Lysine Demethylase JMJD1B (KDM3B)

A Target Enabling Package (TEP)

Gene ID / UniProt ID / EC	JMJD1B 51780 (KDM3B) Homologues: JMJD1A 55818 (KDM3A), JMJD1C 221037 (KDM3C)
Target Nominator	SGC Internal Nomination
SGC Authors	Pavel Savitsky, Stephanie B. Hatch, Udo Opperman, Susanne Muller-Knapp, Catrine Johansson, Anthony Tumber, Tobias Krojer, Romain Talon, Srikannathasan Velupillai, Mike Fairhead, Paul Brennan
Collaborating Authors	Clarence Yapp[1], Patrick Collins[2]
Target PI	Paul Brennan (SGC Oxford)
Therapeutic Area(s)	Cancer
Disease Relevance	JMJD1 family linked with cancer
Date approved by TEP Evaluation Group	17th June 2016
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Affiliations	1. Laboratory of Systems Pharmacology, Harvard University 2. Diamond Light Source, Harwell Science and Innovation Campus

USEFUL LINKS

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SUMMARY OF PROJECT

There are 3 members of the Lysine Demethylase JMJD1 (KDM3) family, JMJD1A-C. SGC Oxford has expressed, purified and crystallized the catalytic domains of JMJD1A, JMJD1B and JMJD1C 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

The JMJD1 family are histone demethylases specific for H3K9me2/me1. There are 3 members of the family JMJD1A-C. The expression of JMJD1A is very significant in testes and has been implicated in demethylation of H3K9me2 of AR target genes. The JMJD1 family has been linked with cancer, for example the hypoxia-inducible epigenetic regulators JMJD1A and G9a provide a mechanistic link between angiogenesis and tumor growth (1), there is a dynamic change of chromatin conformation in response to hypoxia enhancing the expression of GLUT3 (SLC2A3) by cooperative interaction of hypoxia-inducible factor 1 and JMJD1A (2) and JMJD1A is a positive regulator of the G1/S transition in cancer cells via transcriptional regulation of the HOXA1 gene (3). The role of JMJD1A, in hepatocellular carcinoma: clinical impact on recurrence after hepatic resection has also been described (4) and the JMJD1A-KLF2-IRF4 axis maintains myeloma cell survival (5).

Objectives of this TEP:

- Demonstrate that JMJD1 proteins can be expressed, purified and crystallized as recombinant proteins.
- Identify chemical starting points for at least one JMJD1 family member.

RESULTS – THE TEP

The Lysine Demethylase JMJD1 Family

There are three members of the JMJD1 family JMJD1A-C.

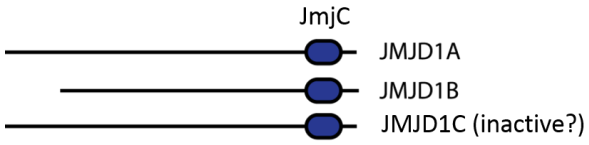


Fig. 1 The Lysine Demethylase JMJD1 family

Purified proteins

Using *E. coli* as the expression host, we expressed and purified samples of the catalytic domains of JMJD1A-C.

Structures

We have obtained the following crystal structures high resolution diffraction structures which have been deposited at the PDB:
JMJD1B: 1 structure (PDB: 4C8D)
JMJD1C: 2 structures (PDB: 5FZO, 2YPD)

