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Targeting USP1-dependent KDM4A protein stability as a potential prostate cancer therapy

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Abstract

The histone demethylase lysine-specific demethylase 4A (KDM4A) is reported to be overexpressed and plays a vital role in multiple cancers through controlling gene expression by epigenetic regulation of H3K9 or H3K36 methylation marks. However, the biological role and mechanism of KDM4A in prostate cancer (PC) remain unclear. Herein, we reported KDM4A expression was upregulated in phosphatase and tensin homolog knockout mouse prostate tissue. Depletion of KDM4A in PC cells inhibited their proliferation and survival in vivo and vitro. Further studies reveal that USP1 is a deubiquitinase that regulates KDM4A K48-linked deubiquitin and stability. Interestingly, we found c-Myc was a key downstream effector of the USP1-KDM4A/androgen receptor axis in driving PC cell proliferation. Notably, upregulation of KDM4A expression with high USP1 expression was observed in most prostate tumors and inhibition of USP1 promotes PC cells response to therapeutic agent enzalutamide. Our studies propose USP1 could be an anticancer therapeutic target in PC.

KEYWORDS

deubiquitination, KDM4A, prostate cancer, tumorigenesis, USP1

Abbreviations: AR, androgen receptor; ARE, androgen-responsive element; DUB, deubiquitinating enzyme; H3K9, histone H3 on lysine 9; H3K36, histone H3 on lysine 36; HA-ub, HA-tagged ubiquitin; IB, immunoblot; IHC, immunohistochemistry; IP, immunoprecipitation; KDM4A, lysine-specific demethylase 4A; PC, prostate cancer; PTEN, phosphatase and tensin homolog; qPCR, quantitative PCR.

Authors Cui, Lei, and Guan contributed equally to this work.

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1 | INTRODUCTION

Prostate cancer is a leading cause of death in American men, behind only lung cancer. Androgen deprivation therapy is the standard-of-care treatment for advanced disease when surgical approaches or radiation fail.¹ Androgen receptor, a member of the steroid hormone receptor family of ligand-activated nuclear transcription factors, plays a key role in PC initiation, progression, and resistance to androgen deprivation therapy.²⁻⁴ Androgen receptor drives PC growth by binding to AREs of target genes and recruiting either coactivators or corepressors.⁵ It has yet to be determined which and how AR coactivators regulate AR activity.

KDM4A (also known as JMJD2A or JHDM3A) reportedly serves as an AR coactivator through H3K9 or H3K36 demethylation at promoters or enhancers of some AR target genes.⁶⁻⁸ KDM4A also functions in promoting tumorigenesis by impairing DNA damage repair and inducing genomic instability.⁹ These findings suggest KDM4A plays a vital role in development and progression of PC. It was recently reported that KDM4A is overexpressed in several types of cancer cells, such as breast tumors¹⁰ and lung cancer.¹¹ However, the regulatory mechanism of KDM4A expression upregulation in cancers remains unknown. Here, we aim to identify the posttranslational modification controlling KDM4A stabilization and the mechanism of KDM4A in regulating AR activity, which can be exploited for potential therapeutic interventions.

Deubiquitinating enzymes are proteases that reverse protein ubiquitination, a process which is significant for normal homeostasis. Several DUBs are deregulated in certain types of human cancer and increasingly being regarded as candidates for drug discovery.¹² One of the best-characterized human DUBs, USP1, is overexpressed in gastric cancer, melanoma, cervical, sarcoma, and lung cancer.¹² The best-characterized function of USP1 is as a regulator of the Fanconi anemia pathway, with USP1-mediated deubiquitination of FANCD2 and FANCI.¹³⁻¹⁶ In addition to these DNA repair-related functions, USP1 contributes to preventing bHLH-mediated differentiation, and thus maintains stem cell characteristics in osteosarcoma cells by deubiquitinating ID proteins.¹⁷ However, the novel substrates and regulators of USP1 in cancers remain largely unclear. Numerous works are required to characterize the substrates of USP1 and to better understand the mechanisms of USP1 in certain types of human cancer.

In the present study, we discovered that USP1 regulates cancer cell proliferation and response to therapeutic drugs through the KDM4A/AR-c-Myc pathway. Mechanistically, USP1 deubiquitinated and stabilized KDM4A, which promoted recruitment of AR to the c-Myc gene enhancer. In addition, inhibition of USP1 decreased PC proliferation and promoted response to therapeutic agent enzalutamide in a KDM4A-dependent manner. Notably, KDM4A is upregulated in most prostate tumors from patients, which is positively correlated with high expression of USP1, suggesting that KDM4A might function as a potential prognostic marker and USP1 could be an anticancer therapeutic target in PC.

2 | MATERIALS AND METHODS

2.1 | Cell lines and reagents

HEK293T cells and PC cell lines CWR22RV1 (hereafter named RV1), LNCAP, VCAP, LN95, C4-2B, DU145, and PC3 were purchased from ATCC. All cell lines were tested and authenticated by karyotyping analysis on 2 June 2018. Expression plasmids containing pCMV-Flag-KDM4A, pCMV-Flag-USP1, and pCMV-Myc-USP1 were constructed as previously described,¹⁸ and pCMV-Myc-USP1 C90S mutant (a deubiquitinating enzyme activity-disrupted mutant, in which a Cys to Ser point mutation was introduced) was generated using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) and validated by DNA sequencing. The HA-ub plasmid and mutation constructs were kindly provided by Professor Jianfei Qi from the University of Maryland Cancer Center.

KDM4A (C37E5) rabbit mAb #5328, USP1 (D37B4) rabbit mAb #8033, c-Myc (E5Q6W) rabbit mAb #18583, AR (D6F11) XP rabbit mAb #5153, di-methyl-histone H3 (Lys9) (D85B4) XP rabbit mAb #4658, and NKX3.1 (D2Y1A) XP rabbit mAb #83700 were purchased from Cell Signaling Technology. Anti-ub (sc-8017) Abs were purchased from Santa Cruz Biotechnology. Antibodies against HA (H9658), FLAG (F1804), and β -actin (A1978) were purchased from Sigma. The USP1 inhibitor ML323 was from Selleck Chemicals. Cycloheximide was purchased from Apexbio.

