

Lysine Demethylase JMJD2D (KDM4D)

A Target Enabling Package

Gene ID	JMJD2D 55693 (KDM4D) Homologues: JMJD2A 9682 (KDM4A), JMJD2B 23030 (KDM4B), JMJD2C 23081 (KDM4C),
Target Nominator	SGC Internal Nomination
Authors	Pavel Savitsky, Clarence Yapp, Stephanie B. Hatch, Udo Opperman, Susanne Muller-Knapp, Catrine Johansson, Anthony Tumber, Patrick Collins, Tobias Krojer, Romain Talon, Srikannathasan Velupillai, Mike Fairhead, Paul Brennan
Target PI	Paul Brennan (Oxford)
Therapeutic Area	Cancer
Disease Relevance	JMJD2 family linked with cancer
Date	17 th June 2016; last update 23 rd May 2017

SUMMARY OF PROJECT

There are 4 members of the Lysine Demethylase JMJD2 (KDM4) family. SGC Oxford has expressed, purified and crystallized the catalytic domains of JMJD2A, JMJD2B, JMJD2C and JMJD2D as part of the probe programme. Fragment screening and X-ray crystallography identified a large number of binders, some of which were progressed into a medicinal chemistry programme. Despite significant effort molecules with probe properties were not obtained. Consequently it has been decided to put the information generated into the public domain.

SCIENTIFIC BACKGROUND

JMJD2 family members are Histone Lysine Demethylases that specifically demethylate 'Lys-9' and 'Lys-36' residues of histone H3, requiring 2-oxoglutarate and Fe²⁺ cofactors for catalytic activity. There are 4 members of the family JMJD2A-D with a further two members JMJD2E and F considered as pseudogenes. Lysine demethylases affect transcription of regulatory genes, including those involved in oncogenic transformation and tumor maintenance, and the role of JMJD2 proteins in cancer is now unequivocally established [reviewed by Oppermann et al Epigenomics 2014] for example hypoxia-driven copy gains are dependent on JMJD4A and are blocked by inhibition of JMJD4A [Black et al. Genes & Dev. 2015], up-regulation of JMJD4A promotes transient site-specific copy gain (TSSG) in cells [Black et al, J Biol Chem. 2016], coding SNP-A482 within the JMJD2A gene is associated with differential outcome in patients with NSCLC and promotes JMJD2A turnover [van Rechem et al Cancer Discovery 2015] and JMJD2A overexpression leads to localized copy gain of 1q12, 1q21, and Xq13.1 without global chromosome instability; JMJD2A-amplified tumors have increased copy gains for these same regions [Black et al Cell 2013]; depletion of JMJD2B impairs the estrogen-induced G1/S transition of the cell cycle in vitro and inhibits breast tumorigenesis in vivo [Shi et al PNAS, 2011] and downregulation of JMJD2D inhibits cell proliferation in wild-type and even more so in p53^{-/-} HCT116 colon cancer cells, suggesting that JMJD2D is a pro-proliferative molecule [Kim et al PLoS One 2012].

Objectives of this TEP:

- Demonstrate that JMJD2 proteins can be expressed, purified and crystallized as recombinant proteins.
- Identify chemical starting points for at least one JMJD2 family member.

RESULTS – The TEP

The Lysine Demethylase JMJD2 Family

There are 4 members of the JMJD2 family JMJD2A-D.

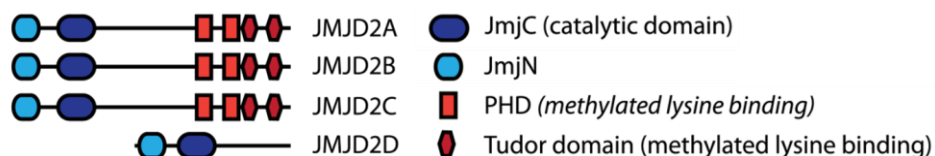


Fig. 1 The Lysine Demethylase JMJD2 family

Purified proteins

Using *E. coli* as the expression host, we expressed and purified samples of the catalytic domains of JMJD2A-D

Structures

We have obtained the following crystal structures high resolution diffraction structures which have been deposited at the PDB:

JMJD2A: 21 structures (including peptides, cofactors, metabolites and inhibitors)