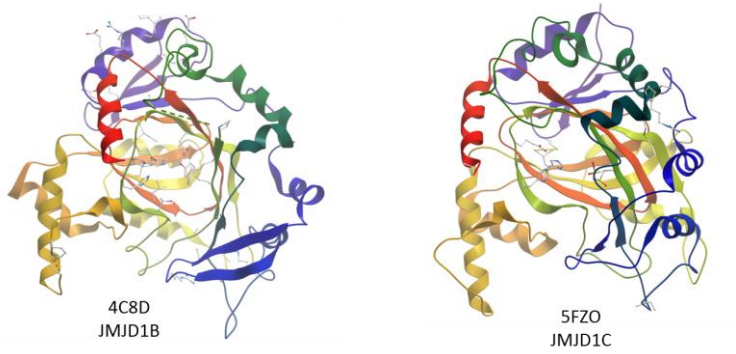


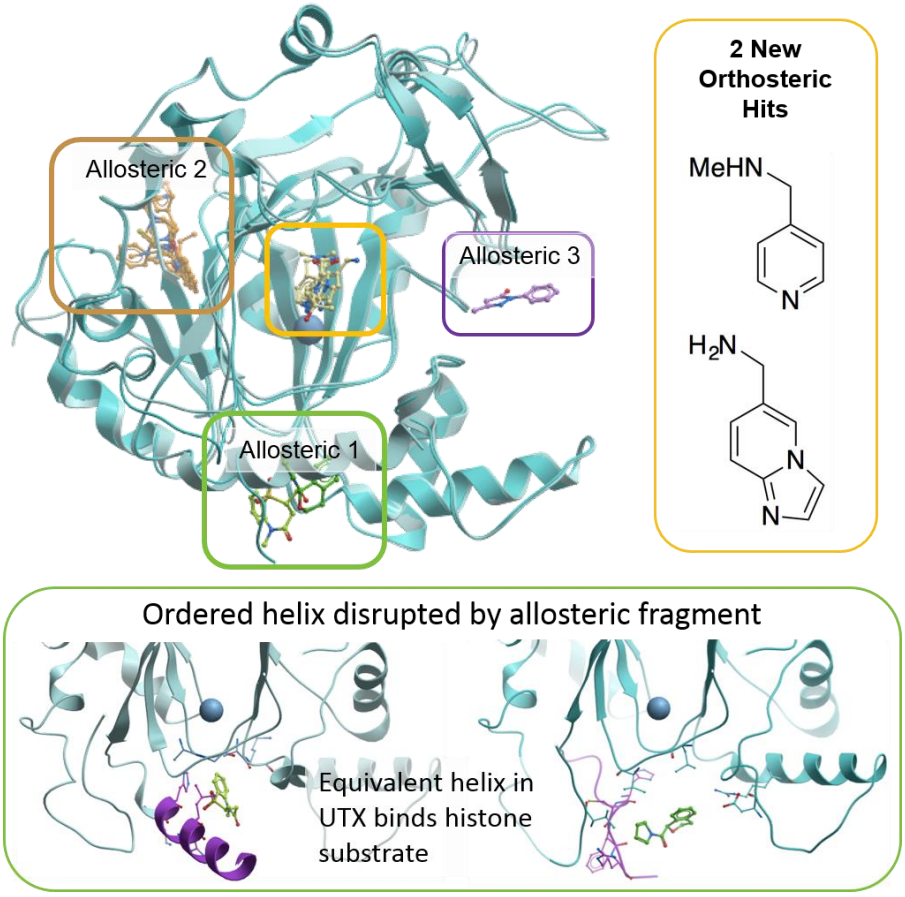
Fig. 2 Structures of the catalytic domain of the JMJD1 family members JMJD1B and JMJD1C

Binding assays

We have developed AlphaScreen and RapidFire MS assays for broad set of 2-OG enzymes including at least 1 member from each KDM sub-family (KDM).

Chemical starting points

We have identified 1-10 µM hits from KDM cross-screening and 12 fragment hits from a JMJD1B soaking campaign.



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. eCollection 2017).

Future work

- Develop chemical probes for JMDJ1B based on fragment hits and cross-screening hits

Key SGC-Oxford contributors

- Paul Brennan
- Oleg Fedorov
- Susanne Muller-Knapp

Collaborations

- SGC Pharma partner

CONCLUSION

We have generated protein, assays, crystal structures and chemical matter that has been shown to bind to, and inhibit the actions of the JMJD1 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

Structure Files

PDB ID	Structure Details
4C8D	Crystal structure of JmjC domain of KDM3B
5FZO	Crystal structure of JMJD1C catalytic domain
2YPD	Crystal structure of JMJD1C Jumonji domain

Materials and Methods

Experimental Procedures

JMJD1A

Expression

Media:

Virus amplification: Sf900 III (Gibco) + 2% FBS.

Expression: Insect-Xpress (Lonza).

Expression protocol:

0.6L of Sf9 insect cells in 3L glass non-baffled flasks was infected with 1.8mL of virus P2 (expression can be performed in 1L of cells but the yield calculated per 1L is higher when performed in 0.6L). Cell density at infection time: 2e6 /mL. Protein was expressed for 72h at 27^{o}C with 100rpm shaking.

