



Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases

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Covalent modifications of histones, such as acetylation and methylation, play important roles in the regulation of gene expression. Histone lysine methylation has been implicated in both gene activation and repression, depending on the specific lysine (K) residue that becomes methylated and the state of methylation (mono-, di-, or trimethylation). Methylation on K4, K9, and K36 of histone H3 has been shown to be reversible and can be removed by site-specific demethylases. However, the enzymes that antagonize methylation on K27 of histone H3 (H3K27), an epigenetic mark important for embryonic stem cell maintenance, Polycomb-mediated gene silencing, and X chromosome inactivation have been elusive. Here we show the JmjC domain-containing protein UTX (ubiquitously transcribed tetratricopeptide repeat, X chromosome), as well as the related JMJD3 (jumonji domain containing 3), specifically removes methyl marks on H3K27 *in vitro*. Further, the demethylase activity of UTX requires a catalytically active JmjC domain. Finally, overexpression of UTX and JMJD3 leads to reduced di- and trimethylation on H3K27 in cells, suggesting that UTX and JMJD3 may function as H3K27 demethylases *in vivo*. The identification of UTX and JMJD3 as H3K27-specific demethylases provides direct evidence to indicate that similar to methylation on K4, K9, and K36 of histone H3, methylation on H3K27 is also reversible and can be dynamically regulated by site-specific histone methyltransferases and demethylases.

trimethylated lysines (11). Histone demethylases that specifically remove methyl marks on K4, K9, and K36 of histone H3 (H3K4, H3K9, and H3K36) have been identified within this family (12).

Trimethylation of H3K27 (H3K27me3) is considered an epigenetic mark important for maintaining embryonic stem (ES) cell pluripotency and plasticity during embryonic development, Polycomb-mediated gene silencing, and X chromosome inactivation (6, 13–16). During ES cell differentiation into neural precursors, neural-specific genes become activated concomitant with at least a 100-fold decrease of H3K27 methylation (13, 15), suggesting the existence and active involvement of H3K27 demethylases. However, demethylases that can reverse methylation on histone H3K27 have not been reported.

The UTX (ubiquitously transcribed tetratricopeptide repeat, X chromosome) protein, encoded by the X chromosome, contains six TPR (tetratricopeptide repeat) domains in the N-terminal half and the JmjC domain in the C-terminal half (Fig. 1A). The *UTX* gene escapes X chromosome inactivation and is ubiquitously expressed in mice and humans (17). UTX belongs to a subfamily of JmjC domain-containing proteins that also includes UTY (ubiquitously transcribed tetratricopeptide repeat, Y chromosome) and JMJD3 (12). The UTY protein is the Y-linked homolog of UTX and shares 88% homology with UTX. The third member of this family, JMJD3, although lacking TPR domains, shares extensive sequence homology with UTX and UTY both within and outside of the JmjC domain. UTX family members have been predicted to be histone demethylases because all three of them contain the signature JmjC domain (12). However, the experimental evidence has been lacking.

We and others recently found that UTX was present in the MLL3- and MLL4 (also known as ALR)-containing histone H3K4 methyltransferase complexes isolated from cell nuclei (8, 18). As the first step toward understanding the significance of the physical association of a putative histone demethylase with histone methyltransferase complexes, we sought to determine whether UTX is a histone demethylase and, if so, the substrate specificity of UTX. By using recombinant proteins that were overexpressed in and purified from mammalian cells, we show that the JmjC domain-containing proteins UTX and JMJD3, but not the related UTY, function as histone H3K27 demethylases and specifically remove methyl marks on H3K27 *in vitro*. Further, we demonstrate that the demethylase activity of UTX requires a catalytically active JmjC domain. Finally, overexpression of UTX and JMJD3 decreases di- and trimethylation on H3K27 in cells, suggesting that UTX and JMJD3 may function as H3K27 demethylases *in vivo*. Our data

histone methylation | transcriptional regulation

Covalent modifications of histones, such as acetylation, methylation, ubiquitination, and phosphorylation, have been shown to play important roles in the regulation of both global and tissue- and developmental stage-specific gene expression (1–3). Histone lysine methylation has been implicated in both gene activation and repression, depending on the specific lysine (K) residue that becomes methylated and the state of methylation (mono-, di-, or trimethylation). For example, di- and trimethylation at K4 of histone H3 (H3K4) are associated with gene activation, whereas di- and trimethylation at K27 of histone H3 (H3K27) are associated with gene repression (4–6). Histone methylation is dynamically regulated by site-specific histone methyltransferases and demethylases (7). In human cells, Set1-like histone methyltransferase complexes specifically methylate H3K4, whereas the Polycomb repressive complex 2 (PRC2) methylates H3K27 (6, 8, 9).