2.2 | Cell survival assay

Prostate cancer cells were transfected with the indicated plasmids. Cells (1×10^4) were plated in 96-well culture plates and cultured in RPMI-1640 medium supplemented with 10% FBS. Ninety-six-well plates were read in an Epoch2 microplate reader (BioTek Instruments). The cell survival ratio was calculated by MTS assay (Promega).

2.3 | Soft agar colony formation assays

Prostate cancer cells were transfected with the indicated plasmids and then 1×10^4 cells were plated in 0.25% (w/v) agarose with a base layer of 0.5% (w/v) agarose. Both layers contained complete 1640 medium. After 2 weeks, colonies were counted by using a light microscope with at 4 \times magnification with a numerical aperture 0.10 objective lens (ECLIPSE 80i; Nikon).

2.4 | Immunoprecipitation

Cells were lysed in NETN buffer (50 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 1% NP-40, 1 mmol/L EDTA) with Proteinase Inhibitor Cocktail (MedChemExpress) added before use. The resulting lysate was then precleaned using 25 mL protein G Sepharose beads

(GE Healthcare) at 4°C. Immunoprecipitation mixtures, including protein lysates, blocked protein G Sepharose, and the indicated Abs or IgG control derived from the same species as the indicated Ab, were incubated on a rotating wheel at 4°C overnight. Sepharose beads with the bound immunoprecipitates were collected and subjected to 4 washes with the cold NETN buffer and then analyzed by western blot assay.

2.5 | Chromatin immunoprecipitation

Prostate cancer cells were cultured in complete RPMI-1640 medium and infected with lentiviral constructs as indicated in figure legends. Cells were pretreated as described in the figure legends before being cross-linked with 1% formaldehyde and then sonicated. Soluble chromatin was immunoprecipitated as described previously.¹⁹ Primer used for ChIP was c-Myc enhancer 5'-CCAGCGAATTATTCAGAA-3' and 5'-AATTACCATTGACTTCCTC-3'. ReChIP analysis was carried out as described previously.¹⁹ Briefly, first-round Ab was added to chromatin extracts and incubated overnight at 4°C followed by addition of 60 mL salmon sperm/protein A agarose (Upstate Biotechnology) to recover immunocomplexes. The bound protein complexes were eluted by 10 mmol/L DTT at room temperature for 30 minutes, and the elution was then diluted 10 times with re-ChIP buffer (1% Triton X-100, 2 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L Tris [pH 8.1]) and subsequently reimmunoprecipitated by addition of the second-round or control IgG Abs overnight at 4°C. Recovery and preparation of DNA were carried out, followed by PCR using the oligonucleotides described above.

2.6 | Protein identification by mass spectrometry

Whole cell lysates from RV1 cells stably expressing Flag-KDM4A were prepared using IP-lysis buffer. Immunoprecipitation of Flag-KDM4A was undertaken as described in the figure legend. The precipitated proteins were eluted with 3× Flag peptides. The eluted samples were subject to in-solution trypsin digestion, liquid chromatography-mass spectrometry (MS) analysis and database search by the proteomic service of Shenzhen University. Identities of the coprecipitated proteins were determined as described previously.²⁰

2.7 | In vivo and in vitro deubiquitylation of KDM4A

For in vivo KDM4A ubiquitylation assay, cells were transfected with the indicated plasmids, then treated with 20 μmol/L MG132 for 8 hours. Cells were collected and lysed in NETN buffer plus 0.1% SDS, 20 μmol/L MG132, and protease inhibitors. Lysates were incubated with anti-KDM4A Ab for 3 hours and protein A/G agarose beads for a further 8 hours at 4°C. The precipitated proteins were then released from the beads by boiling for 10 minutes in SDS-PAGE loading buffer and were subjected to IB with the anti-HA-ub Ab. For preparation of ubiquitinated KDM4A as the substrate for the in vitro

deubiquitylation assay, 293T cells were transfected with both Flag-KDM4A and HA-ub for 2 hours, then treated with 10 μmol/L MG132 for 6 hours. Ubiquitylated KDM4A was purified from the cell extracts with anti-Flag beads and then incubated with the recombinant GST-USP1-WT or GST-USP1-C90S protein in a deubiquitylation buffer for 2 hours at 37°C. Reactions were subjected to IB analysis.

2.8 | Glutathione S-transferase-tagged protein purification and GST pull-down assays

GST-USP1-WT and GST-USP1-C90S in the bacterial expression plasmid pGEX-4T-1 were expressed in the *Escherichia coli* strain BL21 by induction with 0.4 mmol/L isopropyl β-d-1-thiogalactopyranoside at 16°C and purified with GST beads (Sigma-Aldrich) according to the manufacturer's protocol. For GST pull-down assay, bacterial-expressed GST or GST-USP1-WT bound to glutathione-Sepharose 4B beads (GE Healthcare) was incubated with Flag-KDM4A expressed in HEK293T cells for 2 hours at 4°C. After reaction, complexes were washed at least 4 times with GST-binding buffer, eluted by boiling in SDS-PAGE loading buffer, and subjected to IB with the indicated Abs.

2.9 | Immunohistochemistry

Normal prostate tissue samples and prostate tumor tissue samples were collected at the Affiliated Cancer Hospital and Institute of Guangzhou Medical University. Tissue sample collection was approved by the Internal Review and Ethics Boards of Guangzhou Medical University. Tissue microarray chips containing normal prostate tissue samples and prostate tumor tissue samples were obtained from Shanghai OUTDO Biotech Co. Immunohistochemical staining was carried out as described previously.²¹ The immunostaining was blindly scored by 2 pathologists. The IHC score was calculated as described previously.¹⁹ The χ^2 test and Pearson's correlation coefficient were used for statistical analysis of the correlation between USP1 and KDM4A expression.

2.10 | Animal models and mouse xenograft assays

All in vivo studies were undertaken in accordance with the Chinese Animals (Scientific Procedures) and approved by the Animal Welfare and Ethical Review Board at Guangzhou Medical University. The WT mice and mice with prostate-specific deletion of PTEN (stock number 006440; Jackson Laboratories) were kindly provided by Professor Jianfei Qi from the University of Maryland Cancer Center. All mice were housed under pathogen-free conditions, and killed after 3 months were killed when the standard situations occurred.