Extraction

Extraction buffers:

Lysis buffer: 50mM HEPES-KOH, 0.3M KCl, 5% glycerol, 10mM imidazole pH 7.4, protease inhibitors cocktail set VII (Calbiochem)

Extraction procedure:

Cells were spun at 900xg for 10min and resuspended in 30 mL of lysis buffer per litre. Cells were broken by sonication (3min, 35% amplitude, 5s on 10s off). Insoluble fraction removed by centrifugation for 30 minutes at 55 000xg.

Purification

Column 1

IMAC: Sepharose 6 FF resin charged with nickel (GE/Amersham Biosciences).

Lysis: 50mM HEPES, 0.3 KCl, 5% glycerol, 10mM imidazole, pH 7.5

Wash: 50mM HEPES, 0.3M KCl, 5% glycerol, 40mM imidazole, pH 7.5

Elution: 50mM HEPES, 0.3M KCl, 5% glycerol, 250mM imidazole, pH 7.5 + protease inhibitors cocktail set VII (Calbiochem)

Procedure:

Proteins were batch bound to 0.5mL resin for one hour at 4^{O}C. Resin was spun at 500 x g and supernatant removed. Resin was re-dissolved in 20 column volumes of binding buffer and centrifuged again. Resin was again re-dissolved in 10 CV of wash buffer and transferred to gravity column. Proteins were eluted with 5CV of elution buffer. Fractions containing proteins were analysed on SDS-page and pooled together.

Column 2:

Gel Filtration: HiLoad 16/60 Superdex 200 prep grade, 120 mL (GE/ Amersham Biosciences). Buffer: 20mM Tris-HCl, 0.5M NaCl, 5% glycerol pH 7.6

Procedure:

Pooled fractions were filtered and loaded on gel filtration column (flow 1.2ml/min). Fractions corresponding to monomeric protein were pooled together.

Concentration and storage

The protein was concentrated using an Amicon Ultracel centrifugal concentrator (50 kDa MWCO) to 2 mg/ml by A280 and extinction coefficient. Aliquots were frozen in liquid nitrogen and kept in -80oC.

Yield
1.9mg from 0.6L

JMJD1B
Expression
Media:
Virus amplification: Sf900 III (Gibco) + 2% FBS.
Expression: Insect-Xpress (Lonza).
Expression protocol:
4x1L of Sf9 insect cells in 3L glass non-baffled flasks was infected with 3mL of virus P2. Cell density at infection time: 2e6 /mL. Protein was expressed for 72h at 27^{o}C with 100rpm shaking.

Extraction
Extraction buffers:
Lysis buffer: 50mM HEPES-KOH, 0.3M KCl, 5% glycerol, 10mM imidazole pH 7.4, protease inhibitors cocktail set VII (Calbiochem)
Extraction procedure:
Cells were spun at 900xg for 10min and resuspended in 30 mL of lysis buffer per litre. Cells were broken by sonication (3x3min, 35% amplitude, 5s on 10s off). Insoluble fraction removed by centrifugation for 30 minutes at 55 000xg.

Purification
Column 1
IMAC: Sepharose 6 FF resin charged with nickel (GE/Amersham Biosciences).
Lysis: 50mM HEPES, 0.3 KCl, 5% glycerol, 10mM imidazole, pH 7.5
Wash: 50mM HEPES, 0.3M KCl, 5% glycerol, 40mM imidazole, pH 7.5
Elution: 50mM HEPES, 0.3M KCl, 5% glycerol, 250mM imidazole, pH 7.5 + protease inhibitors cocktail set VII (Calbiochem)
Procedure:
Proteins were batch bound to 3mL resin for one hour at 4^{O}C with gentle rotation. Resin was spun at 500 x g and supernatant removed. Resin was re-dissolved in 60 column volumes of binding buffer and centrifuged again. Resin was again re-dissolved in 10 CV of wash buffer and transferred to gravity column. Proteins were eluted with 5CV of elution buffer. Fractions containing proteins were analysed on SDS-page and pooled together.

Column 2
Gel Filtration: HiLoad 16/60 Superdex 200 prep grade, 120 mL (GE/ Amersham Biosciences).
Buffer: 20mM HEPES, 0.3M KCl, 5% glycerol pH 7.6, 0.5mM TCEP
Procedure:
Pooled fractions were filtered and loaded on 2x gel filtration column (flow 1.2ml/min). Fractions corresponding to monomeric protein were pooled together (it's impossible to separate some dimer-monomer fraction of the peak).

Concentration and storage
The protein was concentrated using an Amicon Ultracel centrifugal concentrator (50 kDa MWCO) to 1.3 mg/ml by A280 and extinction coefficient. Aliquots were frozen in liquid nitrogen and kept in -80oC.