Histone methylation was once considered enzymatically irreversible until the first histone lysine demethylase LSD1 was identified (10). LSD1, a FAD-dependent nuclear amine oxidase, can demethylate mono- and dimethylated K4 and K9 of histone H3 (H3K4me1/2 and H3K9me1/2), depending on the proteins associated (10). Subsequently, a large family of JmjC domain-containing histone lysine demethylases has been isolated (11). JmjC domain mediates Fe(II)- and α -ketoglutarate-dependent histone demethylation (12). This family of demethylases demethylate methylated lysine residues on histones through a mechanism that is fundamentally different from the methylamine oxidation reaction catalyzed by LSD1 and thus can demethylate

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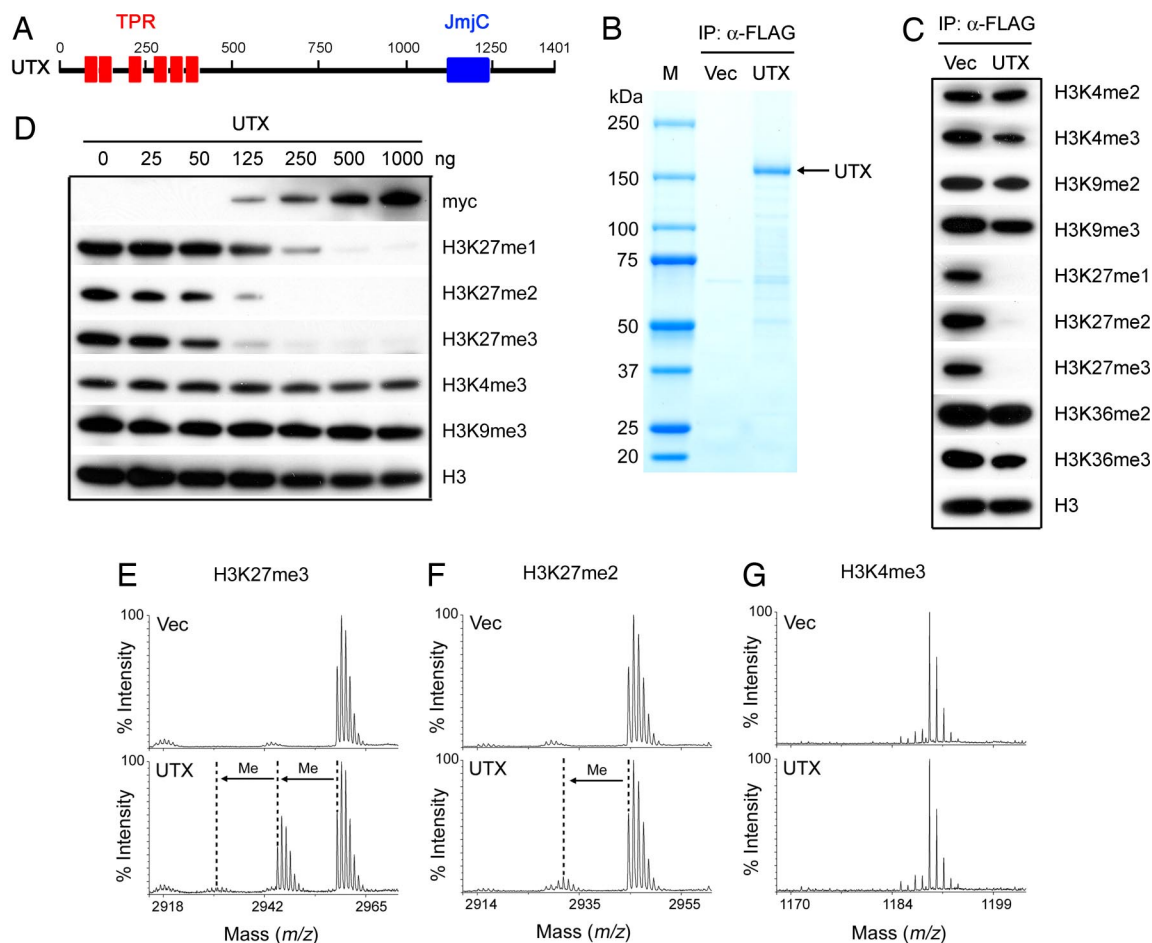


