalyzed by KDM5C(1-789) (e) and KDM5B(1-755)ΔAP (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 panels e and f using the formula \( \text{appk}_{cat} = k_i \times [S]/[E] \), where \( i = 1, 2, 3 \) and \( [S]/[E] = 20 \).

Figure 4. Inhibition of GSK-J1 and JIB-04.
(a-d) Inhibition of demethylation activity of KDM5B(1-755)ΔAP and KDM5C(1-789)ΔAP on H3(1–24)K4me3 substrate by GSK-J1 (a-b) and JIB-04 (c-d). (e-f) Cell growth inhibitory effects of GSK-J4, -J5 (e) and JIB-04 (f) in human breast cancer cell lines. MDA-MB231 (red) or MCF7 (blue) cells were treated with the indicated concentrations of JIB-04 or GSK-J4 (solid line) or GSK-J5 (dashed line), or vehicle equivalent (DMSO, dotted line), and percent of untreated control cell growth was determined after 72 hours by the sulforhodamine B assay. Data represents the mean ± standard error of three or four independent experiments performed in triplicate.

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 grey) with helices packed on the outer surfaces of the major and minor sheets. The yellow circle indicates the bound metal Mn(II). (b-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 H483, E485, H571, αKG (two ligands) and a water molecule. (d) Omit electron densities, contoured at 5\( σ \) and 3\( σ \) above the mean, are shown for Mn(II) (magenta mesh) and αKG (green mesh), respectively. (e) The corresponding helix αC (magenta) in the independently determined structure of KDM5C ARID domain (PDB 2JRZ). (f-g) ITC measurements of binding of αKG (f) or NOG (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.

Figure 6. Structural comparison of KDM5A and KDM6 family demethylases.
(a) Superimposition of KDM5A (colored) and KDM6A (in grey; PDB 3AVR). Note that the two terminal amino groups, K501 of KDM5A and K1137 of KDM6A, occupy the same space and both are in close contact with αKG. (b) A model of tri-methylated lysine (Kme3) in the active site of KDM5A, adopted from the structure of KDM6A in complex with histone H3 peptide (residues 17-33) trimethylated at K27 (PDB 3AVR). (c) A model of GSK-J1 in the active site of KDM5A, adopted from superimposition of the structure of KDM6B in complex with GSK-J1 (PDB 4ASK). Note that GSK-J1 partially overlaps with αKG via the propanoic acid moiety and the pyridyl-pyrimidine biaryl of GSK-J1 makes a bidentate interaction with the metal ion (silver ball). (d) Superimposition of KDM5A (colored) and KDM6B (in gray) in complex with GSK-J1. Note that C481 of KDM5A (an invariant residue among KDM5 family) replaces P1388 of KDM6B, which packs directly against the aromatic ring of tetrahydrobenzazepine.
Figure 7. Sequence alignment of KDM5 family members
White-on-black residues are invariant between the four sequences examined, while 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. 1a-b. The Rice Jmj703 (another H3K4me3 demethylase) was included for comparison (47).
Table 1. Statistics of X-ray data collection and refinement

| Data collection       | KDM5A(1-588)ΔAP |
|-----------------------|-----------------|
| Space group           | C2              |
| Cell dimensions       | α=γ=90°, β=92.6° |
| a (Å)=                | 115.4           |
| b (Å)=                | 61.6            |
| c (Å)=                | 46.6            |
| Beamline (SERCAT)     | APS 22-ID       |
| Wavelength (Å)        | 1.00000         |
| Resolution (Å) *      | 32.6-2.24 (2.32-2.24) |
| R<sub>merge</sub>      | 0.241 (0.603)   |
| Rpim                  | 0.068 (0.262)   |
| CC<sub>1/2</sub>      | 0.801 (0.842)   |
| CC*                   | 0.943 (0.956)   |
| <I/σI>                | 13.3 (3.7)      |
| Completeness (%)      | 99.5 (97.0)     |
| Redundancy            | 11.7 (4.5)      |
| Observed reflections  | 183,848         |
| Unique reflections    | 15,751 (1538)   |
| Refinement            |                 |
| Resolution (Å)        | 2.24            |
| No. reflections       | 15,751          |
| d R<sub>work</sub> / e R<sub>free</sub> | 0.179/ 0.220 |
| No. Atoms             | 2529            |
| Protein               | 2406            |
| Mn(II), αKG           | 11              |
| Solvent               | 112             |
| B Factors (Å²)        |                 |
| Protein               | 34.6            |
| Mn (II), αKG          | 28.6            |
| Solvent               | 38.5            |
| R.m.s. deviations     |                 |
| Bond lengths (Å)      | 0.004           |
| Bond angles (*)       | 0.7             |