Mutant protein for Crystallography and Crystallisation Conditions

Protocols for JMJD1BA-c078 (Q1601H:G1606R)
1. Protein expression and purification
Vector: pNIC28-Bsa4
Cell line: BL21[DE3]-pRARE2

Tags and additions: N-terminal, TEV protease cleavable hexahistidine tag
Final protein sequence
MHHHHHHSSGVDLGTENLYFQ*SMTSHSWLCDGRLLCLHDPSNKNNWKIFRECWKQGQPVLVSGVHKKLKSELWKPEAF
SQEFGDQDVDLVNCRNCAIISDVKVRDFWDGFEIICKRLRSEDGQPMVLKLKDWPPGEDFRDMMPTRFEDLMENLPLPEY
TKRDGRLNLASRLPSYFVRPDLGPKMYNAYGLITAEDRRVGTTNLHLDVSDAVNVMVYVGIPIGEGAHDEEVLKTIDEGDAD
EVTKERIHDHKEKPGALWHIYAAKDAEKIRELLRKVGEEQGQENPPDHDPIHDQSWYLDQTLRKRLYEEYGVQGWAIVQFL
GDAVFIPAGAPHQVHNLYSCIKVAEDFVSPEHVKHCFRLTQEFRHLSNTHT
(underlined sequence contains vector encoded His-tag and TEV protease cleavage site*)

Cell Lysis
Lysis Buffer: 10 mM HEPES, 500 mM NaCl, 5 % glycerol, 0.5 mM TCEP, 10 mM imidazole pH 7.5
Cell pellet (180 g) was dissolved in 500 mL of lysis buffer and lysozyme and benzonase were added to 0.5 mg/mL and 1 µg/mL respectively. After 30 minutes stirring in the cold room 30 mL of 10 % Triton X-100 was added and stirring continued for a further 30 minutes. Cells were split across 24 x 50 mL Falcoln tubes and frozen at -20 °C overnight. Cells were thawed at room temperature and the volume of each tube brought to 50 mL with Lysis Buffer. Cells were centrifuged for 1 h at 5,000 g (4 °C).

Column 1: His GraviTrap columns (24 x 1 ml volume in a gravity-flow column)
Wash Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5 % glycerol, 10 mM imidazole, 0.5 mM TCEP
Elution Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5 % glycerol, 500 mM imidazole, 0.5 mM TCEP
The clarified cell extract was added to a 24 x 1 ml His GraviTrap columns pre-equilibrated with Wash Buffer. The columns were then washed with 2 x 5 ml Wash Buffer. The protein was eluted with 2.5 ml Elution Buffer.

Column 2: PD-10 desalting columns (24 x 8.3 ml volume in a gravity-flow column)
Wash Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5 % glycerol, 10 mM imidazole, 0.5 mM TCEP
Each 2.5 mL His GraviTrap fraction (24 x) was applied directly to a PD-10 column (24 x) for desalting and eluted with 3.5 mL of Wash Buffer

Tag cleavage
1 mg of TEV protease was added to every 10 mg of the eluted protein and the digestion was performed overnight at 4 °C.

Column 3: His GraviTrap columns (24 x 1 ml volume in a gravity-flow column)
Wash Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5 % glycerol, 10 mM imidazole, 0.5 mM TCEP
The TEV cleaved protein (3.5 mL) was applied to a His GraviTrap column pre-equilibrated in Wash Buffer for removal of His-tag, TEV and uncleaved protein. The column was washed with a further 2.5 mL of Wash Buffer for a final pool of 6 mL. The various desalted protein fractions were combined (24 x 6 mL) and 16 mL of 1 M L-arginine + 1 M L-glutamate was added to help avoid precipitation. The protein was concentrated to 25 mg/mL using a 30 kDa MWCO concentrator.

Column 4: Yarra SEC 2000 300x21.2 mm column (300x21.2 mm column, 104 mL volume)
Gel Filtration Buffer: 10 mM HEPES pH 7, 500 mM NaCl, 5 % glycerol, 0.5 mM TCEP
The column was equilibrated with Gel Filtration buffer and the protein loaded, the peak corresponding to JMJD1BA was taken and concentrated to 25 mg/ml using a 30 kDa MWCO concentrator, flash frozen in liquid nitrogen and stored at -80°C.