Seven-week-old male BALB/c nude mice were from Charles River Laboratories in China. A total of 1×10^6 RV1 cells stably expressing plasmids were injected s.c. into male BALB/c nude mice, using the standard procedure, by two researchers blinded to the experimental

FIGURE 2 KDM4A knockdown inhibits cell proliferation and colony formation in vitro and tumor growth in vivo. A-C, RV1, LNCAP, and PC3 cells stably expressing control (Ctrl) or KDM4A shRNAs (#1 and #2) were subjected to detection of KDM4A expression. D, Cell viability after stably expressing control (Ctrl) or KDM4A shRNAs (sh#1 and sh#2) was measured by MTT assay (mean \pm SEM of 3 independent experiments). $**P < .01$. E, KDM4A knockdown decreased the colony formation by RV1 cells on the cell culture plate. RV1 cells (Ctrl or KDM4A knockdown) were seeded at low density on the plate, and maintained for 2 wk. Numbers of colonies were counted in 5 high-power fields (mean \pm SEM of 3 independent experiments). $**P < .01$. F, KDM4A knockdown decreased the colony formation by RV1 cells in soft agar. RV1 cells (Ctrl or KDM4A knockdown) were grown in soft agar for 3 wk. Growth conditions and colony number quantification are as described in (E) (mean \pm SEM of 3 independent experiments). $**P < .01$. G, RV1 cells were transduced with control or KDM4A shRNA for 24 h. Cells were then maintained in growth media containing 5% charcoal-stripped FBS with or without 1 nmol/L R1881 (synthetic androgen). Proteins were collected and analyzed by western blot. H, RNAs from (G) were collected and analyzed by quantitative RT-PCR for selected androgen receptor (AR) target genes (mean \pm SEM of 3 independent experiments). $*P < .05$; $**P < .01$. I, Indicated RV1 cells from (G) were seeded at low density on the plate, and maintained for 2 wk. Numbers of colonies were counted in 5 high-power fields (mean \pm SEM of 3 independent experiments). $*P < .05$; $**P < .01$. J, Cells from (G) were grown in soft agar for 3 wk. Growth conditions and colony number quantification are described in (G). K, L, RV1 cells stably expressing Ctrl or shKDM4A RNAs were injected into BALB/c nude mice. Tumor images and weights are shown (mean \pm SD of 5 mice). All statistical analyses were undertaken with ANOVA. $*P < .05$; $**P < .01$. M, Representative staining of Ki-67 on tumor sections derived from above. Staining was developed by DAB (brown) and counterstained by hematoxylin (blue). Percentages of Ki-67-positive cells are shown. $**P < .01$ for Ctrl vs shKDM4A (ANOVA)

groups. Dr Song-Hui Xu and Dr Shu-Zhong Cui, who were completely blinded to the experimental groups, injected the cells into the mice. After three weeks, we measured the tumors and analyzed the data.

The mice used in these experiments were bred and maintained under defined conditions at the Animal Experiment Center of the College of Medicine (SPF grade), Guangzhou Medical University. The animal experiments were approved by the Laboratory Animal Ethics Committee of the Guangzhou Medical University and conformed to

the legal mandates and national guidelines for the care and maintenance of laboratory animals.

2.11 | Statistical analysis

Statistical analysis was carried out using GraphPad software, version 5. Data are presented as the means \pm SEM or SD. Student's *t* test was