*Values in parenthesis correspond to highest resolution shell;
<sup>a</sup> R<sub>merge</sub> = Σ | I - <I>| / |I|, where I is the observed intensity and <I> is the averaged intensity from multiple observations;
<sup>b</sup> The definitions for CC<sub>1/2</sub>, CC* and Rpim, see reference (56);
<sup>c</sup> <I>/σI> = averaged ratio of the intensity (I) to the error of the intensity (σI);
<sup>d</sup> R<sub>work</sub> = Σ | F<sub>obs</sub> - F<sub>cal</sub> | / Σ | F<sub>obs</sub> |, where F<sub>obs</sub> and F<sub>cal</sub> are the observed and calculated structure factors, respectively;
<sup>e</sup> R<sub>free</sub> was calculated using a randomly chosen subset (5%) of the reflections not used in refinement.
Table 2.

Comparison of IC_{50} values of GSK-J1 against KDM5 family under various αKG concentrations

| KDM5   | Residues | IC_{50} (µM) | Reference/Note |
|--------|----------|--------------|----------------|
| 5A     | -        | -            | This study     |
| 5B     | (1-755)ΔAP | 94           | Assay conditions^a |
| 5C     | (1-789)ΔAP | 11           | FDH-coupled assay |
|       |          |              |                |
| IC_{50} (µM) | -      | 0.95 μM      | SGC^b          |
| Method |          |              | AlphaScreen detection antibody |
|        |          | 1.76 μM      |                |
|        |          |              |                |
| IC_{50} (µM) | 6.8    | 0.17         | (18)           |
| Residues | (1-1090) | (1-809)      | Assay conditions: |
|         | (C-terminal deletion) | (N-terminal half) | Not available |
| Method |          |              | AlphaLISA based assay |
|        |          | 0.55         |                |
|        |          |              |                |
| IC_{50} (µM) | -      | >100         | (17)           |
| Residues | -       | -            | Assay conditions: |
|         |         |              | Not available |
| Method |          |              | MALDI mass spectrometry |
|        |          |              |                |

^a Assay conditions: 1 mM αKG, 50 µM Fe(II), [E]=0.5 µM, [S]=15 µM, t=10 min in 50 mM HEPES pH 7.0 (or 50 mM MES pH 6.6)

^b Adopted from http://www.thesgc.org/chemical-probes/GSKJ1; assay conditions: 5 µM αKG, 10 αM Fe(II), [E]=0.5 nM, [S]=0.1 µM, t=15 min in 50 mM HEPES pH 7.5
Horton et al. (2022). Supplementary Table 1. KDM5 Family Expression Constructs

