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SMYD5 is a histone H3-specific methyltransferase mediating mono-methylation of histone H3 lysine 36 and 37

Mohammad B. Aljazi[1], **Yuen Gao**[1], **Yan Wu**[1], **Jin He**[1,*]

[1]Department of Biochemistry & Molecular Biology, College of Nature Sciences, Michigan State University, East Lansing, MI 48824

Abstract

Although posttranslational modifications (PMTs) on some histone H3 lysine residues are well studied, the PMTs of histone H3 lysine 37 in mammalian cells remain largely unknown. In this study, we provide evidence to show that SMYD family member 5 (SMYD5) is a histone H3-specfic methyltransferase that catalyzes mono-methylation of H3 lysine 36 and 37 (H3K36/K37me1) *in vitro*. The site-mutagenesis analysis shows that a species-conserved histidine in its catalytic SET domain is required for its histone methyltransferase activity. Genetic deletion of *Smyd5* in murine embryonic stem cells (mESCs) partially reduces the global histone H3K37me1 level in cells, suggesting SMYD5 is one of histone methyltransferases catalyzing histone H3K37me1 *in vivo*. Hence, our study reveals that SMYD5 is a histone H3-specific methyltransferase that mediates histone H3K36/K37me1, which provides a biochemical basis for further studying its functions in mammalian cells.

Keywords

SMYD5; Methyltransferase; Histone; Lysine; Modification

1. Introduction

Histone H3 methylation at transcriptional regulatory regions plays critical role in regulating gene expression[1, 2]. Specifically, Polycomb repressive complex 2 (PRC2)-mediated tri-methylation of histone H3 lysine 27 (H3K27me3) at gene promoters silences gene expression[3, 4], while Trithorax-group (TxG) proteins MLL1/MLL2-mediated tri-methylation of histone H3 lysine 4 (H3K4me3) and ASH1L-mediated di-methylation of histone H3 lysine 36 (H3K36me2) facilitate transcription[5-7]. Although the PTMs on these

*To whom correspondence should be addressed Phone: 517-353-0613, hejin1@msu.edu.

Author contributions

J.H. conceived the project. M.B.A., Y.G., Y.W., and J.H. performed the experiments. M.B.A. and J.H. interpreted the data and wrote the manuscript.

Declaration of competing interest

Authors declares that they have no conflict of interests.

histone lysine residues and their functions in transcriptional regulation are well studied, PTMs of other histone H3 lysine residues, such as histone H3 lysine 37 (H3K37), are not fully characterized.

SMYD5 (SET and MYND domain-containing protein 5) protein contains a methyltransferase catalytic SET domain and a MYND zinc finger domain[8, 9]. A previous study has reported that SMYD5 is a histone lysine methyltransferase (KMT) that specifically mediates tri-methylation of histone H4 lysine 20 (H4K20me3)[10]. Functionally, SMYD5 is involved in transcriptional repression of cytokine genes in macrophages, mESC pluripotency and differentiation, as well as primitive and definitive hematopoiesis in zebrafish[10-12].

To study the function of SMYD5 in mammalian cells, we intended to confirm its enzymatic activity towards histone H4 as reported in the previous study[10]. Unexpectedly, our results showed that SMYD5, instead of using histone H4 as its substrate, mediated mono-methylation of histone H3 lysine 36 and lysine 37 (H3K36/K37me1) *in vitro*. The mutagenesis analysis identified a species-conserved histidine in the SET domain was required for is its enzymatic activity. Genetic deletion of *Smyd5* in mESCs partially reduced the global histone H3K37me1 level, suggesting SMYD5 was likely to be one of histone KMTs that mediated histone H3K37me1 in mammalian cells.

