Supplemental Information

Insights into the action of inhibitor enantiomers against histone lysine demethylase 5A

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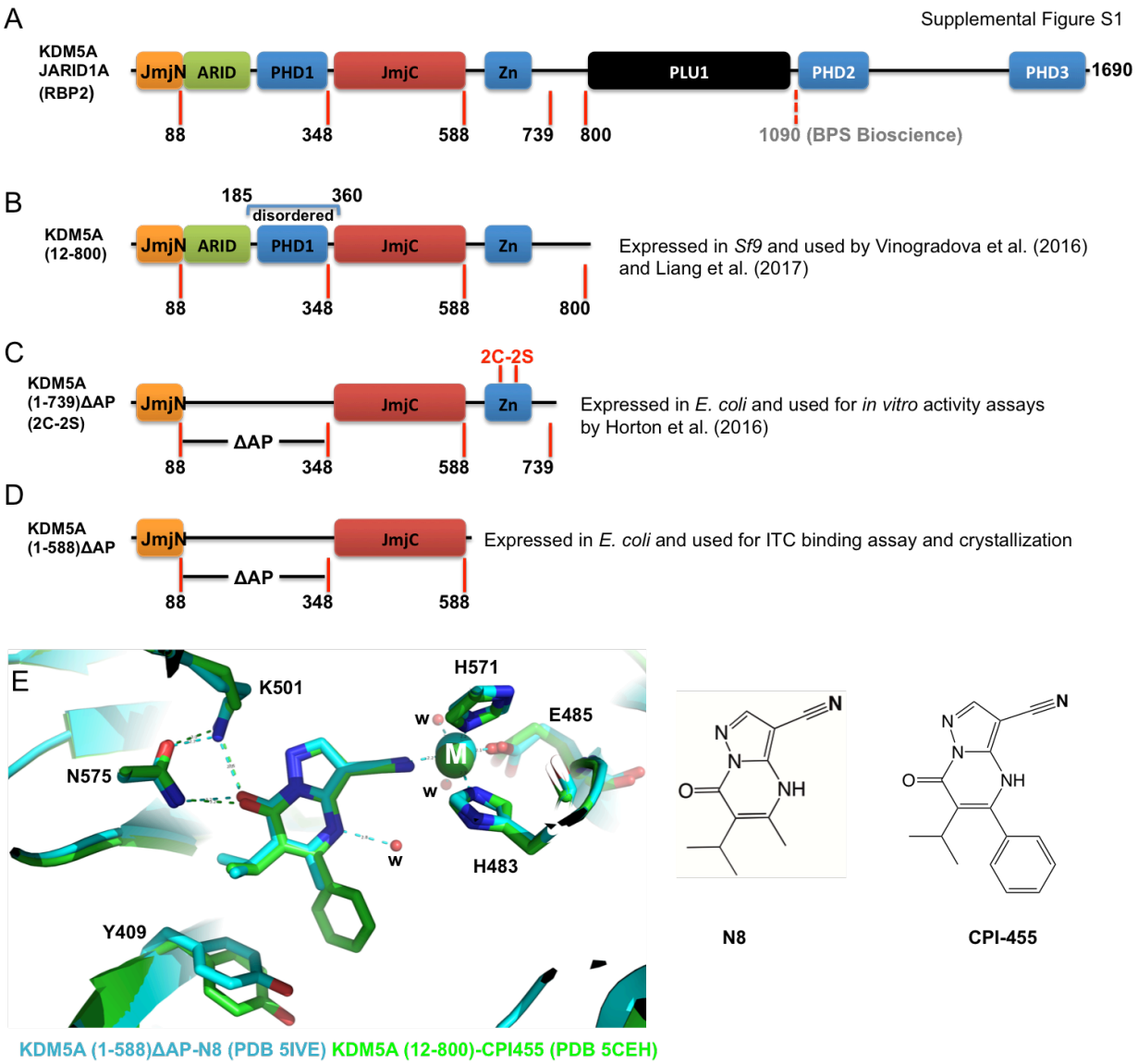
Supplemental Figures S1-S7

Supplemental Table S1-S2



Supplemental Figure S1. Minimal KDM5A domain for in vitro demethylase activity and structural study

(A) Schematic representation of KDM5A. JmjN and JmjC represent the two parts of the catalytic Jumonji domain; DNA binding ARID (AT-rich interactive domain); PHDs (plant homeodomain) bind to histone H3 peptides, either unmethylated or methylated at Lys-4 or Lys-9.

(B-D) Expression constructs of KDM5A used in the published studies and this study.

(**E**) Superimposition of KDM5A linked Jumonji domain [residues (1-588)ΔAP; cyan] and KDM5A (residues 12-800; green) showing nearly identical interactions with **N8** (PDB 5IVE) [1] or **CPI-455** compound (PDB 5CEH) [2].