JMJD2C: 1 structure

JMJD2D: 5 structures and 41 fragment hits have been crystallized

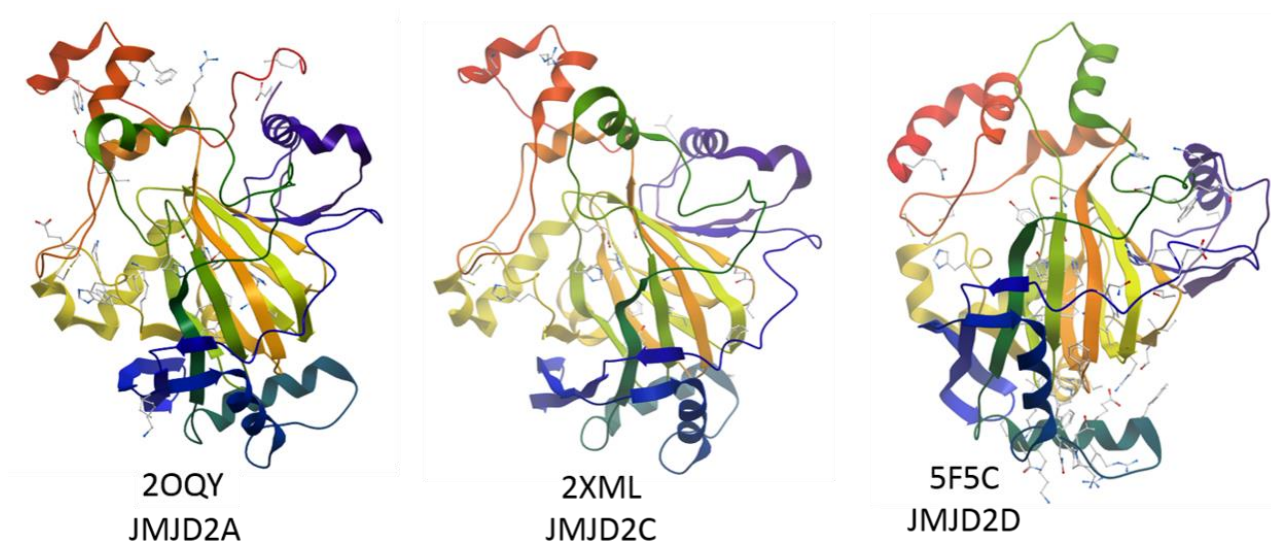


Fig. 2 Structures of the catalytic domain of the JMJD2 family members

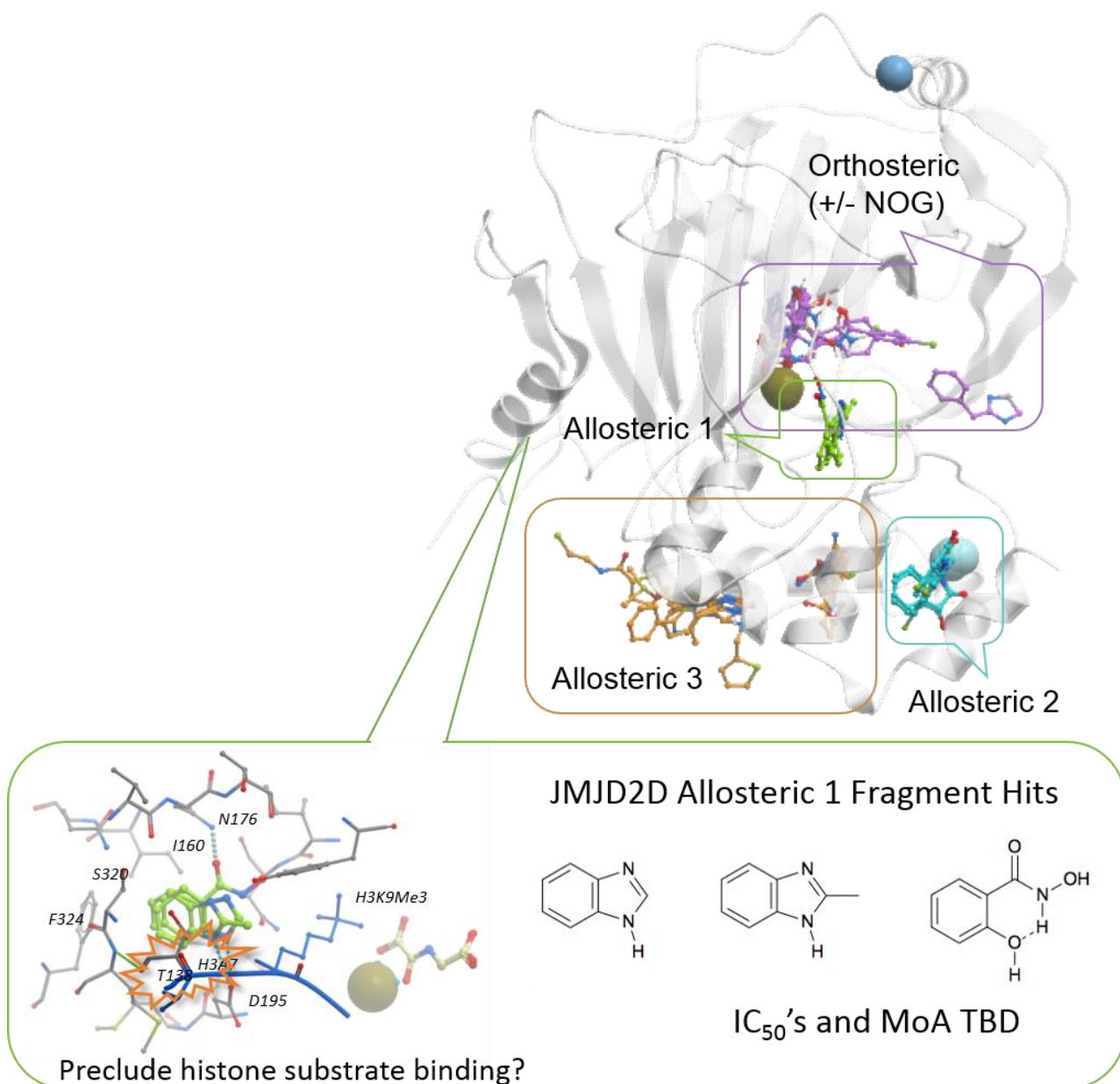
The catalytic JmjC domain adopts a β -barrel fold that is highly homologous among the JMJD2 family members. The JmjC domain and C-terminal region of the enzyme contain a Zn-binding motif composed of Histamine and Cysteine residues, a structural feature conserved throughout the JMJD2 family. In these structures the active site of JMJD2 family members is typically occupied by a Ni(II) atom which occupies the Fe(II) binding site.