2. Protein crystallization
JMDJD1BA was crystallized by mixing 100 nl of 25 mg/ml protein in 10 mM HEPES pH7.5, 500mM NaCl, 5% Glycerol, 0.5 mM TCEP with 50nl of reservoir solution containing 25 % PEG3350, 200 mM $MgCl_2$, 0.1 M bis-tris, pH 6.5. Crystals appeared after 4-7 days from sitting drop plates at 4°C. JMJD1BA crystallized in space group $P2_1$ with unit cell dimensions of a = 58 Å, b = 94 Å, c = 93 Å, corresponding to two JMJD1BA molecules in the asymmetric unit. The crystals typically diffract between 1.6 and 1.8 Å.

Protocols for JMJD1CA-c095 (Q2495R)

1. Protein expression and purification

Vector: pNIC28-Bsa4

Cell line: BL21[DE3]-pRARE2

Tags and additions: N-terminal, TEV protease cleavable hexahistidine tag

Final protein sequence

MHHHHHHSSGVDLGTENLYFQ*SMIPHSWICEKHILWLKDYKNSSNWKLFKECWKQGQPAVVSGVHKKMNISLWKAESI
SLDFGDHQADLLNCKDSIISNANVKEFWDGFEEVSKRQKNKSGETVVLKLKDWPSGEDFKTMMPARYEDLLKSLPLPEYCNP
EGKFNLASHLPGFFVRPDLGPRLCSAYGVVAAKDHDIGTTNLHIEVSDVVNILVYVGIAKGNGILSKAGILKKFEEEDLDDILRK
RLKDSSEIPGALWHIYAGKDVDKIREFLQKISKEQGLEVLPEHDPIRDQSWYVNKKLRQRLLEEYGVRTCTLIQFLGDAIVLPAG
ALHQVQNFHSCIQVTEDFVSPEHLVESFHLTRELRLLKE

(underlined sequence contains vector encoded His-tag and TEV protease cleavage site*)

Cell Lysis

Lysis Buffer: 10 mM HEPES, 500 mM NaCl, 5 % glycerol, 0.5 mM TCEP, 10 mM imidazole pH 7.5

Cell pellet (50 g) was dissolved in 200 mL of lysis buffer and lysozyme and benzonase added to 0.5 mg/mL and 1 µg/mL respectively. After 30 minutes stirring in the cold room, 30 mL of 10 % Triton X-100 was added and stirring continued for a further 30 minutes. Cells were split across 6 x 50 mL Falcon tubes and frozen at -20 °C. Cells were thawed at room temperature and the volume of each tube brought to 50 mL with Lysis Buffer. Cells were centrifuged for 1 h at 5,000 g (4 °C).

Column 1: His GraviTrap columns (4 x 1 ml volume in a gravity-flow column)

Wash Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5 % glycerol, 10 mM imidazole, 0.5 mM TCEP

Elution Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5 % glycerol, 500 mM imidazole, 0.5 mM TCEP

The clarified cell extract was added to a 4 x 1 ml His GraviTrap columns pre-equilibrated with Wash Buffer. The columns were then washed with 2 x 5 ml Wash Buffer. The protein was eluted with 2.5 ml Elution Buffer.

Column 2: PD-10 desalting columns (4 x 8.3 ml volume in a gravity-flow column)

Wash Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5 % glycerol, 10 mM imidazole, 0.5 mM TCEP

Each 2.5 mL His GraviTrap fraction (4 x) was applied directly to a PD-10 column (4 x) for desalting and eluted with 3.5 mL of Wash Buffer

Tag cleavage

1 mg of TEV protease was added to every 10 mg of the eluted protein and the digestion was performed overnight at 4 °C.

Column 3: His GraviTrap columns (4 x 1 ml volume in a gravity-flow column)

Wash Buffer: 10 mM HEPES pH 7.5, 500 mM NaCl, 5 % glycerol, 10 mM imidazole, 0.5 mM TCEP

The TEV cleaved protein (3.5 mL) was applied to a His GraviTrap column pre-equilibrated in Wash Buffer for removal of His-tag, TEV and uncleaved protein. The column was washed with a further 2.5 mL of Wash Buffer for a final pool of 6 mL. The various desalted protein fractions were combined (4 x 6 mL) and 3 mL of 1 M L-arginine + 1 M L-glutamate was added to help avoid precipitation. The protein was concentrated to 25 mg/mL using a 30 kDa MWCO concentrator.