Previously we defined the minimal requirements for *in vitro* enzymatic activity of KDM5A to be a linked JmjN-JmjC domain coupled with the immediate C-terminal helical Zn-binding domain. This was constructed by deleting the internal ARID and PHD1 domains (ΔAP)

between JmjN and JmjC and deleting the C-terminal half beyond the Zn-binding domain, generating KDM5A(1-739)ΔAP (panel C) [3]. In addition, we used a shorter construct, KDM5A(1-588)ΔAP, without the immediate C-terminal helical Zn-binding domain, for structural work with bound cofactor and inhibitors (panel D). Although the linked JmjN-JmjC domain of KDM5A(1-588)ΔAP is not active on its own, structural comparisons with longer construct of related KDM5B, which includes the C-terminal domain and a similar ΔAP internal deletion, showed that the addition of the C-terminal helical Zn-domain does not interfere with either the folding of the linked JmjN-JmjC domain or its active site conformation [1]. For example, structural comparisons between the **N8**-bound structure of KDM5A(1-588)ΔAP (PDB 5IVE) [1] and that of **CPI-455**-bound KDM5A(12-800) (PDB 5CEH) [2], which includes the entire N-terminal half, demonstrated that the chemically related compounds **N8** and **CPI-455** bind in the active site of KDM5A in exactly the same manner (panel E). The appraisal shows that the internal ARID-PHD1 (PHD1 is disordered in the structure) and the C-terminal helical Zn-domain (panel B) does not interfere with either the folding of the linked JmjN-JmjC domain or its active site conformation. Here we use the internal deletion (ΔAP) constructs, KDM5A(1-588)ΔAP and KDM5A(1-739)ΔAP, for the structural and in vitro binding assays, and enzymatic inhibition assays, respectively. The shorter construct provides an opportunity for studying KDM5 demethylase inhibitors in complex with the linked JmjN-JmjC domain of KDM5A at near atomic resolution by X-ray crystallography.

Supplemental References

(1) Horton, J. R.; Liu, X.; Gale, M.; Wu, L.; Shanks, J. R.; Zhang, X.; Webber, P. J.; Bell, J. S.; Kales, S. C.; Mott, B. T.; Rai, G.; Jansen, D. J.; Henderson, M. J.; Urban, D. J.; Hall, M. D.; Simeonov, A.; Maloney, D. J.; Johns, M. A.; Fu, H.; Jadhav, A.; Vertino, P. M.; Yan, Q.; Cheng, X. Structural Basis for KDM5A Histone Lysine Demethylase Inhibition by Diverse Compounds. *Cell Chem Biol* **2016,** *23*, 769-781.

(2) Vinogradova, M.; Gehling, V. S.; Gustafson, A.; Arora, S.; Tindell, C. A.; Wilson, C.; Williamson, K. E.; Guler, G. D.; Gangurde, P.; Manieri, W.; Busby, J.; Flynn, E. M.; Lan, F.; Kim, H. J.; Odate, S.; Cochran, A. G.; Liu, Y.; Wongchenko, M.; Yang, Y.; Cheung, T. K.; Maile, T. M.; Lau, T.; Costa, M.; Hegde, G. V.; Jackson, E.; Pitti, R.; Arnott, D.; Bailey, C.; Bellon, S.; Cummings, R. T.; Albrecht, B. K.; Harmange, J. C.; Kiefer, J. R.; Trojer, P.; Classon, M. An inhibitor of KDM5 demethylases reduces survival of drug-tolerant cancer cells. *Nat Chem Biol* **2016,** *12*, 531-538.

(3) Horton, J. R.; Engstrom, A.; Zoeller, E. L.; Liu, X.; Shanks, J. R.; Zhang, X.; Johns, M. A.; Vertino, P. M.; Fu, H.; Cheng, X. Characterization of a Linked Jumonji Domain of the KDM5/JARID1 Family of Histone H3 Lysine 4 Demethylases. *J Biol Chem* **2016,** *291*, 2631-2646.

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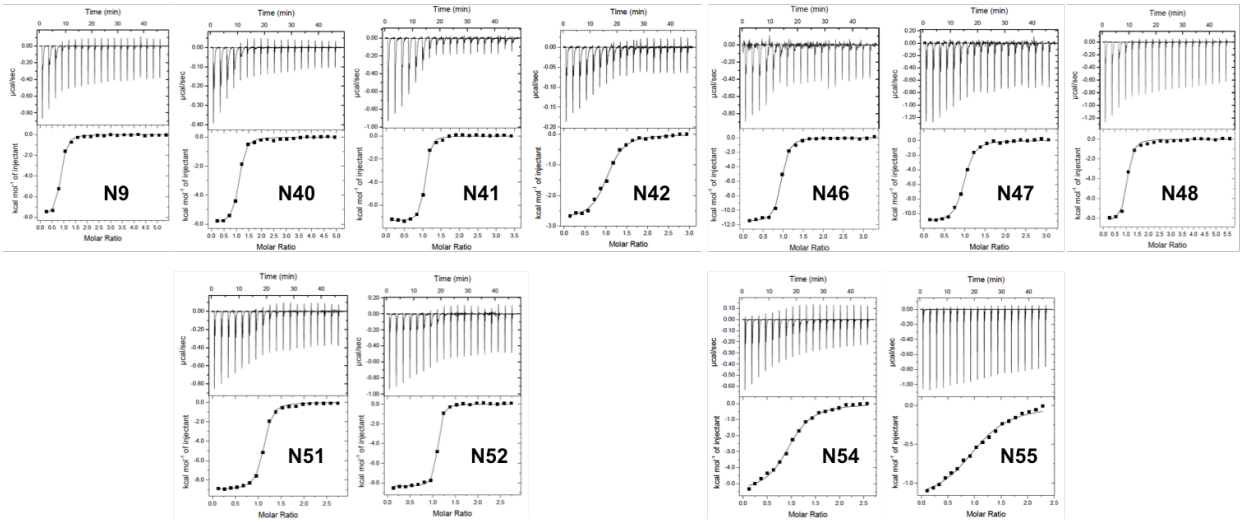