Fig. 1. UTX specifically demethylates histone H3K27 *in vitro*. (A) Schematic representation of human UTX protein with six TPR (tetratricopeptide repeat) domains and one JmjC domain. (B) Coomassie blue staining of purified UTX protein. 293T cells were transfected with plasmids expressing FLAG- and myc-tagged UTX or vector only (Vec). Whole-cell extracts were prepared with 1% Nonidet P-40-containing buffer and were incubated with anti-FLAG M2-agarose. After extensive washing, bound proteins were eluted with FLAG peptide, resolved on SDS/PAGE 4–15% followed by Coomassie blue staining. IP, immunoprecipitation. M, protein marker. (C) Eluate corresponding to $\approx 1 \mu\text{g}$ of purified UTX was incubated with $2.5 \mu\text{g}$ of calf core histone in histone demethylase assay. Assay products were analyzed by Western blotting, with antibodies indicated on the right. Where indicated, me1 is monomethyl, me2 is dimethyl, and me3 is trimethyl. (D) Increasing amounts of purified UTX were subjected to histone demethylase assay. (E–G) Mass spectrometry analysis of UTX activity toward H3K27me3 (E), H3K27me2 (F), and H3K4me3 (G) peptides. Three micrograms of UTX was incubated with $1 \mu\text{g}$ of peptide in histone demethylase assay followed by MALDI-TOF mass spectrometry analysis. The masses of the H3K27me3, H3K27me2, and H3K4me3 peptides were 2,958.7, 2,944.7, and 1,189.7 Da, respectively.

provide direct evidence to indicate that, similar to methylation on K4, K9, and K36 of histone H3, the methylation on K27 of histone H3 is also reversible and can be removed by site-specific histone demethylases.

Results

UTX Specifically Demethylates Histone H3K27 *in Vitro*. To determine whether the JmjC domain-containing protein UTX is a histone demethylase and, if so, the substrate specificity of UTX, full-length UTX with C-terminal FLAG tag and N-terminal myc tag was overexpressed in 293T cells by transient transfection. Whole-cell lysates were prepared with 1% Nonidet P-40-containing lysis buffer and incubated with anti-FLAG M2-agarose. After extensive washing with lysis buffer, bound proteins were eluted with FLAG peptide, resolved on SDS/PAGE. As shown in Fig. 1B, the ≈ 160 -kDa FLAG-tagged UTX was the predominant protein purified from 293T cells, and it could be readily detected by an anti-UTX antibody in Western blot (data not shown). Approximately $1 \mu\text{g}$ of purified UTX was incubated with $2.5 \mu\text{g}$ of calf core histones in a demethylase assay. As shown in Fig. 1C, UTX was associated with robust histone demethylase activity *in vitro* and specifically removed mono-, di-, and trimethyl marks on

histone H3K27 (H3K27me1/2/3) without significantly affecting methylation on H3K4, H3K9, and H3K36. The H3K27 demethylase activity of UTX was dose-dependent. UTX preferred H3K27me2 and H3K27me3 over H3K27me1 as substrates, and 125 ng of UTX was sufficient to remove the majority of di- and trimethyl marks on H3K27 in $2.5 \mu\text{g}$ of calf core histone (Fig. 1D).

To confirm the Western blotting results and also to provide direct evidence that UTX can function as H3K27-specific demethylase *in vitro*, $3 \mu\text{g}$ of purified UTX was incubated with $1 \mu\text{g}$ of synthetic H3K27me2, H3K27me3, or H3K4me3 peptide in the histone demethylase assays, and the reaction mixtures were analyzed by MALDI-TOF mass spectrometry. UTX efficiently converted H3K27me3 to H3K27me2 (Fig. 1E) but was unable to demethylate H3K4me3 peptide (Fig. 1G). UTX was only able to convert H3K27me2 peptide to H3K27me1 weakly (Fig. 1F), suggesting that for converting H3K27me2 to H3K27me1, the intact histone H3 protein in core histones may be the preferred substrate over the synthetic H3K27me2 peptide (compare Fig. 1D with Fig. 1F). Nevertheless, these results are consistent with Western blotting data and indicate that UTX specifically demethylates H3K27 *in vitro*.