Column 4: Yarra SEC 2000 300x21.2 mm column (300x21.2 mm column, 104 mL volume)

Gel Filtration Buffer: 10 mM HEPES pH 7, 500 mM NaCl, 5 % glycerol, 0.5 mM TCEP

The column was equilibrated with Gel Filtration buffer and the protein loaded, the peak corresponding to JMJD1CA was taken and concentrated to 32 mg/ml using a 30 kDa MWCO concentrator, flash frozen in liquid nitrogen and stored at -80°C.

2. Protein crystallization

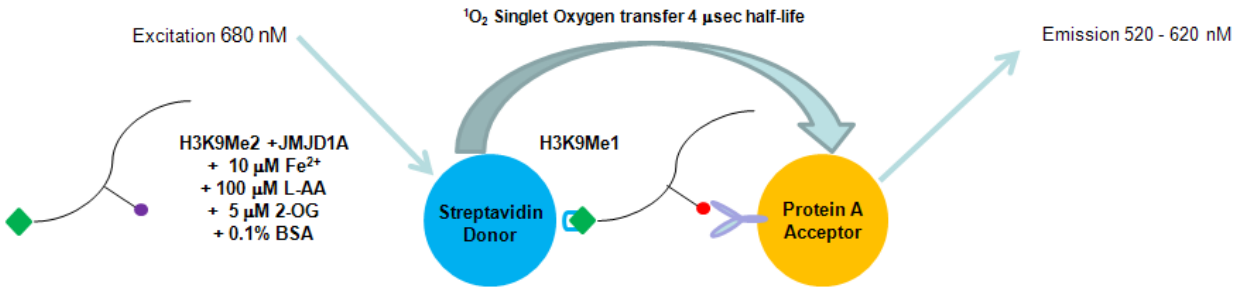
JMDJD1CA was crystallized by mixing 100 nl of 32 mg/ml protein in 10 mM HEPES pH7.5, 500mM NaCl, 5% Glycerol, 0.5 mM TCEP with 50 nl of reservoir solution containing 25 % PEG6000, 10 % ethylene glycol, 10 mM ZnCl, 0.1 Mtris, pH 7.5. Crystals appeared after 2-4 days from sitting drop plates at 4°C. JMJD1CA crystallized

in space group P 2_1 2_1 2 with unit cell dimensions of a = 111 Å, b = 165 Å, c = 46 Å, corresponding to two JMJD1CA molecules in the asymmetric unit. The crystals typically diffract between 1.7 and 1.9 Å.

Assay Conditions

Overview and Principle

The JMJD1A/B demethylase assay uses the peptide ARTKQTARK(2me)STGGKAPRKQLA-GGG Spacer-Biotin (Histone H3 Lys 9 di-methyl) as a substrate and relies on detection of product H3K9Me1-biotin bound to streptavidin donor beads by a rabbit polyclonal anti-H3K9Me1 antibody coupled to protein-A acceptor beads.



For both JMJD1A and JMJD1B, screens use low nM concentrations of enzyme (0.25 nM enzyme routinely used in the assay) and nM concentrations of peptide substrate (H3K9Me2 routinely used at 60 nM in assay screens). Assays are performed in 384-well proxiplates.

100 nl Transfer of Compound Dilution using ECHO550 Acoustic Dispenser

↓

5 ml dispense of 0.5 nM (2X final assay concentration) using Thermo Multidrop

↓

Plate sealed + 15 min incubation at RT

↓

5 ml dispense of 120 nM Biotin H3K9Me2 using Thermo Multidrop

↓

Plate sealed + 5 minute incubation at RT

↓

5 ml dispense of 30 mM EDTA + 800 mM NaCl using Thermo Multidrop

↓

5 ml dispense of AlphaScreen Beads (Manually using Thermo Matrix Pipette)

AlphaScreen Beads

Store at 4°C

H3K9Me2 substrate

H3K9Me2-biotin substrate is stored at -20°C in 24 ml aliquots at a concentration of 100 mM.

JMJD1A and JMJD1B Enzyme

JMJD1A and B are stored in 1 mM aliquots at -80°C and diluted to 100 nM stock for assays.