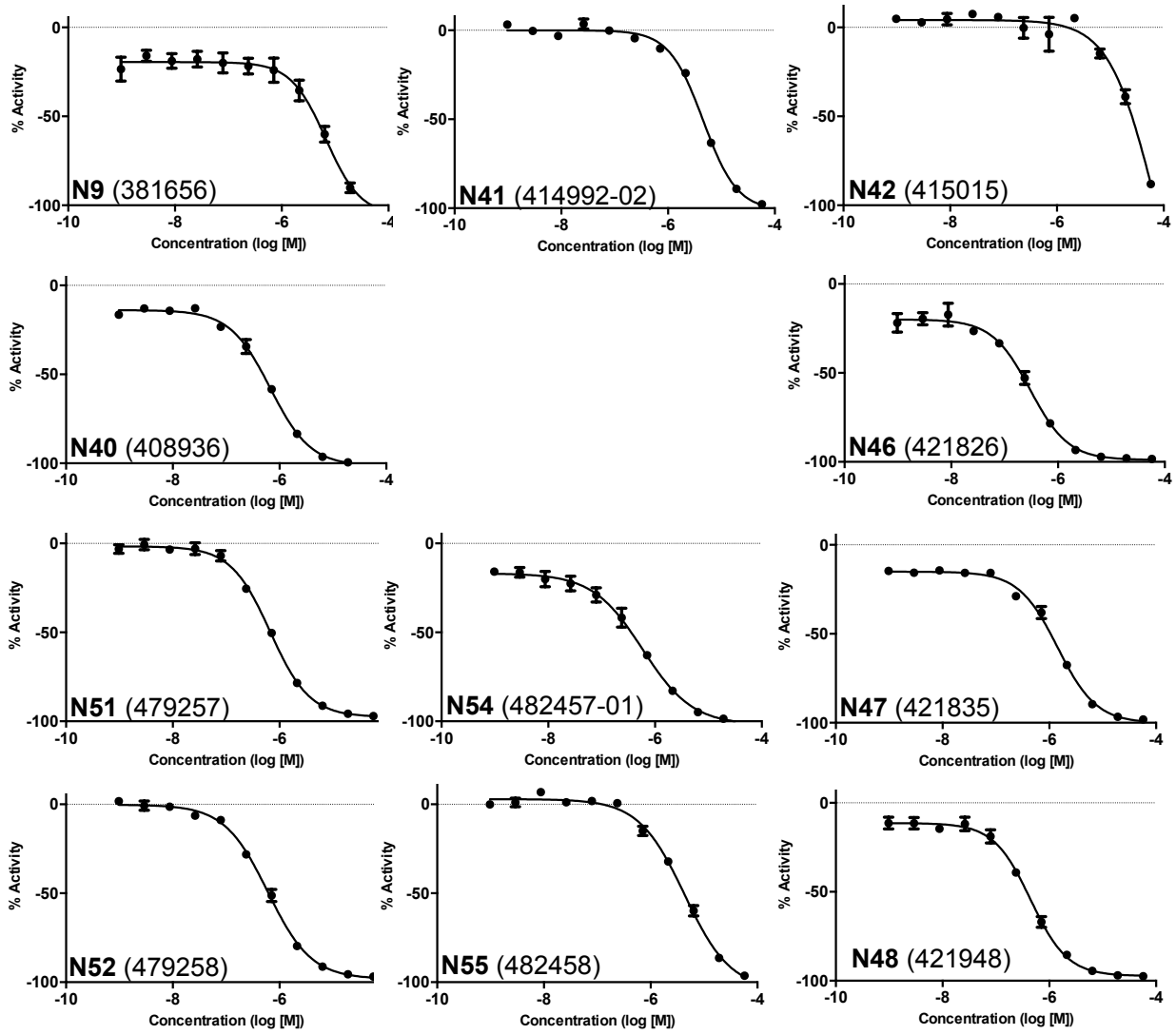
KDM5A	N9	N40	N51	N52	N41	N42	N46	N47	N48	N54	N55
[E] (μM)	25	25	30	30	50	25	30	30	25	80	80
[I] (μM)	680	600	420	420	830	420	420	420	600	1100	1000
K_D (μM)	0.53±0.06	0.18± 0.02	0.22±0.03	0.06±0.01	0.21±0.02	0.47±0.05	0.15±0.01	0.41±0.03	0.25±0.02	2.4±0.4	>12
N	0.96±0.02	1.00± 0.01	1.060±0.005	1.050±0.003	0.994±0.004	1.02±0.01	0.93±0.02	0.97±0.02	0.95±0.02	0.95±0.02	~1.2
ΔH (kcal/mol)	-7.5±0.3	-5.9± 0.4	-9.0±0.5	-8.3±0.5	-7.3±0.5	-2.8±0.6	-11.6±0.7	-11.1±0.7	-8.5±0.7	-5.6±0.5	-1.2
-TΔS (kcal/mol)	-T*(3.0)=-0.9	-T*(10.9)=-3.2	-T*(0.03)=-0.09	-T*(4.9)=-1.4	-T*(5.8)=-1.7	-T*(19.3)=-5.7	-T*(-8.12)=2.4	-T*(-8.45)=2.5	-T*(1.7)=-0.5	-T*(6.9)=-2.0	-T*(18.5)=-5.5
ΔG (kcal/mol)	-8.4	-9.1	-9.0	-9.7	-9.0	-8.5	-9.2	-8.6	-9.0	-7.6	-6.7