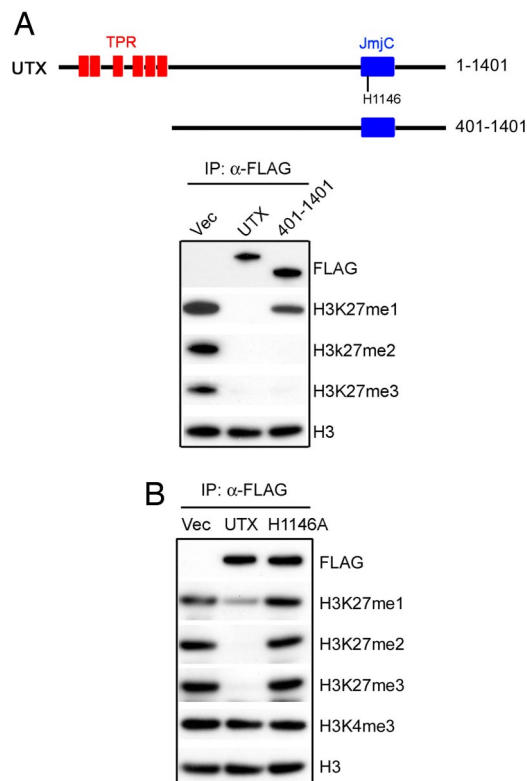


Fig. 2. The H3K27 demethylase activity of UTX requires a catalytically active JmjC domain. (A) The TPR domains are required for the optimal demethylase activity of UTX on H3K27me1 but are dispensable for UTX activity on H3K27me2/3. (Upper) Schematic representation of full-length and 401-1401 aa fragment of human UTX protein. The location of the conserved Fe(II)-binding H1146 within JmjC catalytic domain is indicated. (Lower) Plasmids expressing FLAG-tagged UTX, either full-length or 401-1401 aa fragment, were transfected into 293T cells followed by protein isolation and histone demethylase assay on calf core histones as described in Fig. 1. IP, immunoprecipitation. (B) Mutation of the Fe(II)-binding H1146 in the JmjC domain abrogates the H3K27 demethylase activity of UTX. Plasmids expressing FLAG-tagged wild-type or H1146A mutant UTX were transfected into 293T cells followed by protein isolation and histone demethylase assay as described above.

The H3K27 Demethylase Activity of UTX Requires a Catalytically Active JmjC Domain. Previous publications on characterization of other JmjC domain-containing histone demethylases have pointed to the critical role of the JmjC domain for enzymatic activity (12). The JmjC domain has been shown to form an enzymatically active pocket that contains conserved residues for binding with cofactors Fe(II) ion and α -ketoglutarate (19). UTX contains TPR domains in its N-terminal half and the JmjC domain in its C-terminal half. When the TPR domains were deleted, the remaining 401-1401 aa of UTX maintained its ability to demethylate H3K27me2 and H3K27me3 but had reduced activity toward H3K27me1, suggesting that the TPR domains were only required for the optimal demethylase activity of UTX on H3K27me1 (Fig. 2A). To ensure that the enzymatic activity was endowed in UTX, a point mutation (H1146A) in the first histidine residue of the Fe(II)-binding motif of the JmjC domain was generated. When expressed at a level similar to that of wild-type UTX, the H1146A mutant failed to show any H3K27 demethylase activity (Fig. 2B). Thus, the H3K27 demethylase activity of UTX requires a catalytically active JmjC domain.

Overexpression of UTX Decreases Endogenous Histone H3K27 Methylation in Cells. We next tested whether overexpression of UTX would change endogenous histone H3K27 methylation in cells.