2. Materials and Methods

2.1. Mouse embryonic stem cell culture

The wild-type E14 mESC line were maintained on the 0.1% gelatin coated plates in the serum-containing mESC culture condition included DMEM medium (Life Technologies) supplemented with 100U/ml penicillin/streptomycin (Life Technologies), 15% fetal bovine serum (Sigma), 1x nonessential amino acid, 1x sodium pyruvate (Life Technologies), 1x GlutaMAX (Life Technologies), 1x β-mercaptoethanol (Life Technologies) and 1000 units/ml leukemia inhibitory factor (ESGRO, EMD Millipore).

2.2. Crispr/Cas9-mediated *Smyd5* gene knockout in mESCs

The experiments were carried out according to our previous report[13]. Briefly, the mouse *smyd5* gene gRNA (5'-GCTCTGGGTGTGGTAGAATC-3') was cloned into pX330 vector obtained from Addgene. The target vector and pEF1a-pac vector were co-transfected (5:1 ratio) to E14 mESCs using Xfect according to the manufacture's instruction (TaKaRa, Inc). 48 hours after transfection, Puromycin (1μg/ml) was added to the medium to select transfected cells. The individual clones were manually picked and expanded. The correct knockout clones were selected based on the Sanger sequencing on the targeting sites of genomic DNA, cDNA, and Western blot analysis.

2.3. Recombinant SMYD5 protein purification

Wild-type and mutant SMYD5 cDNAs were cloned into pFASTBAC as described previously[14]. Each baculovirus expressing recombinant proteins was generated and amplified following the manufacturer's protocol (Invitrogen). To purify the recombinant proteins, infected insect SF9 cells were collected and resuspended in F lysis buffer (20 mM

Tris–HCl, at pH 7.9, 500 mM NaCl, 4 mM $MgCl_2$, 0.4 mM EDTA, 2 mM dithiothreitol, 20% glycerol and 0.1% NP40) with proteinase inhibitors. Cells were homogenized with pestle A. The supernatant was recovered by centrifuging and was adjusted to 300 mM NaCl by adding dilution buffer (20 mM Tris–HCl, at pH 7.9), and subsequently incubated with M2 agarose (Sigma) for 4 h at 4 °C. After washing with F washing buffer (20 mM Tris–HCl at pH7.9, 150 mM NaCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 15% glycerol and 0.01% NP40), the bound proteins were eluted with Flag peptide (0.2 mg ml^{-1}).

2.4. Recombinant histone H3 and H4 peptide purification

Recombinant histone H3 and H4 peptides were purified according to a previous report[15]. Briefly, BL21 cells were transformed with the human histones H3 and H4 expression plasmids and grown at 37°C to a density of OD600 0.6 in LB in shaking cultures. Histone expression was induced by addition of 1 mM IPTG for 2 h. The bacteria pellet was resuspended in SAU buffer (40 mM NaOAc pH 5.2, 6 M urea, 1 mM EDTA pH 8, 5 mM β-Mercaptoethanol, 10 mM lysine) supplemented with 200 mM NaCl (SAU 200) and protease inhibitors (1 mM PMSF, 1 mg/l Aprotinin, 1 mg/l Leupeptin, 0.7 mg/l Pepstatin). Cells were lysed by three passes through a French Press and sonication on ice. The extract was cleared by centrifugation for 20–30 min at 41,000 g and filtration through a glass-fiber prefilter (HPF Millex, Millipore). The pre-cleared cell extract was passed over HiTrap Q HP column (GE Healthcare) followed by a HiTrap SP HP column (5 ml; GE Healthcare) that was pre-equilibrated in SAU 200 buffer. The SP column was washed with 200 mM NaCl for 5 column volumes. Histones were eluted with a NaCl gradient. Pooled histone-containing fractions were dialyzed against cold water over night in dialysis tubing with a molecular weight cut-off of 6000–8000 Da. After dialysis, the sample was passed over a 1ml Q HP column. The flow-through containing purified histone peptides was collected and saved in −80°C for later use.