| Protein | Residues | Construct (pXC #) | Expression | Solubility | Yield (mg/L) | Comments |
|---------|----------|-------------------|------------|------------|--------------|----------|
| KDM5A   | 1-808    | +                 | -          | -          | -            |          |
|         | 1-808ΔAP (88-346) | +                 | -          | -          | 1428        |          |
|         | 1-759ΔAP (88-346) | +                 | -          | +          | <0.1        |          |
|         | 1-739ΔAP (88-346) | +                 | -          | -          | 1430        |          |
|         | 1-588    | +                 | -          | -          | 1445        |          |
|         | 1-588ΔAP (88-346) | +                 | -          | -          | 1446        |          |
| KDM5B   | 1-824    | +                 | -          | -          | 1187        |          |
|         | 1-755    | +                 | -          | -          | 1279        |          |
|         | 1-755ΔAP (101-363) | +                 | +          | ~2         | 1404        |          |
|         | 1-604    | +                 | -          | -          | 1450        |          |
|         | 1-604ΔAP (101-363) | +                 | +          | ~2         | 1427        |          |
| KDM5C   | 1-839    | ++                | ++         | -          | 911         | See Ref. (43) |
|         | 1-789    | ++                | ++         | -          | 908         |          |
|         | 1-789ΔAP (83-378) | ++                | +          | ~2         | 1405        |          |
|         | 1-769ΔAP (83-378) | ++                | +          | ~2         | 1451        |          |
|         | 1-618    | +                 | -          | -          | 1448        |          |
|         | 1-618ΔAP (83-378) | +                 | -          | -          | 1447        |          |

Figure 1
**KDM5C (1-789)ΔAP**

- $K_m = 6 \pm 1 \mu M$
- $V_{max} = 0.96 \pm 0.07 \mu M/min$
- $k_{cat} = 1.92 \pm 0.07 \text{min}^{-1}$

**KDM5B (1-755)ΔAP**

- $K_m = 9 \pm 1 \mu M$
- $V_{max} = 0.93 \pm 0.04 \mu M/min$
- $k_{cat} = 1.90 \pm 0.04 \text{min}^{-1}$

**KDM5C (1-789)ΔAP**

- $K_m = 3.3 \pm 0.2 \mu M$
- $V_{max} = 1.35 \pm 0.02 \mu M/min$
- $k_{cat} = 2.71 \pm 0.02 \text{min}^{-1}$

**KDM5B (1-755)ΔAP**

- $K_m = 4.0 \pm 0.5 \mu M$
- $V_{max} = 1.00 \pm 0.03 \mu M/min$
- $k_{cat} = 2.00 \pm 0.03 \text{min}^{-1}$

**Figure 3**

- KDM5B: $\text{app}k_{cat} = 16 \pm 1 \text{min}^{-1}$
- $7.4 \pm 0.6 \text{min}^{-1}$
- $<0.06 \text{min}^{-1}$

- KDM5C: $\text{app}k_{cat} = 11 \pm 1 \text{min}^{-1}$
- $1.04 \pm 0.04 \text{min}^{-1}$
- $<0.05 \text{min}^{-1}$
Figure 4

(a) KDM5B(1-755)ΔAP
IC$_{50}$=94±1 µM

(b) KDM5C(1-789)ΔAP
IC$_{50}$=11±1 µM

(c) KDM5B(1-755)ΔAP
IC$_{50}$=5±1 µM

(d) KDM5C(1-789)ΔAP
IC$_{50}$=42±1 µM

(e) % Control cell growth vs. Concentration (µM)

(f) % Control cell growth vs. Concentration (µM)
ARID domain (PDB 2JRZ)

KDM5A RVQRLNELEAMT RVR-PREAFGFEQAVREYTLQSFGE MA
(disordered 12-residue linker)

Figure 5

(f) KDM5A(1-588)ΔAP

(E) = 0.4 mM
[
αKGM] = 8 mM
K_D = 79 ± 6 µM
N = 1.08 ± 0.02

(g) KDM5A(1-588)ΔAP

(E) = 0.4 mM
[
αKGM] = 8 mM
K_D = 47 ± 2 µM
N = 1.03 ± 0.01

(h) KDM5B(1-604)ΔAP

(E) = 0.2 mM
[
αKGM] = 4 mM
K_D = 49 ± 1 µM
N = 1.02 ± 0.01
Characterization of a linked Jumonji domain of the KDM5/JARID1 family of histone H3 lysine 4 demethylases
John R. Horton, Amanda Engstrom, Elizabeth L. Zoeller, Xu Liu, John R. Shanks, Xing Zhang, Margaret A. Johns, Paula M. Vertino, Haian Fu and Xiaodong Cheng

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