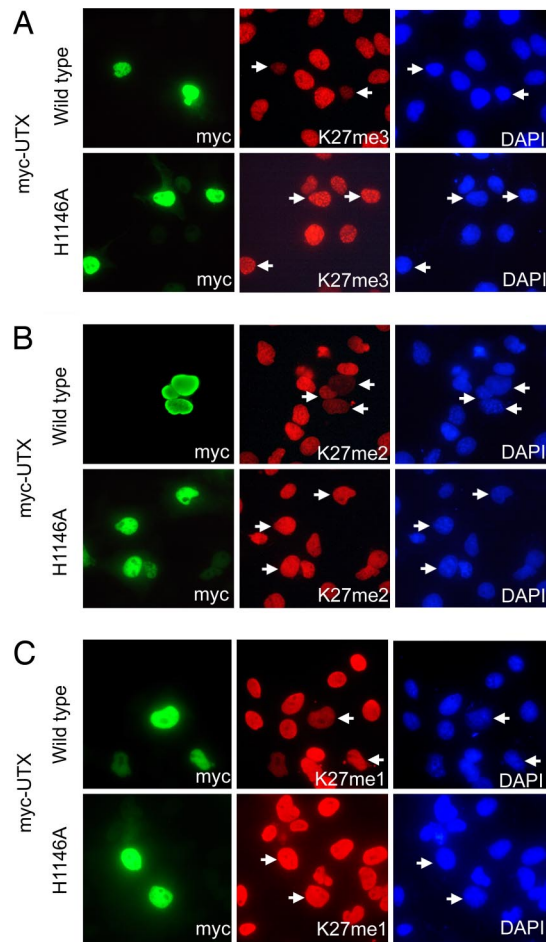


Fig. 3. Overexpression of wild-type UTX but not the H1146A mutant results in reduced H3K27 methylation in cells. COS-7 cells transfected with myc-tagged wild-type or H1146A mutant UTX were stained with anti-myc and anti-H3K27me3 (A), anti-H3K27me2 (B), or anti-H3K27me1 antibody (C). The green corresponds to anti-myc antibody staining; the red corresponds to the staining by specific methylation antibody; and the blue corresponds to DAPI staining of nuclei. Cells with UTX overexpression (indicated by arrows) were scored for the signal intensity of H3K27me3, H3K27me2, and H3K27me1 under the microscope, and the quantitation results are shown in Table 1. The experiments were repeated at least three times, and a representative result is shown.

COS-7 cells were transfected with myc-tagged UTX. After a 36-h incubation, cells were stained with anti-myc antibody to detect transfected cells and with anti-H3K27me1, anti-H3K27me2, or anti-H3K27me3 antibody to examine the level of endogenous H3K27 methylation. As shown in Fig. 3 and Table 1, the levels of H3K27me1, H3K27me2, and H3K27me3 were significantly reduced in 18.7%, 39%, and 36.8% of UTX-overexpressing cells, respectively. Consistent with the requirement for a catalytically active JmjC domain for UTX demethylase activity *in vitro* (Fig. 2B), overexpression of the H1146A mutant failed to decrease methylation of H3K27 in cells. These data suggest that ectopic UTX was capable of depleting endogenous H3K27 methylation in cells, likely through enzymatic demethylation. Interestingly, knockdown of UTX expression by $\approx 80\%$ in a female mouse embryonic fibroblast cell line that carried the *UTX* gene but not the Y-linked *UTY* gene did not change the global level of H3K27 methylation, suggesting that the endogenous UTX may only regulate H3K27 methylation at target genomic loci in cells [supporting information (SI) Fig. 5].

Table 1. UTX overexpression results in decreased histone H3K27 methylation in cells

Change	H3K27me1		H3K27me2		H3K27me3	
	WT	H1146A	WT	H1146A	WT	H1146A
None	75.8% (85/112)	91.5% (75/82)	60.3% (82/136)	97.9% (95/97)	60.4% (64/106)	94.3% (66/70)
Increase	5.3% (6/112)	4.8% (4/82)	0.7% (1/136)	2.1% (2/97)	2.8% (3/106)	5.7% (4/70)
Decrease	18.7% (21/112)	3.6% (3/82)	39% (53/136)	0% (0/97)	36.8% (39/106)	0% (0/70)

COS-7 cells were transfected with myc-tagged wild-type (WT) or H1146A mutant UTX. After a 36-h incubation, cells were stained with anti-myc antibody and anti-H3K27me1, anti-H3K27me2, or anti-H3K27me3 antibody as described in Fig. 3. Cells with UTX overexpression were scored for the signal intensity of H3K27me1, H3K27me2, and H3K27me3 under the fluorescence microscope, and the quantitation results are shown.

JMJD3 Is a H3K27-Specific Demethylase. UTX belongs to a subfamily of JmjC domain-containing proteins that also includes UTY and JMJD3 (Fig. 4A). UTY and UTX share high sequence homology. JMJD3 lacks the TPR domains that are present in UTX and UTY, but it shares high sequence homology with UTX and UTY both within and outside the JmjC domain (Fig. 4A). To test whether JMJD3 and UTY are also H3K27 demethylases, FLAG-tagged JMJD3 and UTY were overexpressed in and affinity-purified from 293T cells followed by histone demethylase assay on calf core histones. As shown in Fig. 4B, JMJD3 specifically demethylated H3K27me2 and H3K27me3 without significantly affecting trimethylation on H3K4, H3K9, and H3K36. Consistent with the *in vitro* demethylase assay results, overexpression of JMJD3 significantly decreased the levels of endogenous H3K27me2 and H3K27me3 in transfected COS-7 cells where JMJD3 was overexpressed (Fig. 4D). Surprisingly, although UTY is the Y-linked homolog of UTX and shares high sequence homology with UTX, purified UTY protein failed to show any H3K27 demethylase activity *in vitro* (Fig. 4C). A similar result was obtained when the JmjC domain-containing C-terminal 828-1347 aa of UTY and the corresponding 881-1401 aa of UTX were tested in such an assay (data not shown). We cannot rule out the possibility that UTY may still be a H3K27 demethylase *in vivo* and may require additional cofactors for histone demethylase activity *in vitro*. Nevertheless, our data indicate that UTX and JMJD3 are histone H3K27-specific demethylases.