2.5. *In vitro* histone methyltransferase assays

To test SMYD5 methyltransferase activity *in vitro*, assays were conducted by preparing 20 ul reactions on ice in methyltransferase buffer (50 mM Tris-HCl pH 8.0, 2 mM MgCl2, 1 mM DTT, 10% glycerol) or Bicine buffer (50 mM Bicine pH 9.0, 2.5 mM EDTA, 4 mM EDTA, 0.5 mM DTT, 5% glycerol), with 1 – 5 μg full-length or short-form SMYD5 enzyme, 3 μg recombinant human histone H3 and H4 peptide, and 1 μCi of [^{3}H]SAM (PerkinElmer Life Sciences). Reactions were incubated for 16 hours at 30°C. Methyltransferase assay samples were resolved by SDS-PAGE gels and transferred on PVDF then stained with Coomassie Blue. The membrane was dried and exposed to a Kodak Tridium Storage Phosphor Screen for 24 – 48 hours and then scanned with a PharosFX Plus Molecular Imager (Bio-Rad).

2.6. Histone extraction

1x 10^6 mESCs lysed in 1 ml buffer A [0.25 M sucrose, 10 mM sodium HEPES (pH 7.5), 3 mM $CaCl_2$, 10 mM NaCl, 1 mM PMSF, 1 mM DTT, 0.25% Nonidet 40] on ice for 30min. The lysate was then centrifuged at 3000 rpm for 10 min to pellet the nuclei. The nuclei were then washed by buffer A one more time before being suspended in 300 ml buffer B [0.25 M sucrose, 10 mM sodium HEPES (pH 7.5), 3 mM $CaCl_2$, 10 mM NaCl, 1 mM PMSF,

1 mM DTT]. 2 N HCl was then added to a final concentration of 0.2 N. The suspension was extracted at 4°C overnight and was then centrifuged for 10 min at 13,000 rpm. The supernatant was mixed with equal volume of 50% TCA and kept on ice for 1 hr. The precipitated proteins were collected and washed with acetone. Proteins were then dissolved in SDS loading buffer and analyzed by Western blotting.

2.7. Western blot analysis

Total proteins were extracted by RIPA buffer and separated by electrophoresis by 10 – 18 % PAGE gel. The protein was transferred to the nitrocellulose membrane and blotted with primary antibodies. The antibodies used for Western Blot included: rabbit anti-SMYD5 (1:1000, in house), rabbit anti-H3 (1:1000, ab1792, Abcam), rabbit anti-H3K36me1 (1:1000, C15410089, Diagenode), rabbit anti-H3K37me1 (1:1000, C15410295, Diagenode), and IRDye 680 donkey anti-rabbit second antibody (1: 10000, Li-Cor). The images were developed by Odyssey Li-Cor Imager (Li-Cor).

2.8. Mass spectrometry analysis

Gel bands were digested in-gel according to Shevchenko, et. al. with modifications[16]. Briefly, gel bands were washed with 100mM ammonium bicarbonate and dehydrated using 100% acetonitrile. Sequencing grade modified typsin was prepared to 0.01ug/uL in 50mM ammonium bicarbonate and ~100uL of this was added to each gel band so that the gel was completely submerged. Bands were then incubated at 37C overnight. Peptides were extracted from the gel by water bath sonication in a solution of 60%ACN/1%TFA and vacuum dried to ~2uL. Peptides were then re-suspended in 2% acetonitrile/0.1%TFA to 20uL. From this, 5uL were automatically injected by a Thermo (www.thermo.com) EASYnLC 1200 onto a Thermo Acclaim PepMap RSLC C18 peptide trap (5um, 0.1mm x 20mm) and washed with buffer A for ~5min. Bound peptides were then eluted onto a Thermo Acclaim PepMap RSLC 0.075mm x 250mm C18 resolving column and eluted over 35min with a gradient of 8%B to 40%B in 24min, ramping to 90%B at 25min and held at 90%B for the duration of the run (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 80% Acetonitrile/0.1% Formic Acid/19.9% Water) at a constant flow rate of 300nL/min. Column temperature was maintained at 50C using an integrated column heater (PRSO-V2, www.sonation.com). Eluted peptides were sprayed into a ThermoFisher Q-Exactive HF-X mass spectrometer (www.thermo.com) using a FlexSpray spray ion source. Survey scans were taken in the Orbi trap (60000 resolution, determined at m/z 200) and the top 15 ions in each survey scan are then subjected to automatic higher energy collision induced dissociation (HCD) with fragment spectra acquired at 15,000 resolution. The resulting MS/MS spectra are converted to peak lists using Mascot Distiller, v2.7 (www.matrixscience.com) and searched against a database containing all *H.sapiens* protein entries available from UniProt (downloaded from www.uniprot.org on 2020-01-15) appended with common laboratory contaminants (downloaded from www.thegpm.org). Searches were performed using the Mascot searching algorithm, v 2.7, on an in-house server. The Mascot output was then analyzed using Scaffold, v4.11.0 (www.proteomesoftware.com) to probabilistically validate protein identifications. Assignments validated using the Scaffold 1% FDR confidence filter are considered true.