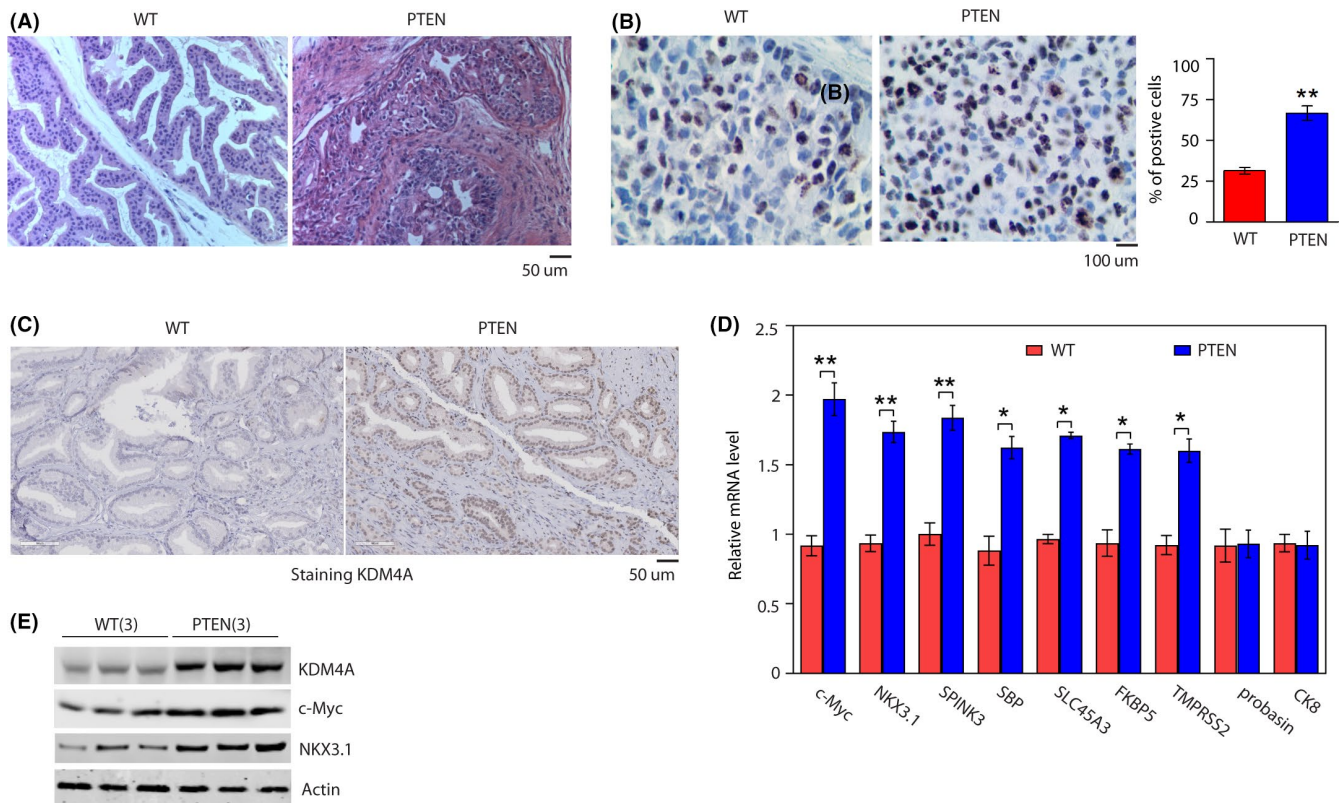
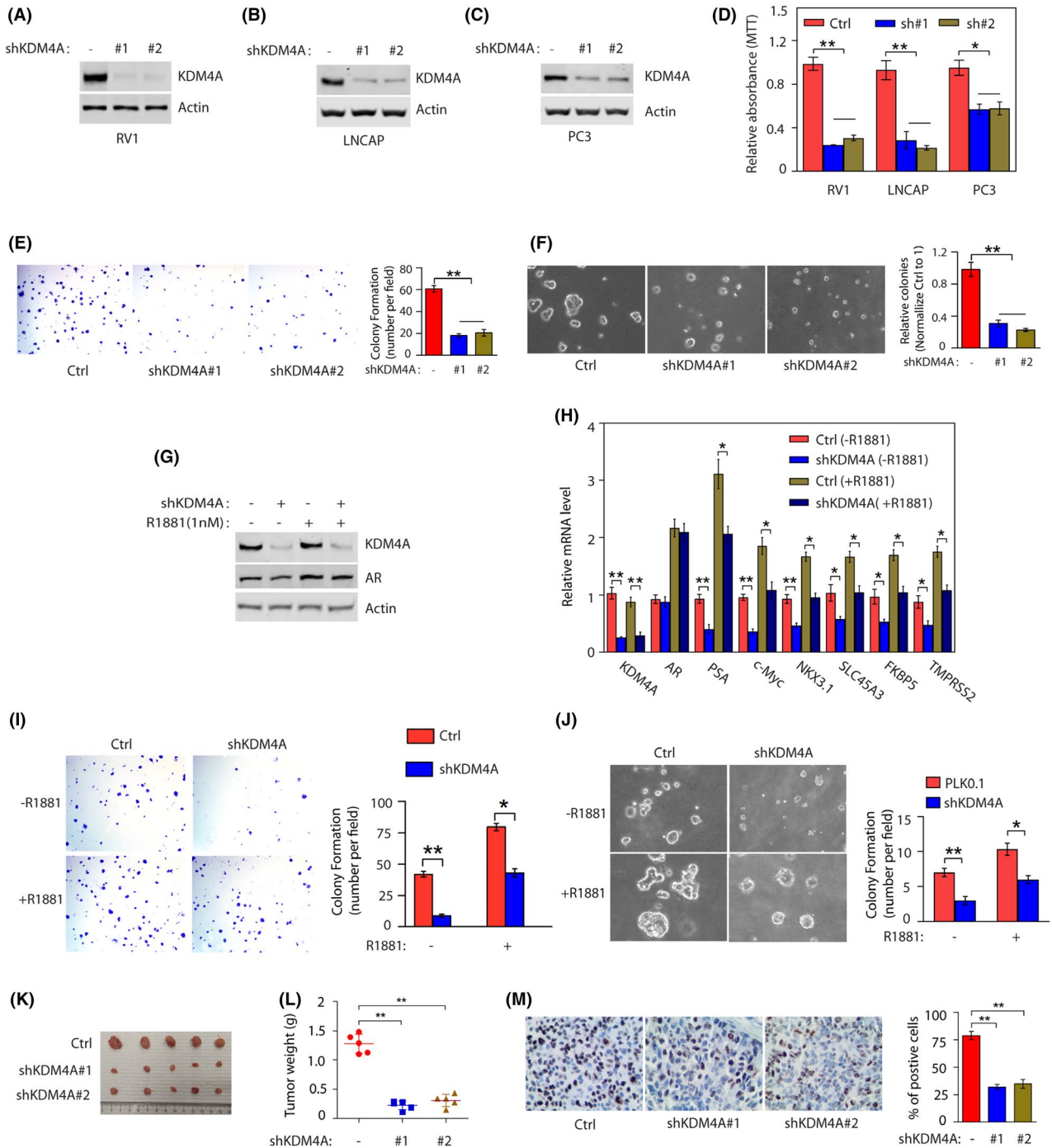


FIGURE 1 KDM4A expression is elevated in phosphatase and tensin homolog (PTEN) knockout mouse prostate tissue. A, H&E stained dorsal prostates from 3-mo-old WT and pbsn⁺ PTEN^{-/-} male mice (PTEN). B, Percentage of Ki-67-positive cells in prostates in (A). $**P < .01$, significant difference between genotypes. C, Representative immunohistochemical images of KDM4A expression in WT and PTEN mouse prostate tissues. D, Transcript levels of selected androgen receptor target genes in the prostates of WT and PTEN mice, RNA for quantitative RT-PCR was isolated from dorsal prostates of 3-mo-old mice. $*P < .05$; $**P < .01$. Data are mean \pm SD. E, Representative western blotting showing indicated protein expression in prostates from WT and PTEN mice (n = 3 mice per genotype)



applied to assess the statistical significance. *P* values less than 0.05 were considered significant. Supplementary materials and methods are provided in Appendix S1.