Discussion

In this work, we identify the JmjC domain-containing proteins UTX and JMJD3 as histone H3K27-specific demethylases. We show (i) that UTX specifically demethylates mono-, di-, and trimethylated H3K27 without significantly affecting methylation on K4, K9, and K36 of histone H3 (Fig. 1); (ii) that the TPR domains are required for the optimal demethylase activity of UTX on H3K27me1 but not for demethylase activity of UTX on H3K27me2/3 (Fig. 2A); (iii) that the demethylase activity of UTX requires an enzymatically active JmjC domain (Fig. 2B); (iv) that JMJD3, which lacks the TPR domains but shares extensive sequence homology with UTX both within and outside the JmjC domain, specifically demethylates di- and trimethylated H3K27 *in vitro* (Fig. 4B); (v) that purified UTY protein has no H3K27 demethylase activity *in vitro* (Fig. 4C); and (vi) that overexpression of UTX and JMJD3 in COS-7 cells leads to decreased di- and trimethylation on H3K27 (Fig. 3, Table 1, and Fig. 4D), suggesting that UTX and JMJD3 may function as H3K27 demethylases *in vivo*. Our results not only identify demethylases that specifically target histone H3K27 but also provide direct evidence to indicate that similar to methylation on K4, K9, and K36 of histone H3, the methylation on K27 of histone H3 is dynamically regulated and can be actively reversed by site-specific histone demethylases.

Histone demethylases display remarkable site- and methylation state-specific activities. Demethylases specifically antagonizing mono-, di-, or trimethylation (me1, me2, and me3, re-

spectively) on each of the K4, K9, and K36 residues of histone H3 have been reported (20).

For methylation on H3K4, the first identified histone lysine demethylase LSD1 reverses H3K4me1/2, whereas the JARID subfamily of JmjC domain-containing demethylases antagonizes H3K4me2/3; for methylation on H3K9, the JMJD1/JHDM2 subfamily of JmjC demethylases reverses H3K9me1/2, whereas the JMJD2/JHDM3 subfamily antagonizes H3K9me2/3; for methylation on H3K36, the JHDM1 demethylase reverses H3K36me1/2, whereas the JMJD2/JHDM3 subfamily of JmjC demethylases antagonizes both H3K36me2/3 and H3K9me2/3 (20). In this work, we demonstrate that the JmjC domain-containing UTX and JMJD3 are H3K27-specific demethylases. UTX can demethylate H3K27me1/2/3, whereas JMJD3 demethylates H3K27me2/3.

The substrate specificity of histone demethylases has been determined mainly using recombinant proteins purified from bacteria, baculovirus, or mammalian cells *in vitro*. However, histone demethylases often exist in protein complexes in cells. The associated proteins/factors can change the substrate specificity of histone demethylases. For example, LSD1 exists in a number of corepressor complexes including CoREST, CtBP, and a subset of HDAC complexes, consistent with the ability of LSD1 to demethylate H3K4 (21). Bacterially purified recombinant LSD1 can demethylate H3K4me1/2 when the substrate is either a histone peptide or free histone but is unable to demethylate H3K4 in nucleosomal substrate (10). Interestingly, association of LSD1 with CoREST complex enables LSD1 to demethylate H3K4me1/2 in nucleosome (21, 22). More interestingly, LSD1 associates with androgen receptor (AR) in human prostate cancer cells in a ligand-dependent manner. Such an interaction converts LSD1 to an H3K9 demethylase and a transcriptional coactivator for AR (23).

We and others have shown that UTX associates with MLL3- and MLL4/ALR-containing histone H3K4 methyltransferase complexes in cell nuclei (8, 18). We have demonstrated in this work that UTX alone is clearly an H3K27 demethylase and specifically demethylates H3K27me1/2/3 when the substrate is either a histone peptide or free histone. However, it remains to be determined whether UTX can demethylate H3K27 in nucleosomal substrate. Further, we cannot rule out the possibility that the association between UTX and the MLL3 and MLL4/ALR histone methyltransferase complexes may change the substrate specificity of UTX and enable UTX to target a lysine residue other than the K27 on histone H3.