3. Results

3.1. SMYD5 mediates methyl group transferring towards histone H3 *in vitro*

The mammalian SMYD5 proteins contain a MYND zinc finger domain, a lysine methyltransferase catalytic SET domain, and an acidic poly-glutamic acid (poly-E) region at its C-terminus (Fig. 1A). A previous study reported SMYD5 possessed a histone methyltransferase activity towards histone H4 lysine 20[10]. To confirm the substrate specificity of its histone methyltransferase activity, we purified recombinant human SMYD5 proteins from baculovirus-infected SF9 insect cells and performed *in vitro* methyltransferase assays using full-length recombinant histone H3 or H4 peptides as substrates. Unexpectedly, the results showed that SMYD5 mediated the methyl group transferring towards histone H3 but not H4 peptides (Fig. 1B).

To confirm our results, we changed the reaction condition to a bicine buffer that was reported to enhance the enzymatic activity of lysine methyltransferases[17]. However, the results showed that under the modified reaction condition, SMYD5 mediated the methyl group transferring only to histone H3 but not to histone H4 peptides (Fig. 1C). Furthermore, to exclude the possibility that the C-terminal poly-E acidic region of SMYD5 interfered its enzymatic activity towards histone H4, we generated a short form of recombinant SMYD5 by removing its C-terminal poly-E region ($SMYD5^{\Delta 384\text{-}418}$) and performed the methyltransferase assays. Same as the full-length SMYD5, the short-form SMYD5 mediated the methyl group transferring to histone H3 only (Fig. 1D). Collectively, these results suggested that SMYD5 was a histone H3-specific methyltransferase and mediated lysine methylation towards histone H3 *in vitro*.

3.2. SMYD5 catalyzes mono-methylation of histone H3 lysine 36 and lysine 37 *in vitro*

To identify the lysine residues of histones that are methylated by SMYD5, we performed the mass spectrometry assays to examine the lysine methylation of histone H3 and H4 peptides after incubating histone peptides in the SMYD5-mediated histone methyltransferase reactions *in vitro*. The results showed that compared to negative controls that were incubated in the SMYD5-free reaction buffer, the histone H3 peptides obtained mono-methylation at both lysine residue 36 and lysine 37 (H3K36/K37me1) after subjected to the SMYD5-mediated methyltransferase reaction (Fig. 2A). In contrast, the mass spectrometry assays did not detect methylation at lysine 20 or other lysine residues of histone H4 peptides, consistent with the results of methyltransferase assays *in vitro* (Fig. 1).

To confirm that histone H3 K36 and K37 were the lysine residues methylated by SMYD5, we generated the full-length histone H3 peptides with single K36, single K37, and double K36/K37 mutated to alanine (H3K36A, H3K37A, H3K36A/K37A) and performed *in vitro* methyltransferase assays using the mutant histone H3 peptides as substrates. The results showed that single lysine mutation (H3K36A or H3K37A) partially reduced the signal intensity. In contrast, the H3K36/K37 double mutant histone H3 peptides (H3K36A/K37A) largely reduced the signal to the baseline (Fig. 2B, C).