Binding assays

We have developed AlphaScreen and/or RapidFire MS assays for JMJD2A-D. In addition we have AlphaScreen and RapidFire MS assays for broad set of 2-OG enzymes including at least 1 member from each KDM sub-family (KDM2-7)

Chemical starting points

We have identified many compounds from SGC chemical probes efforts including advanced leads for JMJD2A-C and allosteric inhibitors for JMJD2D. Numerous hits from fragment screening against JMJD2D



Data for numerous hits from fragment screening can be [found here](#).

We have made approximately 50 analogues of the first two fragments shown above which have demonstrated approximately 50 μ M potency, but have not yet shown if they still bound at the putative allosteric. The third fragment shown above and its analogues show inhibition (best approximately 20 μ M), but it is possible that these are orthosteric, metal chelating inhibitors which are less interesting to follow-up.

IMPORTANT: Please note that the existence of small molecules within this TEP only indicates that chemical matter can bind to the protein in a functionally relevant pocket. As such these molecules should not be used as tools for functional studies of the protein unless otherwise stated as they are not potent enough and not characterised enough to be used in cellular studies. A TEP's small molecule ligands are intended to be used as the basis for future chemistry optimisation to increase potency and selectivity and yield a chemical probe or lead series.

Cellular Assays

Cellular assays developed across the KDM family to assess the effects of KDM inhibitors have been reported in Hatch et al. 2017 (Epigenetics Chromatin. 2017 Mar 1;10:9. doi: 10.1186/s13072-017-0116-6. eCollection 2017;

<https://epigeneticsandchromatin.biomedcentral.com/articles/10.1186/s13072-017-0116-6>)

Future work

- Develop chemical probes for JMDJ2D based on Allosteric 1 hits
- ULTRA-DD

Key SGC-Oxford contributors

- Paul Brennan
- Oleg Fedorov
- Susanne Muller-Knapp

Collaborations

- SGC Pharma partner
- Collaboration with ICR for JMJD2A-B inhibitors (hit to probe to candidate)

CONCLUSION

We have generated protein, assays, crystal structures and chemical matter that has been shown to bind to, and inhibit the actions of the JMJD2 family of lysine demethylases.

FUNDING INFORMATION

The work at the SGC has been supported by the Innovative Medicines Initiative Joint Undertaking (IMI JU) under grant agreement [115766].