The Polycomb-repressive complex 2 (PRC2) uses its SET domain-containing E(z)/EZH2 subunit to trimethylate H3K27 (9). The resulting H3K27me3 mark specifically recruits Polycomb repressive complex 1 (PRC1), an event that is thought to be important for Polycomb-mediated gene silencing (2, 6). It will be important to determine whether overexpression of H3K27 demethylases UTX and JMJD3 antagonize recruitment of PRC1 complex and Polycomb-mediated gene silencing. In addition, an isoform of PRC2 complex can trimethylate K26 of histone H1 (H1K26me3), likely because the flanking sequence of K26 of

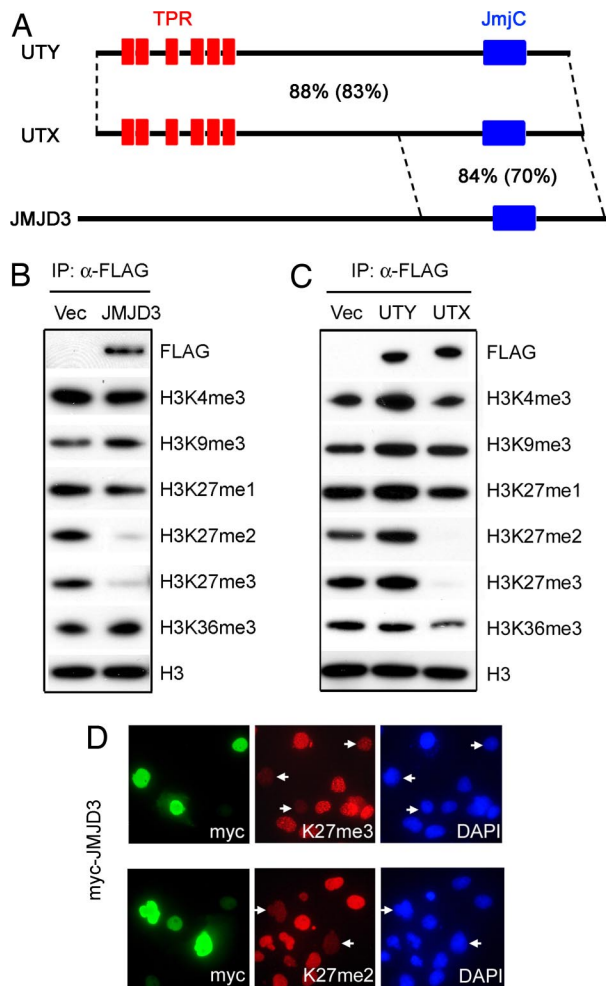


Fig. 4. JMJD3 is a H3K27-specific demethylase. (A) Schematic representation of protein sequence alignment of UTX, UTXY, and JMJD3, with the degree of sequence similarity to UTX shown. The protein sequence identities are shown in parentheses. The JmjC domain containing 1174–1636 aa of JMJD3 shares 84% sequence similarity with the JmjC domain containing 931–1394 aa of UTX. (B) JMJD3 specifically demethylates H3K27me2 and H3K27me3 *in vitro*. Plasmid expressing FLAG-tagged JMJD3 was transfected into 293T cells followed by protein isolation and histone demethylase assay on calf core histones as in Fig. 1. (C) Purified UTXY protein has no H3K27 demethylase activity *in vitro*. Plasmid expressing FLAG-tagged UTXY or UTX was transfected into 293T cells followed by protein isolation and histone demethylase assay as in Fig. 1. Approximately 120 ng of purified UTX and UTXY proteins were used in demethylase assay on calf core histones. (D) Overexpression of JMJD3 results in reduced H3K27me2/3 in cells. COS-7 cells transfected with myc-tagged JMJD3 were stained with anti-myc (green) and anti-H3K27me3 (Upper, red) or anti-H3K27me2 (Lower, red) antibody as described in Fig. 3. The arrows point to cells with JMJD3 overexpression.

human H1 isoform, H1b (H1.4), is nearly identical to that surrounding K27 of histone H3 (24). Because UTX and JMJD3 can demethylate H3K27, it remains to be determined whether UTX and/or JMJD3 can also demethylate H1K26me3.

In summary, we show that the JmjC domain-containing proteins UTX and JMJD3 carry histone H3K27-specific demethylase activity both *in vitro* and in cells. Further work will be needed to identify the target genes of UTX and JMJD3 and to examine whether the endogenous UTX and JMJD3 regulate target gene expression through demethylation of H3K27. It will be interesting to investigate the physiological significance of the physical association between the H3K27 demethylase UTX and the MLL3- and MLL4/ALR-containing H3K4 methyltransferase

complexes in the regulation of gene expression. Finally, it will also be of great interest to find out whether the endogenous UTX and JMJD3 play active roles in the removal of endogenous H3K27me3, an epigenetic mark important for ES cell maintenance, Polycomb-mediated gene silencing, and X chromosome inactivation (6, 13, 15).