Supplemental Figure S2, related to Figure 1C.

The ITC measurements of K_D values by various compounds were carried out under the conditions indicated.

A

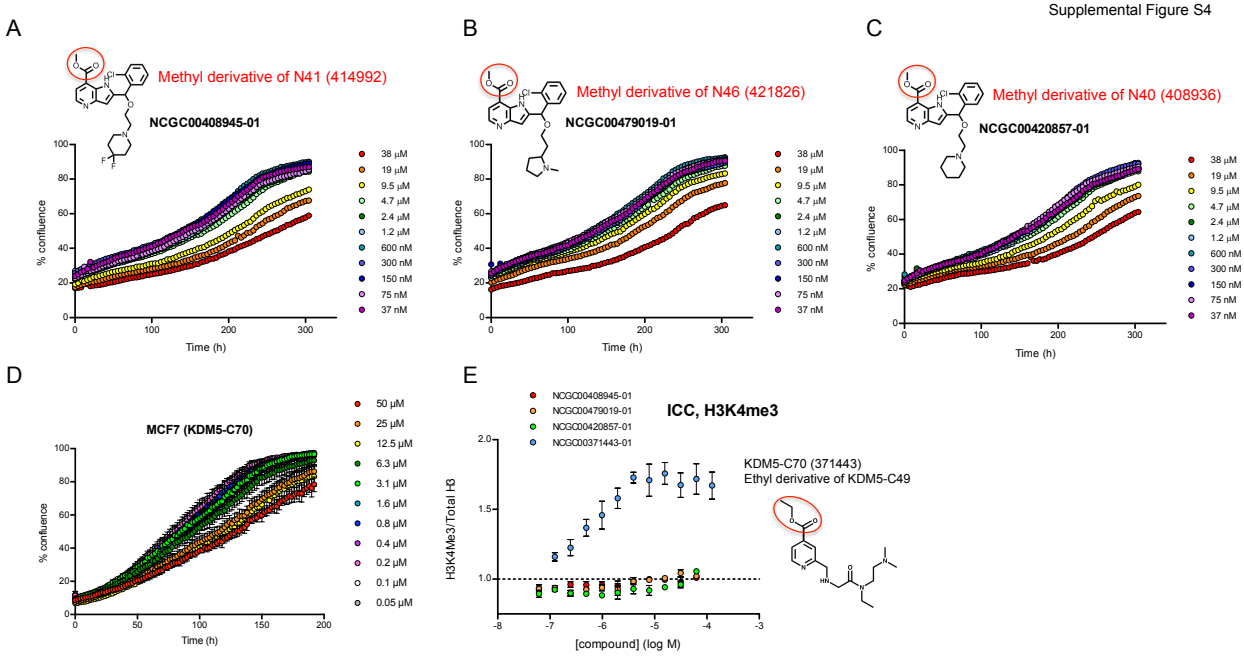
Supplemental Figure S3



Supplemental Figure S3, related to Figure 1E.