ADDITIONAL INFORMATION

JMJD2A	Expression, Purification and Crystallization
Expression method	Media: Starter culture: 2 x LB + 50 µg/mL Kanamycin + 34 µg/mL chloramphenicol Expression culture: 3 x 1L home-made TB + 50 µg/mL Kanamycin + salts added after autoclaving. Induction protocol: Freshly transformed BL21(DE3)-R3-pRARE2 bacteria were used to inoculate starter culture. Each litre of TB in 3L baffled flasks was inoculated with 10 mL of starter culture and grown at 37°C. The protein expression was induced with 0.2 mM IPTG an hour after OD reached OD₆₀₀ = 0.7 and media cooled to 18°C. The cells were collected by centrifugation and frozen at -80°C.
Extraction	Extraction buffers: Lysis buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 10 mM imidazole, protease inhibitor cocktail set III (Calbiochem), 50U of benzonase (EMD Milipore) Extraction procedure: Frozen cell pellets were thawed and resuspended in 200 mL of lysis buffer. The cells were disrupted by high pressure homogenisation (25 kpsi). Cell debris were removed by centrifugation for 60 minutes at 35 000xg
Purification	Column 1 IMAC: HisTrap FF Crude, 1 mL (GE/Amersham Biosciences). Lysis buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 10 mM imidazole, 5% glycerol. Wash buffer: 50 mM HEPES, pH 7.5, 500 mM NaCl, 30mM imidazole, 5% glycerol. Elution buffer 50 mM HEPES pH 7.5, 500 mM NaCl, 250 mM imidazole, 5% glycerol. Procedure: The cell extract was loaded on the column at 1 mL/minute on an AKTA-express system (GE/Amersham). The column was then washed with 30 column volumes of lysis buffer, 10 volumes of wash buffer, and then eluted with elution buffer. The eluted peak of A280 was automatically collected. Column 2 Ion exchange: 2 x 1ml HiTrap HP Q ion exchange column (GE/Amersham Biosciences). Low salt buffer: 25 mM HEPES pH 7.5, 50 mM NaCl, High salt budder: 25 mM HEPES pH 7.5, 1M NaCl, 5% glycerol Dilution buffer: 25mM HEPES pH 7.5 Procedure: The eluted fractions from the Ni-affinity Histrap column were pooled and diluted 10x to 50mM NaCl concentration, then loaded on ion exchange columns at flow of 1ml/min. Both peaks were eluted at salt concentration between 150-200mM. Glycerol and NaCl were added during concentration procedure to reach final buffer formula of 25mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol.
Concentration and storage	The protein was concentrated using an Amicon Ultracel centrifugal concentrator (10 kDa MWCO) to 1 mg/ml by A280 and predicted extinction coefficient. Stock was concentrated to 3mg/ml and all samples were frozen in liquid nitrogen followed by storage in -80°C.
Mass spec	The mass determined for JMJD2AA-p084 was 44266 Da, in agreement with the predicted mass for the his-tagged protein. Protein is also modified by N-glucuronylation and phosphogluconoylation which are typical modifications for His-tagged proteins.
Crystallization	JMJD2A was crystallized in sitting drop plates at 4°C by mixing 100nl of 30 mg/mL protein in 20 mM HEPES pH7.5, 5 % (v/v) glycerol, 500 mM NaCl, 0.5 mM TCEP with 50 nL of reservoir solution containing 0.1 M Bis-Tris pH 5.9, 0.15 M ammonium sulphate, 13% (w/v) PEG3350. Plate-like crystals typically grew within a week. The crystals belong to space group P2 ₁ 2 ₁ 2 with two molecules in the asymmetric unit and unit cell dimensions of a=101.0Å, b=149.6 Å, c=57.9Å. The typical resolution limit of the crystals is between 1.9 and 2.5 Å.

JMJD2C	Expression and Purification
Expression method	Media: Starter culture: SOB + 50 µg/mL Kanamycin + 34 µg/mL chloramphenicol Expression culture: 6 x 1L home-made TB + 50 µg/mL Kanamycin + phosphate salts added after autoclaving. Induction protocol: Freshly transformed BL21(DE3)-R3-pRARE2 bacteria were used to inoculate starter culture. Each litre of TB in 3L baffled flasks was inoculated with 10 mL of starter culture and grown at 37°C, 180rpm. The