To further confirm that SMYD5 mediated the histone H3K36/K37me1 modification, we performed the western blot (WB) analyses using both histone H3K36me1 and histone

H3K37me1-specific antibodies to detect the modification after incubation of histone H3 peptides with SMYD5-containing methyltransferase reaction *in intro*. The results showed both antibodies detected the H3K36me1 and H3K37me1 modifications, and the signal intensity was positively correlated with the SMYD5 concentration in the reactions (Fig. 2D). Collectively, these results suggested that SMYD5 was histone H3-specific methyltransferase catalyzing mono-methylation of histone H3 lysine 36 and lysine 37 *in vitro*.

3.3. A species-conserved histidine in the SET domain is required for SMYD5 methyltransferase activity

To identify the critical amino acids required the methyltransferase activity of SMYD5, we compared the amino acid sequences of catalytic SET domains of SMYD5 proteins from different species. The analysis showed a histidine (H316 of human SMYD5) was conserved in different species (Fig.3A), suggesting its importance for the SMYD5 methyltransferase activity. To test this hypothesis, we generated recombinant human SMYD5 with its histidine mutated to alanine ($SMYD5^{H316A}$) and performed *in vitro* methyltransferase assays to examine its enzymatic activity. The results showed that the mutant SMYD5 lost its methyltransferase activity towards histone H3 peptides, suggesting that the conserved histidine in the SET domain is required for the histone methyltransferase activity of SMYD5.

3.4. Deletion of *Smyd5* in mESCs partially reduces the global histone H3K37me1 level

To examine the SMYD5-mediated histone H3 modifications in mammalian cells, we knockout the *Smyd5* gene in mESCs (Smyd5-KO) by the Crispr/Cas9-mediated gene knockout approach[13]. The deletion of *Smyd5* gene in two cell clones was confirmed by WB analysis at the protein level (Fig. 4A). Further WB analysis showed that compared to wild-type cells, the *Smyd5*-KO mESCs had partially reduced global histone H3K37me1 levels, while the global histone H3K36me1 did not show markedly changes (Fig. 4B), suggesting that SMYD5 was likely to be one of KMTs contributing to the histone H3K37me1 modification in mammalian cells.

4. Discussion

Different from a previous study showing that SMYD5 is a histone H4K30me3-specific methyltransferase[10], our current study demonstrates that that SMYD5 is a histone H3-specific lysine methyltransferase that catalyzes mono-methylation of histone H3 K36 and K37. Our conclusion is supported by *in vitro* methyltransferase assays showing that SMYD5 mediates the methyl group transferring towards histone H3 peptides only under two reaction conditions as well as using both full-length and short-form recombinant SMYD5 proteins for the reactions (Fig. 1A-C). Moreover, the mass spectrometry assays show that histone H3 peptides, but not histone H4 peptides, obtain mono-methylation at its K36 and K37 residues after subjected to the SMYD5-mediated methyltransferase reactions (Fig. 2A), which is further confirmed by the *in vitro* methyltransferase assays showing that mutation of both histone H3 K36 and K37 diminishes the methyl group transferring to histone H3 peptides (Fig. 2B, C). In addition, WB analyses show that H3K36me1 and H3K37me1-specific antibodies detect both modifications of histone H3 peptides after subjected to the SMYD5-

mediated methyl group transferring reactions (Fig. 2D). Compared to the H3K37me1 signal, there existed a background signal of histone H3K36me1 in the WB analysis (Fig. 2D, lane 1), which might be caused by non-specific recognition of histone H3 by the antibody. The collective results suggest that SMYD5 is a histone H3-specific methyltransferase catalyzing mono-methylation of histone H3 K36 and K37. The discrepancy between our results and the earlier report regarding the histone substrates of SMYD5 could be due to the antibody-based methods used for detecting histone modifications in the previous study[10], which might generate a non-specific H4K20me3 signal and mislead the conclusion.