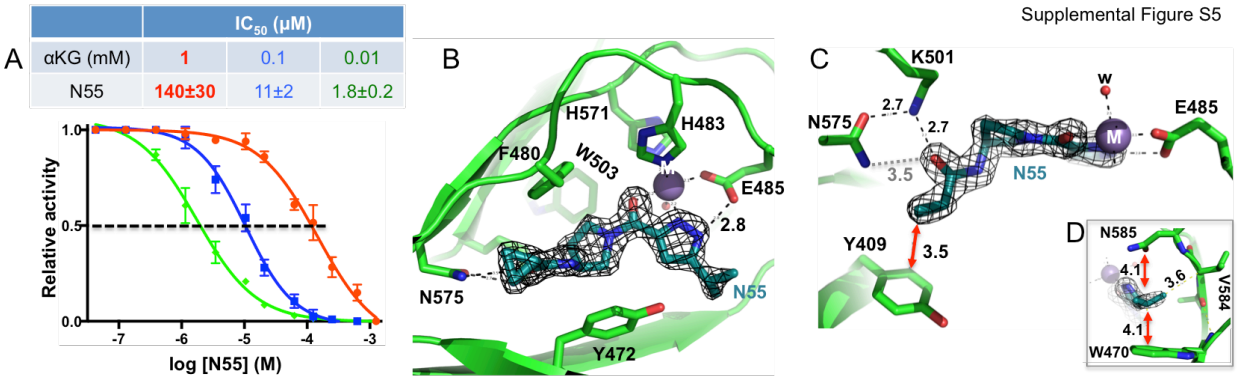
(**A**) Inhibition in AlphaLISA assays (performed in triplicate in 1536-well format) of enzyme activities of the C-terminal Flag-tagged KDM5A residues 1-1090 (BPS Bioscience; cat. 50110). (**B**) Comparison of inhibition of KDM5A (1-1090) and KDM4A catalytic domain in the presence of 11 compounds, under the indicated experimental conditions, for 3 independent experiments.

B	AlphaLISA IC_{50} (µM) at 50 µM [Fe(II)], 25 µM [αKG], 10 nM [E] and 100 nM [S]										
Compound	N9 (381656)	N40 (408936)	N51 (479257)	N52 (479258)	N41 (414992)	N42 (415015)	N46 (421826)	N47 (421835)	N48 (421948)	N54 (482457)	N55 (482458)
KDM5A (1-1090)	6.7±0.7	0.69±0.05	0.67±0.03	0.59±0.02	4.5±0.2	22±1	0.29±0.03	1.4±0.1	0.43±0.00	0.57±0.09	4.3±0.3
KDM4A (1-350)	27±2	14.2±0.6	20.9±0.8	17±2	26±3	27±2	15±2	20.1±0.8	14.8±0.6	20.9±0.8	46±3
Fold of selectivity	4	20	31	29	6	1	50	14	34	37	11



Supplemental Figure S4

BT474 proliferation in cells treated with the methyl ester versions of (**A**) **N41**, (**B**) **N46**, or (**C**) **N40**. Percent confluence was tracked over 304 hours by imaging the cells every 4h using an Incucyte. (**D**) MCF7 proliferation in cells treated with **KDM5C-70**. (**E**) Methyl esters of **N41**, **N46**, or **N40** do not increase H3K4me3 levels as measured by immunocytochemistry, but **KDM5-C70** does. H3K4me3 intensity was normalized to total H3 on a per cell basis (mean +/- SEM, n=3; ≥ 635 cells per replicate).



Supplemental Figure S5, related to Figure 5. Compound (*S*)-**N55**

(A) Inhibition of demethylation of KDM5A(1-739)ΔAP by (*S*)-**N55** at three concentration of αK via FDH-coupled assay.

(**B**) Structure of **N55**-KDM5A-Mn(II) showing the binding of **N55** compound in the active site involving two metal ligands (upper right corner), and aromatic interactions via Phe480 (top), Tyr472 (bottom), and Trp503 (in the background away from the viewer).

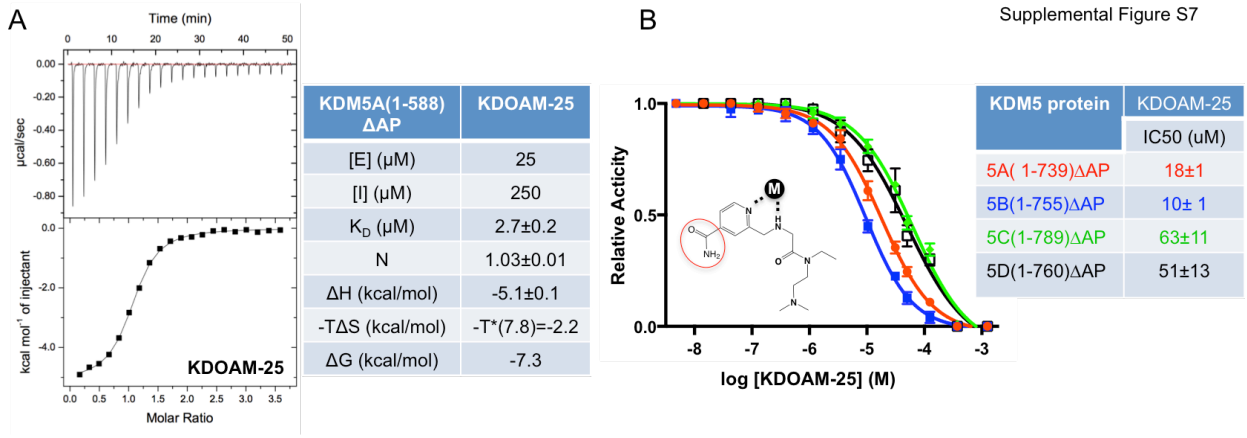
(**C**) The terminal cyclopropyl group forms a stacking interaction with Tyr409 and the associated carbonyl oxygen forms a weak hydrogen bond with Asn575 (left).