	protein expression was induced with 0.2 mM IPTG at OD ₆₀₀ = 2.5 for 18 h at 18°C. The cells were collected by centrifugation and used directly for purification.
Extraction	Extraction buffers: Lysis buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 10 mM imidazole, protease inhibitor cocktail set III (Calbiochem) Extraction procedure: Pellets were thawed and resuspended in 150 mL of lysis buffer +500U benzonase (Novagene). The cells were disrupted by high pressure homogenisation (25 kpsi). Cell debris were removed by centrifugation for 45 minutes at 55 000xg
Purification	Column 1 IMAC 5ml of Ni-Sepharose 6FF in gravity column (GE/Amersham Biosciences). Lysis buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 10 mM imidazole, 5% glycerol. Wash buffer: 50 mM HEPES, pH 7.5, 500 mM NaCl, 30mM imidazole, 5% glycerol. Elution buffer 50 mM HEPES pH 7.5, 500 mM NaC, 250 mM imidazole, 5% glycerol. Procedure: The cell extract was applied to gravity column containing equilibrated resin. Resin washed with 20CV of lysis buffer, 20 CV of wash buffer, and then proteins eluted with elution buffer. Column 2: Gel filtration and TEV cleavage Gel Filtration: HiLoad 16/60 Superdex 75prep grade, 120 mL (GE/ Amersham Biosciences). Buffer: 20mM HEPES, 0.5M NaCl, 5% glycerol, pH 7.5 TEV protease purified in house Procedure: Pooled fractions were filtered and loaded on two gel filtration columns (flow 1.2ml/min). Fractions corresponding to monomeric protein were pooled together. 2mg of TEV protease was added per each 50mg of target protein and incubated over-night. Column 3: Rebinding: Protein rebound to 1ml of Ni-sepharose 6 FF equilibrated in gel filtration buffer. Protein solution was passed twice through gravity column containing resin. Resin washed with 5CV of gel filtration buffer and contaminants eluted with 5CV of elution buffer. F-T fraction and gel filtration fractions contained pure protein and were pooled together.
Concentration and storage	The protein was concentrated using an Amicon Ultracel centrifugal concentrator (10 kDa MWCO) to 5 mg/ml by A280 and predicted extinction coefficient. Frozen in 200ul aliquots in liquid nitrogen and stored at -80C. Protein yield: 3.7mg/1L
Quality control	Mass spectrometry detects proper molecular weight and protein is enzymatically active confirmed by FDH assay. Protein remains active for at least two freeze-thaw cycles confirmed by demethylase assay with RapidFire detector.
Crystallization	

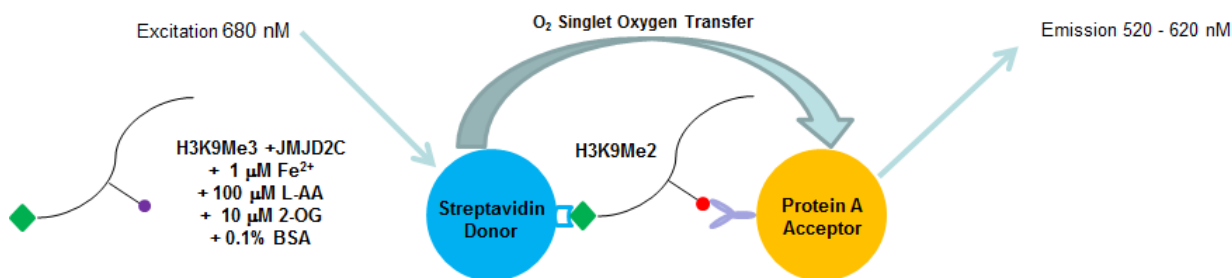
JMJD2D	Expression, Purification and Crystallization
Expression method	Media: Starter culture: TB (home made) + 50 µg/mL Kanamycin + 34 µg/mL chloramphenicol Expression culture: 6 x 1L home-made TB + 50 µg/mL Kanamycin + salts added after autoclaving. Induction protocol: Freshly transformed BL21(DE3)-R3-pRARE2 bacteria were used to inoculate starter culture. Each litre of TB in 6L baffled flasks was inoculated with 10 mL of starter culture and grown at 37°C. The incubator with cultures was cooled down to 18°C after OD reached 0.7. Protein expression was induced with 0.1 mM IPTG after one hour. The cells were collected by centrifugation and frozen at -80°C.
Extraction	Extraction buffers: Lysis buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 10 mM imidazole, protease inhibitor cocktail set III (Calbiochem) Extraction procedure: Frozen cell pellets were thawed and resuspended in 200 mL of lysis buffer. The cells were disrupted by high pressure homogenisation (25 kpsi). Cell debris were removed by centrifugation for 60 minutes at 35 000xg
Purification	Column 1 IMAC Ni-Sepharose 6 FF, 2 mL resin (GE/Amersham Biosciences). Lysis buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 10 mM imidazole, 5% glycerol. Wash buffer: 50 mM HEPES, pH 7.5, 500 mM NaCl, 30mM imidazole, 5% glycerol. Elution buffer 50 mM HEPES pH 7.5, 500 mM NaC, 250 mM imidazole, 5% glycerol.