Although our *in vitro* biochemical assays suggest that SMYD5 is a histone H3K36/K37-specific methyltransferase (Figs 1-3), we notice that the deletion of *Smyd5* in mESCs does not completely abolish histone H3K37me1 in cells (Fig. 4). The discrepancy between *in vitro* and *in vivo* results could be caused by two possible reasons: (i) histone H3K36me1 and H3K37me1 modifications in mammalian cells are catalyzed by both SMYD5 and other histone KMTs, which compensate the loss of SMYD5 function in depositing H3K36/K37me1 in the *Smyd5*-KO cells; or (ii) SMYD5 mediates locus-specific histone H3K36/K37me1, thus loss of SMYD5 has limited effects on the global H3K36/K37me1 level.

Recently, SET7 and SET1/SET2 are found to catalyze histone H3K37 methylation in *S. pombe* and *S. cerevisiae*, respectively[18, 19] , which are functionally involved in gametogenesis and DNA replication. Our current study demonstrates that histone H3K37me1 also exits in mammalian cells and SMYD5 is likely to be one of histone KMTs depositing this histone modification in cells. Although the function of SMYD5 remains largely unelucidated, it is worth to note that SMYD5 is found to localize in both nuclei and mitochondria in mammalian cells[20], suggesting its intra- and extra-nuclear functions. The identification of SMYD5-mediated histone H3K36/K37me1 in this study provides a biochemical basis for further studying the functions of SMYD5 and its-mediated histone modifications in mammalian cells.

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Data availability

Data will be made available on request.

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Highlights

- SMYD5 mediates methyl group transferring towards histone H3 *in vitro*.
- SMYD5 catalyzes mono-methylation of histone H3 lysine 36 and lysine 37 *in vitro*.
- A species-conserved histidine in the SET domain is required for SMYD5 methyltransferase activity.
- Deletion of *Smyd5* in mouse embryonic stem cells reduces the global histone H3K37me1 level.

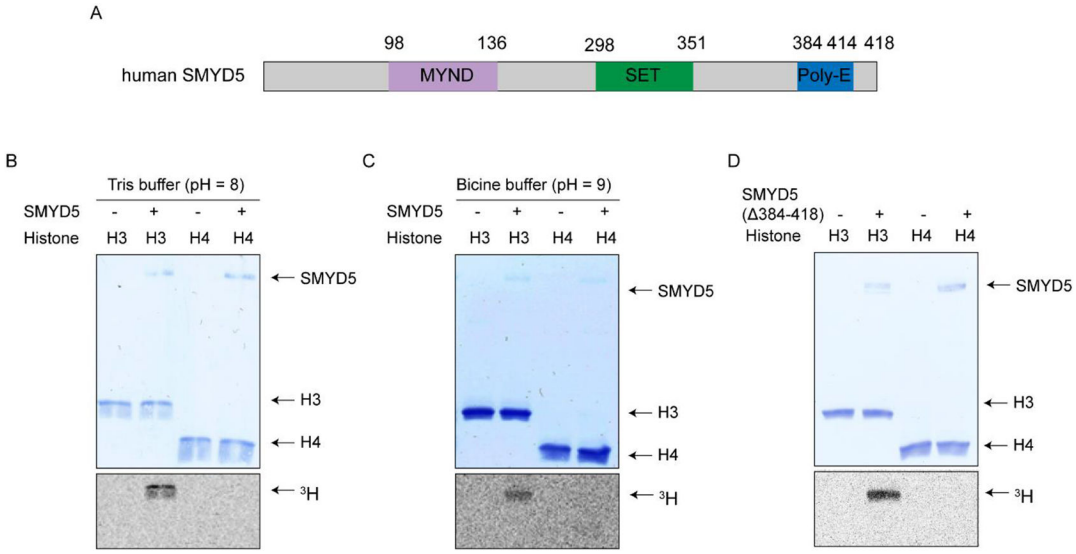

Figure 1. SMYD5 mediates methyl group transferring towards histone H3 *in vitro*.