(**D**) The terminal isopropyl methyl groups form van der Waals interactions with Trp470, Asn585, and Val584.

Supplemental Figure S6. Comparison of (*R*)-**N54** bound KDM5A structures

(**A**) Superimposition of linked JmjN-JmjC domain of KDM5A(1-588)AP (colored yellow; this study) and N-terminal half of KDM5A including ARID, PHD1 (disordered), and C-terminal Zn helical domain (PDB 5V9T) [4].

(**B-C**) Superimposition of KDM5A linked JmjN-JmjC domain [residues (1-588)ΔAP; yellow] and KDM5A (residues 12-800; green) showing nearly identical conformations of (*R*)-**N54** in the active site, except the terminal cyclopropyl group and the conformation of Trp780.



KDM5A(1-588) ΔAP	KDOAM-25
[E] (μM)	25
[I] (μM)	250
K_D (μM)	2.7±0.2
N	1.03±0.01
ΔH (kcal/mol)	-5.1±0.1
-TΔS (kcal/mol)	-T*(7.8)=-2.2
ΔG (kcal/mol)	-7.3

KDM5 protein	KDOAM-25
	IC50 (uM)
5A(1-739)ΔAP	18±1
5B(1-755)ΔAP	10± 1
5C(1-789)ΔAP	63±11
5D(1-760)ΔAP	51±13

Supplemental Figure S7. Compound KDOAM-25

(A) The ITC measurement of K_D value under the conditions indicated.

(B) Inhibition of demethylation of four KDM5 enzymes [1] at 1 mM concentration of αK via FDH-coupled assay.

Table S1. Summary of X-ray data collection from SERCAT beamlines at wavelength=1Å and refinement statistics [in space group *C*2](*)

KDM5A(1-588)ΔAP	**N9** (3 crystals)	**N40**	**N41**	**N42**
PDB Code	6BGU	6BGV	6BGW	6BGX
Beamline (date)	22-ID (03-11-16)	22-ID (12-11-15)	22-ID (11-20-15)	22-BM (12-19-15)
Cell dimensions (Å)	116.53, 62.08, 46.64	116.29, 61.88, 46.74	116.58, 61.83, 46.76	115.37, 61.85, 46.54
α=γ=90°, β (°)	92.3	92.3	92.1	92.4
Resolution (Å)	32.91-1.68 (1.75-1.68)	30.94-1.59 (1.65-1.59)	32.88-1.64 (1.69-1.64)	46.50-1.88 (1.92-1.88)
[a] Rmerge	0.143 (0.857)	0.155 (0.743)	0.177 (0.814)	0.123 (0.808)
Rpim	0.045 (0.284)	0.061 (0.408)	0.066 (0.427)	0.052 (0.432)
$CC_{1/2}$, CC*	(0.885, 0.969)	(0.645, 0.885)	(0.623, 0.876)	(0.681, 0.900)
[b] <I/σI>	17.1 (4.0)	15.0 (3.2)	11.6 (1.9)	21.3 (3.0)
Completeness (%)	100.0 (100.0)	98.4 (96.1)	98.6 (96.8)	96.8 (78.5)
Redundancy	11.0 (10.0)	6.8 (3.4)	7.7 (4.4)	6.3 (3.5)
Observed reflections	410,264	306,172	303,297	163,325
Unique reflections	37,329 (3685)	43,550 (4247)	39,552 (2569)	25780 (2062)
Refinement				
Resolution (Å)	1.68	1.59	1.64	1.88
No. Reflections	37,326	43,547	39,550	25,749
[c] Rwork / [d] Rfree	0.198/ 0.209	0.168 / 0.184	0.190/0.213	0.200/0.236
No. Atoms				
Protein	2451	2440	2463	2415
Mn(II)	1	1	1	1
Inhibitor	24	37	31	30
Solvent	247	290	237	192
B Factors ($Å^2$)				
Protein	24.9	22.3	28.1	29.7
Mn(II)	14.2	10.3	14.7	16.4
Inhibitor	21.6	19.6	27.3	30.2
Solvent	35.9	36.0	40.6	36.4
R.m.s. deviations				
Bond lengths (Å)	0.002	0.002	0.002	0.002
Bond angles (°)	0.5	0.6	0.5	0.5

* Values in parenthesis correspond to highest resolution shell; [a] $Rmerge = \Sigma | I - \langle I \rangle | / \Sigma I$, where I is the observed intensity and <I> is the averaged intensity from multiple observations; [b] <I/σI> = averaged ratio of the intensity (I) to the error of the intensity (σI); [c] $Rwor_k = \Sigma | Fobs - Fcal | / \Sigma | Fobs |$, where Fobs and Fcal are the observed and calculated structure factors, respectively; [d] Rfree was calculated using a randomly chosen subset (5%) of the reflections not used in refinement.

