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A scintillation proximity assay for histone demethylases

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ABSTRACT

Covalent modifications, such as methylation and demethylation of lysine residues in histones, play important roles in chromatin dynamics and the regulation of gene expression. The lysine demethylases (KDMs) catalyze the demethylation of lysine residues on histone tails and are associated with diverse human diseases, including cancer, and are therefore proposed as targets for the therapeutic modulation of gene transcription. High-throughput assays have been developed to find inhibitors of KDMs, most of which are fluorescence-based assays. Here we report the development of a coupled scintillation proximity assay (SPA) for 3 KDMs: KDM1A (LSD1), KDM3A (JMJD1A), and KDM4A (JMJD2A). In this assay methylated peptides are first demethylated by a KDM, and a protein methyltransferase (PMT) is added to methylate the resulting peptide with tritiated *S*-(5′-adenosyl)-L-methionine. The enzyme activities were optimized and kinetic parameters were determined. These robust coupled assays are suitable for screening KDMs in 384-well format (*Z*′ factors of 0.70–0.80), facilitating discovery of inhibitors in the quest for cancer therapeutics.

Covalent histone modifications, including acetylation, methylation, phosphorylation, ubiquitinylation, and sumoylation, play an important role in regulating chromatin dynamics and function [1]. Lysine residues in histones are methylated and demethylated by sequence-specific methyltransferases and demethylases which are important processes in the control of chromatin structure and transcriptional activity. There are at least 60 protein methyltransferases (PMTs)[1] encoded in the human genome, including 51 lysine methyltransferases (PKMTs) and 9 protein arginine methyltransferases (PRMTs) [1]. Methyltransferases recognize specific protein sequences and mono-, di-, or trimethylate-specific lysines or mono- or dimethylate arginine residues. Examples include KMT1C which specifically mono- and dimethylates Lys-9 of histone H3 (H3K9) in euchromatin [2], and KMT3A which specifically mono-, di-, and trimethylates Lys-36 of histone H3 (H3K36) [3].

In contrast, the lysine demethylases (KDMs) "erase" the methyl marks. There are two main classes of histone demethylases which are defined by their mechanisms: flavin adenine dinucleotide (FAD)-dependent demethylases (e.g., KDM1A, KDM1B) and 2-oxoglutarate (2-OG)-dependent demethylases (e.g., KDM3A and KDM4A) [4]. KDM1A, also referred to as LSD1, specifically demethylates mono- or dimethylated H3K4 and H3K9 *via* a redox process [5]. Recent evidence shows that KDM1A plays an important role in a variety of biological processes, including cell proliferation [6], adipogenesis [7], chromosome segregation, and embryonic development [8,9]. Furthermore, KDM1A can also promote tumor development by inhibiting the tumor suppressor activity of p53 [10,11] and KDM1A inhibitors have shown anticancer effects in cells [12,13], supporting their potential as cancer drugs [14,15].

Another class of histone demethylases is the Jumonji domain containing histone demethylases (JKDMs). These JKDMs are Fe^{2+} and 2-OG-dependent oxygenases. Members of this class constitute the largest family of lysine demethylases. In contrast to KDM1A, which is only able to demethylate mono- and dimethylated lysine residues, JKDMs are able to demethylate mono-, di-, and trimethylated lysines. Although these KDMs have highly conserved methylated lysine binding pockets and act *via* a similar catalytic mechanism, they show differences in both degree and sequence specificity in their demethylation reactions [16]. For instance, the KDM4 subfamily (KDM4A, KDM4B, KDM4C, and KDM4D) acts on

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[1] *Abbreviations used:* HMTs, histone methyltransferases; IOX1, 8-hydroxyquinoline-5-carboxylic acid; IPTG, isopropyl-1-thio-D-galactopyranoside; JKDMs, Jumonji domain containing histone demethylases; KDMs, lysine demethylases; 2-OG, 2-oxoglutarate; PKMTs, lysine methyltransferases; PMTs, protein methyltransferases; PRMTs, protein arginine methyltransferases; SPA, scintillation proximity assay.