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 in the order of addition: Antibody > Protein A Acceptor > Streptavidin Donor.
3. Retrieve an aliquot of JMJD1A and 100 mM peptide from frozen 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 5.
5. Compounds for routine screening at 100 mM top concentration in IC_{50}s are stored at a concentration of 10 mM in Labcyte 384-polypropylene or 384-Low Dead Volume (LDV) plates. For IC_{50} determinations an eleven point IC_{50} of the compound is prepared using ECHO dose response software (see section 6.1 for a typical plate layout). For single point screening ECHO plate reformat software is used to prepare assay plates. A DMSO control is included on each assay plate (column 12) and 2, 4-PDCA (100 mM) is dispensed into each well of column 24 (100 nl transfer). Dry dispense 100 nl of the prepared compound IC_{50} dilution into a Proxiplate.
6. Prepare JMJD1A/B enzyme at 0.5 nM and dispense 5 ml into columns 1 -24 of a 384-well proxiplates using a Multidrop Dispenser and seal the plate.
7. Incubate the plate for 15 minutes on the bench at room temperature.
8. Prepare enough substrate solution (Assay buffer containing 200 mM L-Ascorbic Acid, 20 mM FAS, 120 nM H3K9Me2 peptide, 10 mM a-ketoglutarate) and dispense 5 ml of substrate into columns 1 -24 of the assay plate. Seal the plate.
9. Allow the enzyme reaction to proceed at room temperature for 5 minutes.
10. Dispense across the plate 5 ml of assay buffer containing 30 mM EDTA, 800 mM NaCl to stop the reaction.
11. In a dark box, dispense 5 ml 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 an AlphaScreen capable plate reader (BMG Labtech Pherastar FS or Perkin Elmer Envision).

Preparation of Solutions

Assay Buffer:

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

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 H_2O. Store at room temperature.

2-Oxoglutarate (2-OG):

Prepare 2-OG fresh each dat. Make up 10 mM stock solution in deionized H_2O (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 H_2O (8.806 mg/ml) and store at room temperature.

Substrate Solution

			Volume Required For		
	Stock Concentration	**Working Concentration**	**10 ml of Substrate**	**20 ml of Substrate**	**30 ml of Substrate**
L-Ascorbic Acid	50 mM	200 mM	**40 µl**	**80 µl**	**120 µl**
FAS	1 mM	20 mM	**200 µl**	**400 µl**	**600 µl**
H3K9Me2-Biotin	100 mM	0.12 mM	**12 µl**	**24 µl**	**36 µl**
2-OG	10 mM	10 mM	**10 µl**	**20 µl**	**30 µl**
Assay Buffer			**9738 µl**	**19476 µl**	**29214 µl**

Beads Detection Solution:

AlphaScreen donor beads and acceptor beads are mixed and pre-incubated with Anti-H3K9Me1 antibody 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-H3K9Me1 1.6 mg/ml	Final in Assay = 0.2 mg/ml

Make up the beads in dark box in the order: Antibody > Protein A Acceptor > Streptavidin Donor. Cover in foil and keep at room temperature.

Compound and Assay Plate Layouts

Typical IC_{50} Plate Layout:

16 compounds are plated out per plate. Duplicate transfers are performed for each compound concentration. Final DMSO concentration must not exceed 1.0%.

ECHO dose response software is used to prepare each compound IC_{50} approximating to a 1:3 dilution for each compound with 11 concentration points. Maximum volume transferred is 100 nl.

	100.0000	33.3333	11.1111	3.7037	1.2346	0.4115	0.1372	0.0457	0.0152	0.0051	0.0017		100.0000	33.3333	11.1111	3.7037	1.2346	0.4115	0.1372	0.0457	0.0152	0.0051	0.0017	
A	Cpd 1											DMSO	Cpd 9											2, 4-PDCA
B												DMSO												2, 4-PDCA
C	Cpd 2											DMSO	Cpd 10											2, 4-PDCA
D												DMSO												2, 4-PDCA
E	Cpd 3											DMSO	Cpd 11											2, 4-PDCA
F												DMSO												2, 4-PDCA
G	Cpd 4											DMSO	Cpd 12											2, 4-PDCA
H												DMSO												2, 4-PDCA
I	Cpd 5											DMSO	Cpd 13											2, 4-PDCA
J												DMSO												2, 4-PDCA
K	Cpd 6											DMSO	Cpd 14											2, 4-PDCA
L												DMSO												2, 4-PDCA
M	Cpd 7											DMSO	Cpd 15											2, 4-PDCA
N												DMSO												2, 4-PDCA
O	Cpd 8											DMSO	Cpd 16											2, 4-PDCA
P												DMSO												2, 4-PDCA

References

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