Materials and Methods

Plasmids and Antibodies. Full-length human UTX and mouse JMJD3 with C-terminal FLAG tags were amplified by PCR from Mammalian Gene Collection (MGC) clones BC093868 and BC054499, respectively, and were cloned in-frame into the XhoI-XbaI sites downstream of the CMV promoter and in frame with N-terminal 6myc tag in pCS2+NLSMT vector (25). Full-length human UTXY with C-terminal FLAG tag was amplified by reverse-transcriptase PCR from human monocytic leukemia cell line THP-1 and was similarly cloned into pCS2+NLSMT. The H1146A mutant and 401–1401 aa fragment of UTX were generated by PCR. All plasmids were verified by DNA sequencing.

Anti-FLAG (catalog no. F3165), anti-FLAG M2-agarose (A2220), and mouse IgG-agarose (A0919) were from Sigma. Anti-myc (catalog no. SC-40) was from Santa Cruz Biotechnology. Anti-histone H3K9me2 (catalog no. 07–212), anti-H3K27me1 (07–448), anti-H3K27me2 (07–452), anti-H3K27me3 (07–449), and anti-H3K36me2 (07–369) were from Upstate. Anti-histone H3 (catalog no. ab1791), anti-H3K4me2 (ab7766), anti-H3K4me3 (ab8580), anti-H3K9me3 (ab8898), and anti-H3K36me3 (ab9050) were from abcam. Rabbit anti-UTX antibody was generated against His-tagged 550–728 aa of human UTX that was produced as recombinant protein in bacteria. All chemicals were from Sigma unless otherwise indicated.

Cell Culture and Immunofluorescence. 293T and COS-7 cells were cultured in DMEM plus 10% FBS. Transfection was done by using FuGENE 6 (Roche). For immunofluorescence, COS-7 cells plated on chamber slides were transfected with pCS2+NLSMT expressing FLAG- and myc-tagged UTX and JMJD3. After a 36-h incubation, cells were fixed in 4% formaldehyde in PBS, permeabilized in 0.5% Triton X-100 in PBS, and blocked in 10% FBS, 0.1% Saponin in PBS. Secondary antibodies were anti-rabbit IgG–Cy3 conjugate (Sigma, C2306) and Alexa Fluor 488 goat anti-mouse IgG (Invitrogen). Images were acquired by using a Nikon Eclipse 80i fluorescence microscope.

Protein Expression and Purification. 293T cells plated on 100 × 10-cm dishes were transfected with pCS2+NLSMT expressing FLAG-tagged UTX, JMJD3, or UTXY. Forty hours later, cells were collected and dissolved in 4–6× packed cell volume of whole-cell lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 10% (vol/vol) glycerol, 0.5 mM PMSF, and 0.5 mM DTT] supplemented with 1 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.7 μg/ml pepstatin (Roche). After centrifugation, the supernatant was precleared twice with 0.5 ml of mouse IgG-agarose at 4°C for 1 h followed by immunoprecipitation twice with 0.5 ml of anti-FLAG M2-agarose for 6–9 h. After washing with whole-cell lysis buffer for four times, bound proteins were eluted twice with 2.5 ml of elution buffer [50 mM Hepes (pH 7.9), 100 mM KCl, 0.25 mg/ml FLAG peptide] and concentrated with a Vivaspin 500 (Sartorius). The eluate was either resolved on SDS/PAGE 4–15% for Coomassie blue staining or subjected to histone demethylase assay.

Histone Demethylase Assay and MALDI-TOF Mass Spectrometry. Histone demethylase assay was done as described (26) with minor modifications. Briefly, purified proteins were incubated with 1 μg of peptide or 2.5 μg of calf core histones (Sigma H9250) in

the demethylase assay buffer [50 mM Hepes (pH 7.9), 50 mM KCl, 50 mM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6(\text{H}_2\text{O})$, 1 mM α -ketoglutarate, and 2 mM ascorbic acid] for 4 h at 37°C. Assay mixtures containing calf core histone were separated on 4–12% NuPAGE gel (Invitrogen) and analyzed by Western blotting. Assay mixtures containing peptide were subjected to MALDI-TOF mass spectrometry analysis as described (26). H3K27me2 peptide [ATKAARK(me2)SAPATGGVKKPHRYRPG-GK-biotin, catalog no. 12-566] and H3K27me3 peptide [ATKAARK(me3)-

SAPATGGVKKPHRYRPG-GK-biotin, 12–565] were from Upstate. H3K4me3 peptide [ARTK(me3)QTAR-GGC, catalog no. ab1342] was from abcam.

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