3 | RESULTS

3.1 | KDM4A expression is elevated in PTEN KO mouse prostate tissue

To investigate the role of KDM4A in PC, we used the widely characterized PTEN KO mouse (hereafter, PTEN mice) model of PC. In

this model, cre-recombinase is under the control of a modified rat prostate-specific probasin promoter (PB-Cre4)²² that drives deletion of the floxed *Pten* allele in the epithelial cells of the mouse prostate. Prostate cancer development was monitored in the PTEN mice at 12 weeks of age, the spontaneous formation of PC was observed by histopathological analysis (Figure 1A) using Ki-67, a marker of cell proliferation. Ki-67 staining was significantly increased in prostate from the PTEN mice (Figure 1B). To our interest, we found KDM4A protein was significantly increased in the PTEN mice compared to WT mice (Figure 1C,E), but KDM4A mRNA level had no change (Figure S1A). It was previously reported that KDM4A interacts with AR and functions as a key AR coactivator

through its histone demethylase activity.⁶ Here, in PTEN mice we found a significant increase in transcript levels of some AR target genes (*c-Myc*, *NKX3.1*, *SPINK3*, *SLC45A3*, *FKBP5*, and *TMPRSS2*, but not *probasin* or *SBP*), pointing to the possibility that KDM4A regulates the expression of a specific subset of AR target genes (Figure 1D). The increase in lesions observed in PTEN-deficient mice is not likely to be due to probasin-driven SV40 T-antigen expression, as the level of probasin transcript protein were similar in both WT mice and PTEN mice tissues (Figure 1D). In agreement, the dorsal prostates from both genotypes expressed similar transcript levels of the luminal marker CK8 (Figure 1D), suggesting that the reduced expression of AR target genes was not due to a change in cellular differentiation. Furthermore, we confirmed the 2 selected AR target genes, *c-Myc* and *NKX3.1*, protein expression are markedly increased in prostate tissue from PTEN mice compared to WT mice (Figures 1E and S1B). These results suggest that KDM4A could play an oncogenic role in tumorigenesis in PC, and that may link its role to AR signal.

3.2 | KDM4A knockdown inhibits cell proliferation and colony formation in intro and tumor growth in vivo

Given the abnormal expression of KDM4A in PTEN mice, we then assessed whether KDM4A knockdown affects PC cell growth in vitro. We knocked down KDM4A expression in two AR-positive PC cells (RV1 and LNCAP) and one AR-negative PC3 cell line. Separately, both constructs reduced mRNA levels (Figure S2A) and expression of KDM4A protein (Figure 2A-C). Further evaluating the role of KDM4A in PC proliferation, we found that PC cell proliferation was obviously decreased when KDM4A was knocked down in AR-positive PC cells (Figures 2D and S2B), but partially reduced AR-negative PC3 cells, suggesting that KDM4A might require AR to regulate cell proliferation. Concordantly, KDM4A knockdown also decreased the number and size of cell colonies in RV1 (Figure 2E) and LNCAP cells (Figure S2C). Consistent with this result, as determined in RV1 cells by soft agar colony formation assays (Figure 2F). To determine whether KDM4A regulates AR activity in human PC cells, KDM4A-knockdown RV1 cells were cultured in medium supplemented with 5% charcoal-stripped (CS)-FBS (which has low androgen levels) for 2 days then stimulated with the synthetic androgen R1881 (to restore androgens to physiological levels) for 24 hours. We found that protein levels of KDM4A were not affected irrespective of the androgen conditions, but AR protein levels were slightly increased in both RV1 and LNCAP cells after R1881 treatment (Figures 2G and S2D). KDM4A knockdown has no effect on AR protein levels irrespective of the androgen conditions (Figures 2G and S2D). Furthermore, quantitative RT-PCR analysis showed that KDM4A knockdown in RV1 cells reduced mRNA levels of the selected AR target genes under either androgen-deprived or normal conditions (Figure 2H). To further determine the role of KDM4A in PC, we knocked down KDM4A

in PC cells and measured the cell proliferation by colony formation assay or soft agar assay. Compared with control cells, KDM4A knockdown in the AR-positive RV1 or LNCAP cells decreased cell proliferation in the presence or absence of androgen (Figures 2I, J and S2E). Of note, the effect of KDM4A knockdown to reduce cell growth was more apparent under androgen deprivation conditions (Figures 2I, J and S2E).

To examine the in vivo protumor activity by KDM4A, control vector or 2 stably expressed shKDM4A cells were individually injected into right s.c. tissues of Balb/c nude mice. Compared with control cells, KDM4A-knockdown RV1 cells showed a significant reduction in tumor size (Figure 2K) and weight (Figure 2L). We next undertook IHC staining for the proliferation marker Ki-67 on xenograft tumor sections. KDM4A knockdown reduced the percentage of Ki-67-positive cells (Figure 2M). These results suggest that KDM4A knockdown inhibits proliferation in xenograft prostate tumors, indicating that KDM4A participates in PC tumorigenesis.

3.3 | USP1 binds and stabilizes KDM4A

To investigate the mechanism through which KDM4A acts in tumorigenesis, the proteins that interact with KDM4A were identified and USP1, a deubiquitinase, was one of the top-scored proteins identified by MS analysis (Figure 3A) (MS data available upon request). The interaction between KDM4A and USP1 was confirmed by coimmunoprecipitation experiments in 293T cells overexpressing KDM4A (Figure 3B). To determine whether KDM4A and USP1 directly interact, we mixed the purified GST-USP1 with the purified Flag-KDM4A and tested the in vitro binding between the 2 proteins. GST-USP1, but not GST protein, was coprecipitated with Flag-KDM4A, indicating a direct interaction between the 2 proteins (Figure S3A). Furthermore, immunofluorescence staining revealed that endogenous KDM4A and USP1 were mainly colocalized in punctate structures spreading throughout the nucleus (Figure 3C).

Multiple reports showed WDR48 act as a strong activator of USP1 by enhancing the USP1-mediated deubiquitination.^{23,24} To test whether WDR48 is required for the interaction between KDM4A and USP1, we undertook coimmunoprecipitation experiments in RV1 cells using KDM4A or WDR48 Ab pull-down assays. As shown in Figure 3D,E, the interaction between KDM4A and WDR48 was confirmed by reciprocal coimmunoprecipitation experiments. In addition, the association of endogenous KDM4A with USP1 was reduced by WDR48 knockdown treatment in RV1 cells (Figure 3F). As is well known, active transcription foci are distributed in the nucleus in a punctate pattern; therefore, USP1, WDR48, KDM4A, and AR might form a complex to regulate transcription in PC cells.