	Procedure: The cell extract was purified through gravity column containing nickel resin. The column was then washed with 25 column volumes (CV) of lysis buffer, 10 CV of wash buffer, and then eluted with elution buffer. Column 2 TEV cleavage and Gel filtration HiPrep Superdex 200 16/60 (GE/Amersham Biosciences). Buffer : 10 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol. TEV protease produced in house. Procedure: The eluted fractions were pooled and TEV protease was added over-night. The following day protein has been concentrated to 5ml and loaded onto gel filtration column. Fractions corresponding to monomeric peak were pooled together . Column 3 Nickel rebinding: Protein contaminants were rebound to 250ul of Ni-Sepharose using gravity method. After target protein was collected in flow-through the resin was washed with 10CV of gel filtration buffer, lysis buffer and elution buffer each. Flow-through and gel filtration fractions were containing target protein.
Concentration and storage	The protein was concentrated using an Amicon Ultracel centrifugal concentrator (30 kDa MWCO) to 7 mg/ml by A280 and predicted extinction coefficient. Frozen in Liquid nitrogen and stored at -80°C. The purification procedure remains to be optimised.
Expression method	Media: Starter culture: TB (home made) + 50 µg/mL Kanamycin + 34 µg/mL chloramphenicol Expression culture: 6 x 1L home-made TB + 50 µg/mL Kanamycin + salts added after autoclaving. Induction protocol: Freshly transformed BL21(DE3)-R3-pRARE2 bacteria were used to inoculate starter culture. Each litre of TB in 6L baffled flasks was inoculated with 10 mL of starter culture and grown at 37°C. The incubator with cultures was cooled down to 18°C after OD reached 0.7. Protein expression was induced with 0.1 mM IPTG after one hour. The cells were collected by centrifugation and frozen at -80°C.
Crystallization	JMJD2D was crystallized in sitting drop plates at 20°C by mixing 100 nL of 11mg/ml protein in 20 mM HEPES pH7.5, 5 % (v/v) glycerol, 500 mM NaCl, 0.5 mM TCEP with 50 nL of reservoir solution containing 0.1 M Bis-Tris pH 5.9, 0.15 M ammonium sulfate , 11% (w/v) PEG3350. Bi-pyramidal shaped crystals grew within several days. They belong to space group P4₃2₁2 with one molecule in the asymmetric unit and typical unit cell dimensions of a=b=72 Å, c=151Å. The typical resolution limit of the crystals is between 1.1 and 1.5 Å.

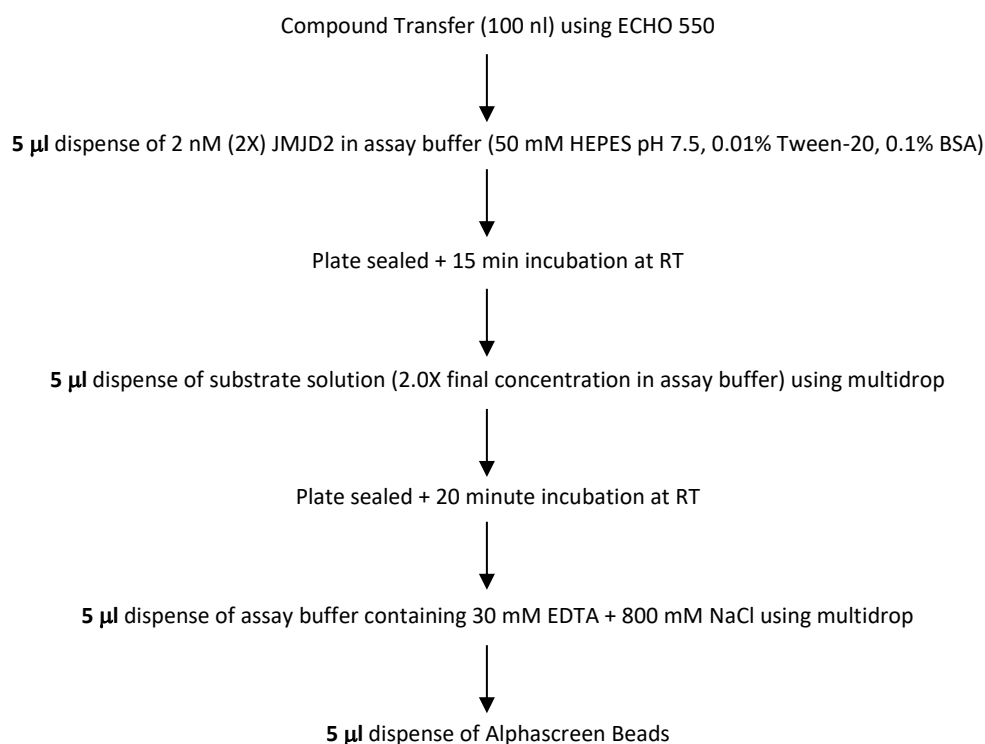
Assays

Overview

The JMJD2C demethylase assay uses the peptide: biotin-ARTKQTARK(Me3)STGGKAPRKQLA-GGK-Biotin (Histone H3 Lys 9 tri-methyl) as a substrate and relies on detection of H3K9Me2-biotin bound to streptavidin donor beads by a monoclonal anti-H3K9Me2 antibody coupled to protein-A acceptor beads.