(A) Diagram showing the MYND domain, the SET domain, and the poly-E acidic region of human SMYD5 protein. (**B**) The results of *in vitro* histone methyltransferase assay (Tris buffer) showing SMYD5 mediates methyl group transferring towards histone H3 but not H4 peptides. (**C**) The results of *in vitro* histone methyltransferase assay (Bicine buffer) showing SMYD5 mediates methyl group transferring towards histone H3 but not H4 peptides. (**D**) The results of *in vitro* histone methyltransferase assay showing short-form SMYD5$^{\Delta 384\text{-}418}$ mediates methyl group transferring towards histone H3 but not H4 peptides.

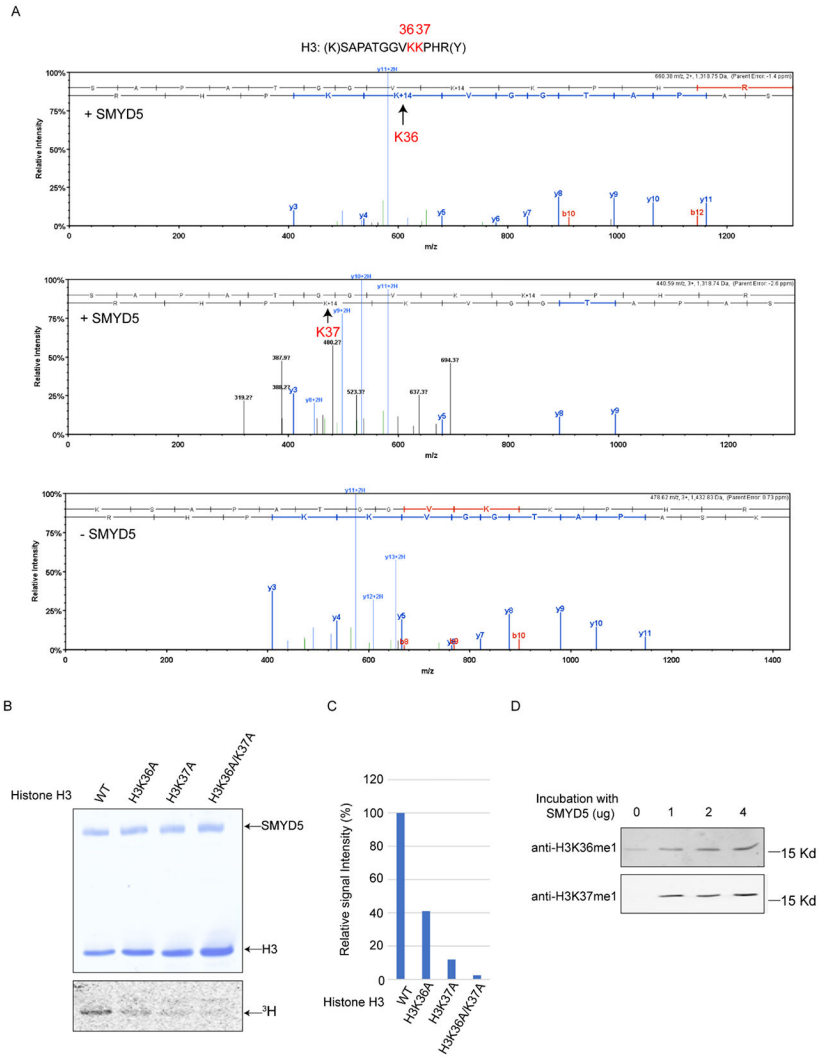

Figure 2. SMYD5 catalyzes mono-methylation of histone H3 lysine 36 and lysine 37 *in vitro*.