The interaction of USP1 and KDM4A led us to test a potential role for the ubiquitination enzyme USP1 in the regulation of KDM4A turnover and function. USP1 knockdown in RV1, LNCAP, and PC3 cells significantly decreased KDM4A protein level (Figure 3G), but did not affect KDM4A mRNA level (Figure S3B). In addition, treating

cells with the proteasome inhibitor MG132 did not significantly reduce KDM4A protein level in cells with downregulation of USP1 (Figure 3H). Furthermore, overexpression of WT USP1 but not the catalytic inactive mutant (CS mutant) in RV1 cells could dramatically increase KDM4A protein level in the cells with depletion of USP1 (Figure 3I). We then hypothesized that USP1 might regulate KDM4A stability. As shown in Figure 3J, KDM4A protein was more stable after overexpression of USP1. Taken together, these results suggest that USP1 directly regulates KDM4A stability.

3.4 | USP1 deubiquitinates KDM4A

To further investigate whether USP1 functions as a deubiquitinase for KDM4A, we undertook a deubiquitination assay by cotransfecting cells with WT USP1 or the CS mutant in the presence of MG132. A significant decrease in polyubiquitylated KDM4A protein was observed in cells transfected with WT USP1, whereas the expression of CS mutant was not able to significantly reduce KDM4A ubiquitination (Figure 4A). Conversely, using 2 different shRNAs of USP1 dramatically increased KDM4A ubiquitination (Figure 4B). Notably, the USP1 inhibitor, ML323,²⁵ significantly increased KDM4A ubiquitination (Figure 4C). To determine whether USP1 functions as a direct deubiquitinase for KDM4A, ubiquitinated KDM4A was incubated with purified GST fusion USP1 (WT) or USP1 (CS) *in vitro*. As shown in Figure 4D, a significant decrease in polyubiquitylated KDM4A protein was observed in cells transfected with GST fusion USP1, whereas GST fusion USP1 (CS) was not able to reduce KDM4A ubiquitination. Furthermore, we found that USP1 overexpression decreased the polyubiquitination levels in WT, K6R, K11R, K27R, K29R, K33R, and K63R HA-ubiquitin mutants but not in K48R mutant (Figure 4E), suggesting USP1 promotes KDM4A K48-linked deubiquitin; however, which E3 ubiquitin ligase mediated KDM4A ubiquitin and degradation is still unclear. Taken together, these results suggest USP1 is a new deubiquitinase that regulates KDM4A K48-linked deubiquitin and stability.

3.5 | c-Myc is a downstream effector of USP1-KDM4A axis in PC cells

Because of the key role of c-Myc in PC and our above findings that KDM4A regulates c-Myc transcription, we determined whether c-Myc is a downstream effector of KDM4A. We knocked down KDM4A in 2 AR-positive PC cells (RV1 and LNCAP) and two AR-negative cells (PC3 and DU145). KDM4A knockdown in the 2 AR-positive PC cells (RV1 and LNCAP) significantly decreased mRNA levels of c-Myc, whereas it had no effect in the 2 AR-negative PC cells (PC3 or DU145) (Figure 5A). Furthermore, western blot analysis showed that downregulation of c-Myc expression, but not AR, following KDM4A knockdown in 2 AR-positive (Figure 5B) or in AR-negative PC cells (Figure S4A), suggesting that KDM4A might

regulate c-Myc expression at both transcriptional and posttranscriptional levels. We previously found that USP1 knockdown decreased levels of KDM4A in PC cells. To evaluate the possible involvement of the USP1-KDM4A axis in modulating c-Myc expression. We test whether KDM4A was able to rescue the effect caused by USP1 depletion. As shown in Figure 5C, USP1 knockdown dramatically reduced levels of KDM4A and c-Myc, whereas the reconstitution of KDM4A was able to efficiently rescue their expression. In addition, our qPCR analysis also showed that USP1 knockdown in AR-positive PC cells decreased c-Myc mRNA levels, whereas the reconstitution of KDM4A was able to efficiently rescue the effect (Figure 5D). To examine whether expression of c-Myc could rescue the effect of USP1 and KDM4A depletion on proliferation and survival of PC cells, we undertook MTT assay. We found reconstitution of c-Myc was able to efficiently increase the proliferation and survival of the RV1 cells with depletion of USP1 or KDM4A (Figure S4B). Taken together, these results indicate that USP1 promotes the expression of c-Myc by regulating KDM4A. We next asked how the USP1-KDM4A axis increases AR-dependent c-Myc transcription. As KDM4A is a coactivator of AR,⁶ and the c-Myc gene enhancer contains an ARE,²⁶ we undertook a ChIP assay using a KDM4A Ab to determine whether KDM4A associates with the ARE on the c-Myc enhancer. We found silencing of either USP1, KDM4A, and/or AR reduced KDM4A binding to c-Myc the enhancer (Figure 5E). To evaluate whether KDM4A regulates AR binding to the c-Myc enhancer, we undertook an AR ChIP assay. Downregulation of either USP1, KDM4A, and/or AR decreased binding of AR to the c-Myc enhancer (Figure 5F). Consistent with the role of KDM4A as the H3K9me2 demethylase, downregulation of either USP1, KDM4A, and/or AR increased levels of the H3K9me2 mark at the c-Myc enhancers (Figure 5G). To determine whether the USP1-KDM4A axis-dependent transcription of c-Myc requires the AR ligand, we maintained the PC cells (control, shUSP1, and shUSP1 + KDM4A) in the media supplemented with CS FBS that contains extremely low levels of androgen for 2 days, followed by treatment of cells with or without synthetic androgen R1881 for 1 day. We found that R1881 induced a significant increase in NKX3.1 (a positive control) transcript in RV1 cells, but it failed to induce the c-Myc transcript, and USP1 knockdown reduced the c-Myc or NKX3.1 transcript similarly, with or without R1881. However, the reconstitution of KDM4A was able to efficiently rescue the effect (Figure 5H). These results suggest that AR ligand is not required for USP1-KDM4A axis-dependent transcription of c-Myc. To further confirm this result, we undertook ChIP assay using AR Ab in RV1 cells (control, shUSP1, and shUSP1 + KDM4A). Even in the absence of R1881, we observed the binding of AR to the c-Myc gene enhancer in RV1 control cells (control), and such binding was not affected by treatment with R1881 (Figure 5I). Knockdown of USP1 equally reduced the binding between AR and c-Myc enhancer in the presence or absence of R1881, and the reconstitution of KDM4A partially rescued the effect (Figure 5I), suggesting that USP1 not only regulates the binding between AR and c-Myc enhancer by KDM4A, but