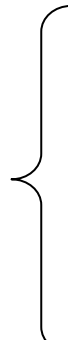


Screens use low nM concentrations of JMJD2 (1.0 nM enzyme routinely used in the assay) and nM concentrations of peptide substrate (H3K9Me3 routinely used at 30 nM in assay screens). Assays are performed in 384-well proxiplates and compounds dispensed using ECHO Acoustic dispensing.



Experimental Conditions

Enzyme Reaction Volume	10 μl	
Final Assay Volume After Addition of EDTA and Alphascreen Beads:	20 μl	
Enzyme Reaction :	Composition Enzyme Reaction Final Concentration in 10 μl:	
pH 7.5	Enzyme	: 1.0 nM
	HEPES pH 7.5	: 50 mM
	Tween-20	: 0.01%
	FAS	: 1 μM
	L-Ascorbic Acid	: 100 μM
	Peptide	: 30 nM
	2-Oxoglutarate	: 10 μM
	BSA	: 0.1 %

Alphascreen Beads (Detection Step) :		Composition of Beads Detection Final Concentration in 20 µl:	
pH 7.5		HEPES pH 7.5	: 50 mM
		Tween-20	: 0.01%
		BSA	: 0.1 %
		EDTA	: 7.5 mM
		NaCl	: 200 mM
		Protein A Acceptor	: 0.02 mg/ml
		Streptavidin Donor	: 0.02 mg/ml
		Anti-H3K9Me2	: 0.05 µg/ml

Material

Reagent	Supplier	Code
HEPES Free Acid >99.5%	Gibco Life Technologies	11344-041
H3(1-21)K9Me3 Peptide	Anaspec	64360
(NH ₄) ₂ [Fe(SO ₄) ₂].6H ₂ O	Sigma-Aldrich	12304
L-Ascorbic Acid	Sigma-Aldrich	A5960
2-OG (α-ketoglutaric acid)	Sigma-Aldrich	K3752
Tween-20	Fisher Scientific	BPE-337-500
BSA Cohn Fraction V	Sigma-Aldrich	**A7030
Anti-H3K9Me2	Abcam	Ab1220
EDTA	Fisher Scientific	BP120
NaCl	VWR Analar Normapur	27810.364
AlphaScreen General IgG	Perkin Elmer	6760617c
384-well proxiplates plus	Perkin Elmer	6008280
Thermowell Sealing Tape	Corning	6570

****Critical** : Always use this batch of BSA

Alphascreen Beads

Store at 4°C

H3K9Me3 substrate

H3K9Me3-biotin substrate is stored at -80°C in 24 µl aliquots at a concentration of 100 µM.

JMJD2 Enzyme

JMJD2C is stored in 1 µM aliquots at -80°C.

Preparation Of Solutions

Assay Buffer

Prepare fresh every week and filter sterilize through a 0.2 micron filter: 50 mM HEPES pH 7.5, 0.01% Tween-20, 0.1% BSA.
Store at 4°C

Assay Stop

Prepare fresh each day. Assay Buffer containing 30 mM EDTA + 800 mM NaCl (4X final concentration).

Ferrous Ammonium Sulphate (FAS)

Prepare FAS fresh each day. Make up 400 mM stock solution (156.856 mg/ml) in 20 mM HCl and then prepare 2 ml of 1 mM FAS in deionized H₂O. Store at room temperature.

2-Oxoglutarate (2-OG)

Prepare 2-OG fresh each day. Make up 10 mM stock solution in deionized H₂O (1.901 mg/ml) and store at room temperature.

L-Ascorbic Acid (L-AA)

Prepare L-AA fresh each day. Make up 50 mM stock solution in deionized H₂O (8.806 mg/ml) and store at room temperature.