(A) The spectrum of mass spectrometry analysis showing histone H3 K36 (top panel) and K37 (middle panel) obtain mono-methylation after subjected to the SMYD5-mediated methyltransferase reaction *in vitro*. The histone H3 peptides incubated in the SMYD5-free reaction buffer serve as negative controls (bottom panel). (**B-C**) *In vitro* histone methyltransferase assays (B) and quantitative analysis (C) showing mutations of histone K36 and K37 largely reduce SMYD5-mediated methyl group transferring to histone H3 peptides. Relative intensity of ^{3}H signal for different histone H3 peptides is normalized to both input (Coomassie staining) and signal intensity of wild-type H3 peptides: (Intensity of ^{3}H / Intensity of H3 input) / (Intensity of ^{3}H of wild-type H3/ Intensity of wild-type H3 input) x 100%. (**D**) WB analysis showing the histone H3K36me1 and H3K37me1-specific antibodies detect the H3K36me1 and H3K37me1 modifications after histone H3 peptides are subjected to the SMYD5-mediated methyltransferase reaction.

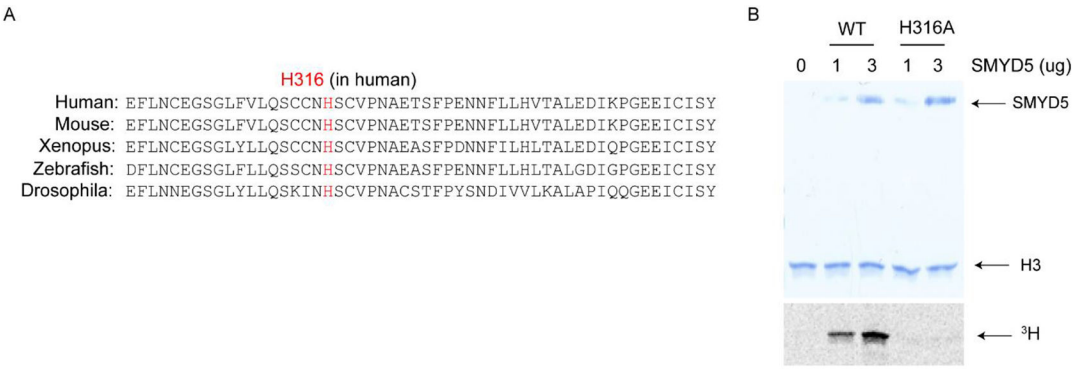

Figure 3. A species-conserved histidine in the SET domain is required for SMYD5 methyltransferase activity.

(A) Sequence alignment showing the conserved histidine in the SET domain of SMYD5 from different species. (**B**) The results of histone methyltransferase assay showing that mutation of histidine (H316A) of human SMYD5 abolishes its histone KMT activity.

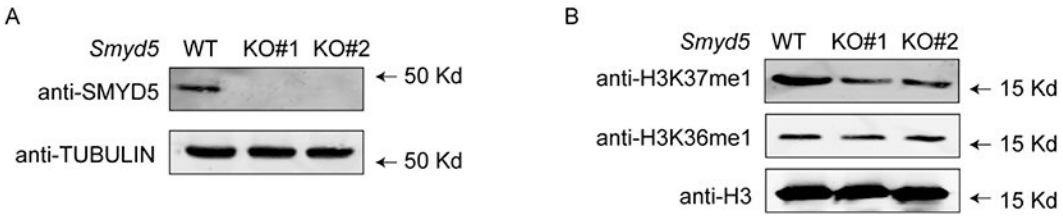

Figure 4. Deletion of *Smyd5* in mESCs partially reduces the global histone H3K37me1 level.

(A) WB analysis showing the SMYD5 expression in wild-type (WT) and *Smyd5*-knockout mESC clone 1 and 2 (KO #1 and KO #2). (**B**) WB analysis showing the global histone H3, H3K36me1, and H3K37me1 levels in wild-type (WT) and *Smyd5*-knockout mESC clone 1 and 2 (KO #1 and KO #2).