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di- and trimethylated H3K9, H3K36, and H1K26, while the KDM2 subfamily (KDM2A and KDM2B) only acts on mono- and dimethylated H3K36 [17]. This subfamily is linked to a variety of cancers [4], and a small molecule JKDM modulator has been shown to selectively inhibit cancer growth [18].

Because of the potential pharmaceutical significance of KDM inhibitors, high-throughput assays have been developed to find small molecule modulators of KDM activity and several commercial assay kits are available [19–22]. Most of these assays and kits use a fluorescence-based format and require no wash or liquid transfer steps, and the homogeneous "mix-and-measure" nature makes these assays simple and robust with relatively low cost. However, these assays usually tend to generate a high number of false positives because of fluorescence interference from labeled substrates, colored and fluorescent compounds, and fluorescent tracers [23–25]. High-throughput mass spectrometry has been used to overcome these shortcomings [26], and is widely used as a confirmatory assay for KDMs [20]; however, this technique has inherent throughput limitations. The ability of LC/MS to separate substrates containing different methylation states is another important consideration and is vital when screening involves the detection of multiple chemical entities [27].

The scintillation proximity assay (SPA) is a homogeneous, versatile assay technology for the rapid and sensitive analysis of a wide range of biological processes. The assay gives fewer false positives, is widely recognized as the "gold standard" for radiometric high-throughput screening [28,29], and has been used for histone methyltransferases (HMTs) [30–32]. Here we report the development of coupled SPA methods for 3 KDMs. In this assay, biotin-labeled methylated peptides are demethylated by a KDM, and subsequently a protein methyltransferase is added to methylate the peptide product with ^{3}H-SAM (*S*-(5′-adenosyl)-L-methionine) after KDMs are quenched by heat shock. We also characterized the kinetic parameters and highlight the broader utility of this approach as a generic assay for all KDMs.

Materials and methods

Iron (II) sulfate (Cat. No. F7002), 2-OG (Cat. No. K1875), ascorbic acid (Cat. No. A5960), and *S*-(5′-adenosyl)-L-methionine (referred to as "cold SAM"; Cat. No. 2408) were purchased from Sigma-Aldrich. Tritiated SAM ("hot SAM"; Cat. No. NET155V250UC) and 384-well FlashPlate coated with streptavidin (Cat. No. SMP410A001PK) were from Perkin Elmer. KDM1A (Cat. No. 50097; purity: ~80%) was from BPS bioscience. Biotin-labeled H3K4me1(1–21) (sequence: ARTK(me)QTARKSTGGKAPRKQLA-GGK-biotin), H3K9me2(1–21) (sequence: ARTKQTARK(me2) STGGKAPRKQLA-GGK-biotin), and H3K9me3(1–21) (sequence: ARTKQTARK(me3)STGGKAPRKQLA-GGK-biotin) were from AnaSpec. Inhibitors RN-1 and IOX1 (8-hydroxyquinoline-5-carboxylic acid) were obtained from Sigma-Aldrich.