Substrate Solution

	Stock Concentration	2.0X Concentration	Volume Required For		
			10 ml of Substrate	20 ml of Substrate	30 ml of Substrate
L-Ascorbic Acid	50 mM	200 µM	40 µl	80 µl	120 µl
FAS	1 mM	2.0 µM	20 µl	40 µl	60 µl
H3K9Me3-Biotin	100 µM	0.06 µM	6.0 µl	12 µl	18 µl
2-OG	10 mM	20 µM	20 µl	40 µl	60 µl
Assay Buffer			9914 µl	14828 µl	29742 µl

Protocol

- 1) Calculate the amount of alphascreen beads required for the experiment.
- 2) Prepare the required amount of beads in a dark box at 4X the final concentration:
Alphascreen donor beads and acceptor beads are mixed and pre-incubated with Anti-H3K9Me2 antibody in assay buffer for at least 1 hour before addition to the assay. Make up at 4X final concentration:
Streptavidin Donor 0.08 mg/ml Final in Assay = 0.02 mg/ml
Protein A Acceptor 0.08 mg/ml Final in Assay = 0.02 mg/ml
Anti-H3K9Me2 0.2 µg/ml Final in Assay = 0.05 µg/ml
- 3) Retrieve an aliquot of JMJD2C and 100 µM peptide from the -80°C and place on ice.
- 4) Weigh out Ferrous Ammonium Sulphate (100 -150 mg), 2-Oxoglutarate (2 - 4 mg) and L-Ascorbic Acid (8 -16 mg) in 2.0 ml eppendorf tubes and prepare the solutions as described in section 4.
- 5) Compounds should be supplied in Labcyte 384-LDV (5.0 – 10 µl per well) or Labcyte 384-PP (25 – 45 µl per well) plates at 10 mM concentration in DMSO. IC50 plates are set up using ECHO dose response software and single shot screening plates are set up using ECHO plate reformat software. Transfer compound to proxiplate using ECHO 550 (maximum DMSO in assay should be 1%). A typical 11-point IC50 plate layout for 16 compounds is shown below with column 12 as a DMSO control and column 24 (100 µM 2, 4-PDCA reference compound)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	1	1	1	1	1	1	1	1	1	b	9	9	9	9	9	9	9	9	9	9	9	a
B	1	1	1	1	1	1	1	1	1	1	1	b	9	9	9	9	9	9	9	9	9	9	9	a
C	2	2	2	2	2	2	2	2	2	2	2	b	10	10	10	10	10	10	10	10	10	10	10	a
D	2	2	2	2	2	2	2	2	2	2	2	b	10	10	10	10	10	10	10	10	10	10	10	a
E	3	3	3	3	3	3	3	3	3	3	3	b	11	11	11	11	11	11	11	11	11	11	11	a
F	3	3	3	3	3	3	3	3	3	3	3	b	11	11	11	11	11	11	11	11	11	11	11	a
G	4	4	4	4	4	4	4	4	4	4	4	b	12	12	12	12	12	12	12	12	12	12	12	a
H	4	4	4	4	4	4	4	4	4	4	4	b	12	12	12	12	12	12	12	12	12	12	12	a
I	5	5	5	5	5	5	5	5	5	5	5	b	13	13	13	13	13	13	13	13	13	13	13	a
J	5	5	5	5	5	5	5	5	5	5	5	b	13	13	13	13	13	13	13	13	13	13	13	a
K	6	6	6	6	6	6	6	6	6	6	6	b	14	14	14	14	14	14	14	14	14	14	14	a
L	6	6	6	6	6	6	6	6	6	6	6	b	14	14	14	14	14	14	14	14	14	14	14	a
M	7	7	7	7	7	7	7	7	7	7	7	b	15	15	15	15	15	15	15	15	15	15	15	a
N	7	7	7	7	7	7	7	7	7	7	7	b	15	15	15	15	15	15	15	15	15	15	15	a
O	8	8	8	8	8	8	8	8	8	8	8	b	16	16	16	16	16	16	16	16	16	16	16	a
P	8	8	8	8	8	8	8	8	8	8	8	b	16	16	16	16	16	16	16	16	16	16	16	a

- 6) Dilute JMJD2C enzyme to 2.0 nM in 50 mM HEPES pH 7.5, 0.01% Tween-20, 0.1% BSA and dispense 5.0 µl into columns 1 -24 of a 384-well proxiplate. Dispenses are performed using a Thermo multidrop.
- 7) Incubate the plate for 15 minutes on the bench at room temperature
- 8) Prepare enough 2.0X substrate solution containing L-Ascorbic Acid (200 µM), FAS (2.0 µM), Peptide (60 nM) and 2-OG (20 µM) and dispense 5 µl of substrate into columns 1 -24 of the assay plate. Seal the plate.
- 9) Allow the enzyme reaction to proceed at room temperature for 20 minutes.
- 10) Dispense across the plate 5 µl of assay stop from section 4.2.
- 11) In a dark box, dispense 5 µl of the alphascreen beads prepared in STEP 2 into every well of the assay plate. Seal the plate with an aluminium plate foil.
- 12) Incubate the plate for 120 minutes at room temperature.
- 13) Read the plate on the BMG Labtech Pherastar FS.