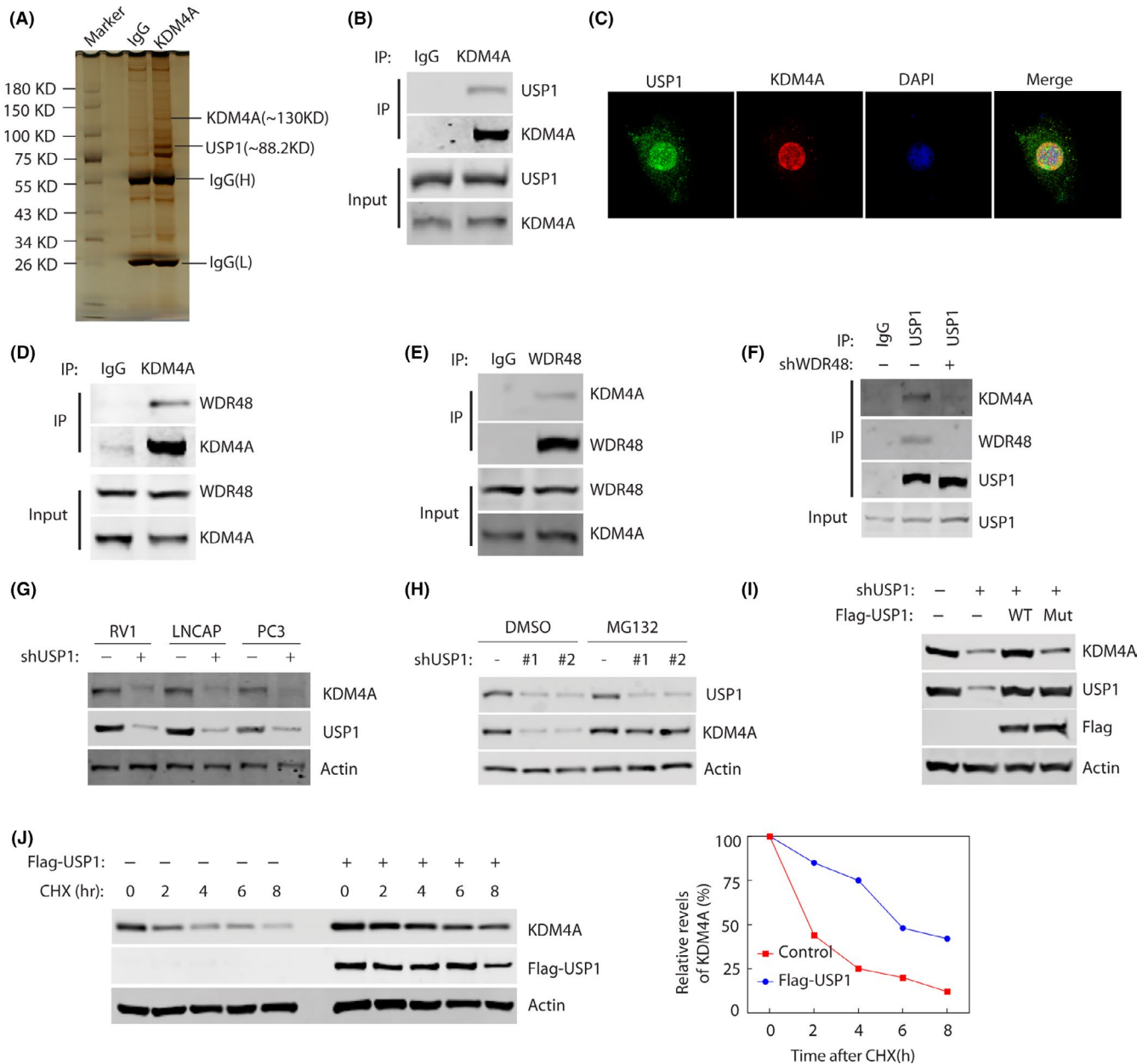


FIGURE 3 USP1 binds and stabilizes KDM4A. A, Proteins that interacted with KDM4A were identified by Co-IP and mass spectrometry assays. B, RV1 cells were transfected with Flag-KDM4A plasmids for 48 h, and lysates were subjected to immunoprecipitation with anti-KDM4A Ab. Bound proteins were analyzed by western blotting with anti-KDM4A or USP1 Abs. C, Representative fluorescence images of KDM4A and USP1 proteins in RV1 cells. Cells were seeded on coverslips for 24 h and then stained for KDM4A (red) and USP1 (green). Nuclei were visualized by DAPI staining (blue). D, E, RV1 cells were lysed and immunoprecipitated (IP) with indicated Abs. Immunocomplexes were subjected to western blotting. F, RV1 cells stably expressing control or WDR48 shRNAs were lysed and IPed with indicated Abs. Immunocomplexes were subjected to western blotting. G, RV1 cells stably expressing control or USP1 shRNAs were subjected to western blotting to examine the indicated proteins. H, Cells were untreated or treated with 10 μ mol/L MG-132 and western blotting was carried out to examine the indicated protein levels. I, 293T cells were transfected with indicated constructs. Indicated proteins were analyzed by western blotting. J, RV1 cells were stably expressed control or Flag-USP1 constructs and then cycloheximide (CHX) pulse-chase assay was undertaken in cells. Right panel, protein levels of USP1 relative to β -actin

also by other transcription factors. Conversely, a low level of AR was associated with NKX3.1 enhancer in the absence of R1881, whereas after R1881 treatment, the binding of AR to NKX3.1 enhancer was highly enhanced (Figure 5I). Taken together, our results reveal that the USP1-KDM4A axis is required for the constitutive association between AR and c-Myc enhancer in the presence or

absence of androgen. This result is consistent with the previous report that AR upregulates the expression of c-Myc in a ligand-independent manner.⁵