Protein expression and purification

DNA fragments encoding human KMT1C residues 913–1193 and KMT1B residues 52–350 were amplified by PCR and subcloned into the SGC in-house pET28a-LIC vector. For KMT7, a DNA fragment encoding residues 1–366 was amplified by PCR and subcloned into the pET28a-LIC-C His vector, downstream of the poly-histidine coding region with M at the C-terminal region. The proteins were overexpressed in *Escherichia coli* BL21 (DE3) pRARE2-V2R by the addition of 1 mM isopropyl-1-thio-D-galactopyranoside (IPTG) and incubated overnight at 15 °C. Harvested cells were resuspended in 20 mM Tris buffer, pH 7.5, with 500 mM NaCl, 5 mM imidazole, and 5% glycerol and flash frozen in the presence of protease inhibitor. The cells were thawed and lysed chemically (CHAPS to final concentration of 0.5%, and 22.5 U/mL benzonase) followed by sonication at a frequency of 8.5 Hz with 10 s on and 10 s off. After clarification of the crude extract by high-speed centrifugation (16,000 rpm for 1 h), the lysate was loaded onto a DE52 column and passed onto the Ni-NTA column. The column was washed and His-tagged protein was eluted by 20 mM Tris, pH 7.5, 500 mM NaCl, 5% glycerol, and 50 and 250 mM imidazole, respectively. For KMT7, the protein was dialyzed against 20 mM Tris buffer, pH 7.5, and 500 mM NaCl. Next 4 mM DTT and 10% glycerol were added to 90% pure KMT7 after concentration. The protein was stored at −80 °C after flash freezing in liquid nitrogen. For KMT1C, thrombin was used to cut the His-tag overnight, the cut protein was passed through an Ni-NTA column, and the flowthrough was loaded onto a Superdex 200 16/60 column which was equilibrated with 20 mM Tris, pH 7.5, and 500 mM NaCl. Fractions with higher than 95% purity, as judged by SDS-PAGE, were pooled, concentrated, and flash frozen. For KMT1B, thrombin was used to cut the His-tag overnight. The cut protein was diluted to 50 mM NaCl prior to injection onto a 6 mL Resource S cation exchange column which was preequilibrated with 3 column volume (CV) 10 mM Tris, pH 6.8, followed by 3 CV of 10 mM Tris, pH 6.8, containing 500 mM NaCl and 3 CV of 10 mM Tris, pH 6.8. The pure KMT1B eluted at around 150–300 mM NaCl. Pure fractions based on SDS–PAGE were pooled, concentrated, and flash frozen.

For KDM3A, a DNA fragment encoding residues 515–1317 was amplified by PCR and subcloned into the SGC in-house pFB-CT10HF-LIC vector. Recombinant baculovirus was produced by transformation of DH10Bac cells. *Spodoptera frugiperda* (Sf9) insect cells in Sf-900 II SFM medium (Life Technologies) were infected with recombinant baculovirus and incubated for 72 h at 27 °C in shake flasks to overexpress the protein. Harvested cells were suspended in buffer containing 50 mM Hepes (pH adjusted using KOH), pH 7.4, 300 mM KCl, 5 mM imidazole, 5% glycerol, 10 mM imidazole, and protease inhibitor cocktail set VII (Calbiochem) and lysed by Dounce homogenization. The protein was purified by Ni-NTA chromatography on Sepharose 6 FF resin charged with nickel (GE Healthcare) followed by gel filtration on Superdex 200 column. The pure protein was stored in 20 mM Hepes, pH 7.4, 300 mM KCl, and 5% glycerol at −80 °C after flash freezing.

For KDM4A, a DNA fragment encoding residues 1–359 was amplified by PCR and subcloned into the SGC in-house pNIC28-Bsa4 vector. The protein was overexpressed in *E. coli* BL21 (DE3) -R3-pRARE2 by the addition of 0.2 mM IPTG and incubated overnight at 18 °C. Harvested cells were frozen at −80 °C. The cells were thawed and suspended in buffer containing 50 mM Hepes, pH 7.5, 500 mM NaCl, 10 mM imidazole, protease inhibitor cocktail set III (Calbiochem), and 50 U of Benzonase (Merck Millipore) and lysed by high pressure homogenization (25 kpsi). The protein was purified by Ni-NTA chromatography on a 1 mL HisTrap FF Crude column (GE Healthcare) followed by ion exchange on a 1 mL HiTrap HP Q column (GE Healthcare). The pure protein was stored in 25 mM Hepes, pH 7.5, 500 mM NaCl, 5% glycerol at −80 °C after flash freezing.

KDM1A assay

The assay was performed in a 384-well format. The 60 nM KDM1A with or without compounds was incubated with 5 μM biotin-labeled H3K4me1(1–21) peptide in 50 mM Tris-HCl, pH 8.0, 0.01% Triton X-100 using an Agilent Bravo automated liquid handling platform. The reaction volume was 10 μl. The plate was covered by aluminum foil and incubated at room temperature for 30 min. The plate was then heated at 80 °C for 15 min in a water bath to denature KDM1A and was centrifuged at 2000 rpm at