Table S1 – Continue

KDM5A(1-588)ΔAP	**N46**	**N47**	**N48**	**N51**
PDB Code	6BGY	6BGZ	6BH5	6BH0
Beamline (date)	22-ID (2-22-16)	22-ID (2-22-16)	22-BM (2-13-16)	22-ID (03-11-16)
Cell dimensions (Å)	116.53, 62.08, 46.64	116.96, 62.20, 46.78	116.37, 61.84, 46.70	116.81 61.95, 46.86
α=γ=90°, β (°)	92.3	92.3	92.3	92.4
Resolution (Å)	30.97-1.22 (1.25-1.22)	33.02-1.69 (1.65-1.69)	32.84-1.65 (1.71-1.65)	32.95-1.99 (1.92-1.99)
[a] Rmerge	0.091 (0.764)	0.111 (0.946)	0.062 (0.501)	0.127 (0.649)
Rpim	0.034 (0.593)	0.049 (0.505)	0.031 (0.293)	0.072 (0.404)
$CC_{1/2}$, CC*	(0.449, 0.787)	(0.648, 0.887)	(0.780, 0.936)	(0.683, 0.901)
[b] <I/σI>	18.9 (1.1)	16.1 (2.0)	22.0 (2.0)	10.9 (2.0)
Completeness (%)	81.7 (18.1)	99.6 (98.6)	98.7 (89.0)	99.7 (98.2)
Redundancy	7.10 (1.6)	5.8 (4.1)	4.8 (3.5)	4.0 (3.2)
Observed reflections	568,989	218,806	189,940	91,539
Unique reflections	80,481 (1184)	37,456 (2459)	39,298 (3505)	23,085 (2242)
Refinement				
Resolution (Å)	1.22	1.69	1.65	1.99
No. Reflections	80,439	37,451	39,287	23,077
[c] Rwork / [d] Rfree	0.167/ 0.193	0.187 / 0.197	0.196/0.216	0.190/0.219
No. Atoms				
Protein	2415	2325	2345	2275
Mn(II)	1	1	1	1
Inhibitor	30	37	30	29
Solvent	192	219	262	158
B Factors (Å^2)				
Protein	29.7	29.5	26.3	33.4
Mn(II)	16.4	16.7	13.2	15.8
Inhibitor	30.2	22.4	23.1	26.0
Solvent	36.4	41.5	37.3	39.7
R.m.s. deviations				
Bond lengths (Å)	0.011	0.002	0.002	0.002
Bond angles (°)	1.0	0.5	0.5	0.5

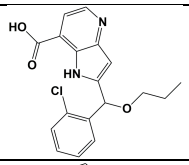
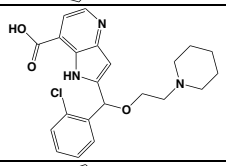
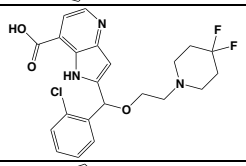
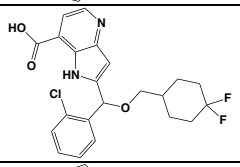
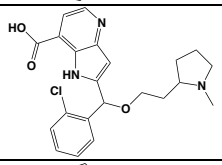
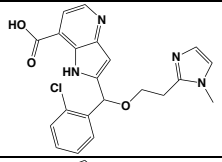
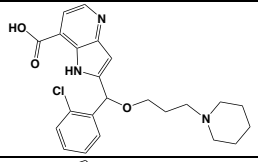
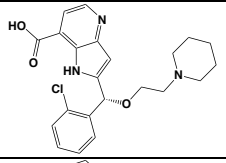
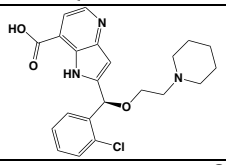
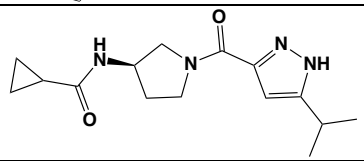
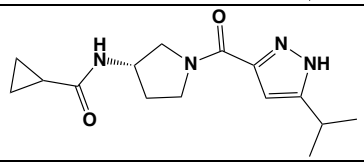
* Values in parenthesis correspond to highest resolution shell; [a] Rmerge = Σ | I - <I>| /Σ I, where I is the observed intensity and <I> is the averaged intensity from multiple observations; [b] <I/σI> = averaged ratio of the intensity (I) to the error of the intensity (σI); [c] Rwork = Σ | Fobs - Fcal | /Σ | Fobs |, where Fobs and Fcal are the observed and calculated structure factors, respectively; [d] Rfree was calculated using a randomly chosen subset (5%) of the reflections not used in refinement.

Table S1 – Continue

KDM5A(1-588)ΔAP	**N52**	**N54**	**N55**	**KDOAM-25**
PDB Code	6BH1	6BH2	6BH3	5IWF
Beamline (date)	22-ID (3-11-16)	22-ID (6-17-16)	22-ID (7-2-16)	22-ID (11-21-15)
Cell dimensions (Å)	116.03, 61.79, 46.64	117.00, 62.50, 46.96	116.67, 62.33, 46.87	117.07 61.92, 46.75
α=γ=90°, β (°)	92.5	92.6	92.6	92.3
Resolution (Å)	27.28-1.93 (2.02-1.93)	33.06-1.45 (1.50-1.45)	32.98-1.70 (1.76-1.70)	32.99-2.29 (2.34-2.29)
[a] Rmerge	0.107 (0.733)	0.110 (0.870)	0.106 (0.924)	0.106 (0.569)
Rpim	0.063 (0.451)	0.045 (0.702)	0.048 (0.603)	0.041 (0.211)
$CC_{1/2}$, CC*	(0.725, 0.917)	(0.389, 0.748)	(0.568, 0.851)	(0.964,0.991)
[b] <I/σI>	13.1 (2.3)	16.1 (2.0)	22.0 (2.0)	17.6 (5.5)
Completeness (%)	99.9 (99.7)	95.6 68.3)	96.7 (73.8)	96.6(100.0)
Redundancy	3.8 (3.4)	6.0 (1.6)	5.3 (2.4)	8.0 (8.3)
Observed reflections	93,180	345,881	189,851	117,428
Unique reflections	24,685 (2411)	57,330 (4064)	35,686 (2696)	14,593 (1005)
Refinement				
Resolution (Å)	1.93	1.45	1.70	2.29
No. Reflections	24,382	57,247	39,287	14,531
[c] Rwork / [d] Rfree	0.167/ 0.193	0.188 / 0.205	0.196/0.216	0.192/0.244
No. Atoms				
Protein	2325	2316	2370	2311
Mn(II)	1	1	1	1
Inhibitor	29	21	21	22
Solvent	172	239	262	101
B Factors (Å^2)				
Protein	33.8	28.4	30.9	33.9
Mn(II)	166	14.1	17.6	19.6
Inhibitor	25.6	16.4	23.1	32.6
Solvent	42.3	40.4	40.9	33.6
R.m.s. deviations				
Bond lengths (Å)	0.005	0.002	0.002	0.003
Bond angles (°)	0.7	0.5	0.5	0.7

* Values in parenthesis correspond to highest resolution shell; [a] Rmerge = Σ | I - <I>| /Σ I, where I is the observed intensity and <I> is the averaged intensity from multiple observations; [b] <I/σI> = averaged ratio of the intensity (I) to the error of the intensity (σI); [c] Rwork = Σ | Fobs - Fcal | /Σ | Fobs |, where Fobs and Fcal are the observed and calculated structure factors, respectively; [d] Rfree was calculated using a randomly chosen subset (5%) of the reflections not used in refinement.

Supplementary Table S2. KDM5 inhibitors used in the study

ID	NCGC ID	Original Source	Chemical structure	Chemical name
N9	00381656	WO2014164708 (Quanticel pharmaceuticals) Histone dementhylase inhibitors		2-((2-chlorophenyl)(propoxy)methyl)-1H-pyrrolo[3,2-b]pyridine-7-carboxylic acid
N40	00408936			2-((2-chlorophenyl)(2-(piperidin-1-yl)ethoxy)methyl)-1H-pyrrolo[3,2-b]pyridine-7-carboxylic acid
N41	00414992			2-((2-chlorophenyl)(2-(4,4-difluoropiperidin-1-yl)ethoxy)methyl)-1H-pyrrolo[3,2-b]pyridine-7-carboxylic acid
N42	00415015			2-((2-chlorophenyl)((4,4-difluorocyclohexyl)methoxy)methyl)-1H-pyrrolo[3,2-b]pyridine-7-carboxylic acid
N46	00421826			2-((2-chlorophenyl)(2-(1-methylpyrrolidin-2-yl)ethoxy)methyl)-1H-pyrrolo[3,2-b]pyridine-7-carboxylic acid
N47	00421835			2-((2-chlorophenyl)(2-(1-methyl-1H-imidazol-2-yl)ethoxy)methyl)-1H-pyrrolo[3,2-b]pyridine-7-carboxylic acid
N48	00421948			2-((2-chlorophenyl)(3-(piperidin-1-yl)propoxy)methyl)-1H-pyrrolo[3,2-b]pyridine-7-carboxylic acid
N51	00479257			(*R*)-2-((2-chlorophenyl)(2-(piperidin-1-yl)ethoxy)methyl)-1H-pyrrolo[3,2-b]pyridine-7-carboxylic acid
N52	00479258			(*S*)-2-((2-chlorophenyl)(2-(piperidin-1-yl)ethoxy)methyl)-1H-pyrrolo[3,2-b]pyridine-7-carboxylic acid
N54	00482457	WO2016/057924 A1 (Genentech/Constellation) Pyrrolidine amide compounds as histone demethylase inhibitors Example 29 (N54) and 28 (N55)		(*R*)-*N*-(1-(3-isopropyl-1*H*-pyrazole-5-carbonyl)pyrrolidin-3-yl-cyclopropanecarboxamide
N55	00482458			*(S)*-*N*-(1-(3-isopropyl-1*H*-pyrazole-5-carbonyl)pyrrolidin-3-yl-cyclopropanecarboxamide