To further test the role of USP1 in KDM4A-dependent tumor growth in vivo, we used a xenograft prostate tumor model in which RV1 cells were injected into nude mice. Compared with

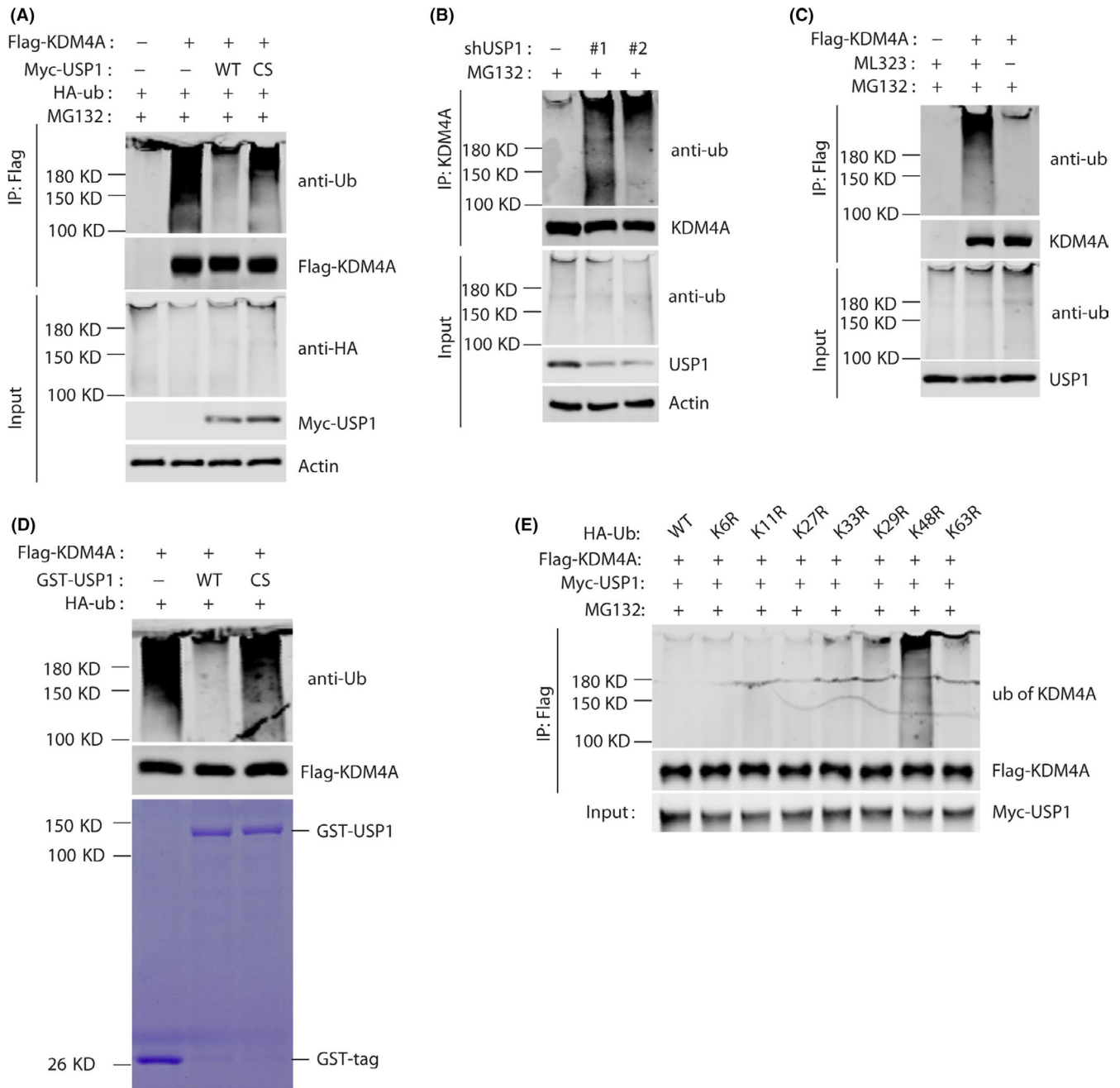


FIGURE 4 USP1 deubiquitinates KDM4A. A, 293T cells were transfected with Flag-KDM4A, Myc-USP1, and HA-tagged ubiquitin (HA-ub) as indicated. Cellular lysates were coimmunoprecipitated by anti-Flag Ab. Polyubiquitylated KDM4A protein was detected by anti-HA-ub Ab. B, RV1 cells stably expressing control or USP1 shRNAs were subjected to ubiquitination assay and the polyubiquitylated KDM4A protein was detected by anti-ub Ab. C, RV1 cells transfected with Flag-KDM4A were treated with or without 10 μ mol/L ML323 (USP1 inhibitor). Polyubiquitylated KDM4A protein was examined as in (B). D, Deubiquitination of KDM4A in vitro by USP1. Ubiquitinated KDM4A was incubated with purified GST fusion USP1 (WT) or USP39 (CS) in vitro and then blotted with indicated Abs. E, 293T cells were transfected with Myc-USP1, Flag-KDM4A, and HA-ub (WT, K6R mutant, K11 mutant, K27 mutant, K29 mutant, K33 mutant, K48 mutant, and K63 mutant). Analysis was carried out as described in (A)

control cells, USP1 knockdown cells showed a significant decrease in tumor size (Figure 5J) and weight (Figure 5K) whereas the reconstitution of KDM4A in USP1 knockdown cells could significantly rescue xenograft tumor formation (Figure 5J,K). In addition, we carried out IHC staining for the proliferation marker Ki-67 on xenograft tumor sections (Figure 5L). USP1 knockdown reduced the

percentage of Ki-67-positive cells, whereas the reconstitution of KDM4A in USP1 knockdown cells could significantly rescue the effect (Figure 5L).

Overall, these findings revealed that c-Myc was a key downstream effector of the USP1-KDM4A axis to drive the growth and survival of PC cells.

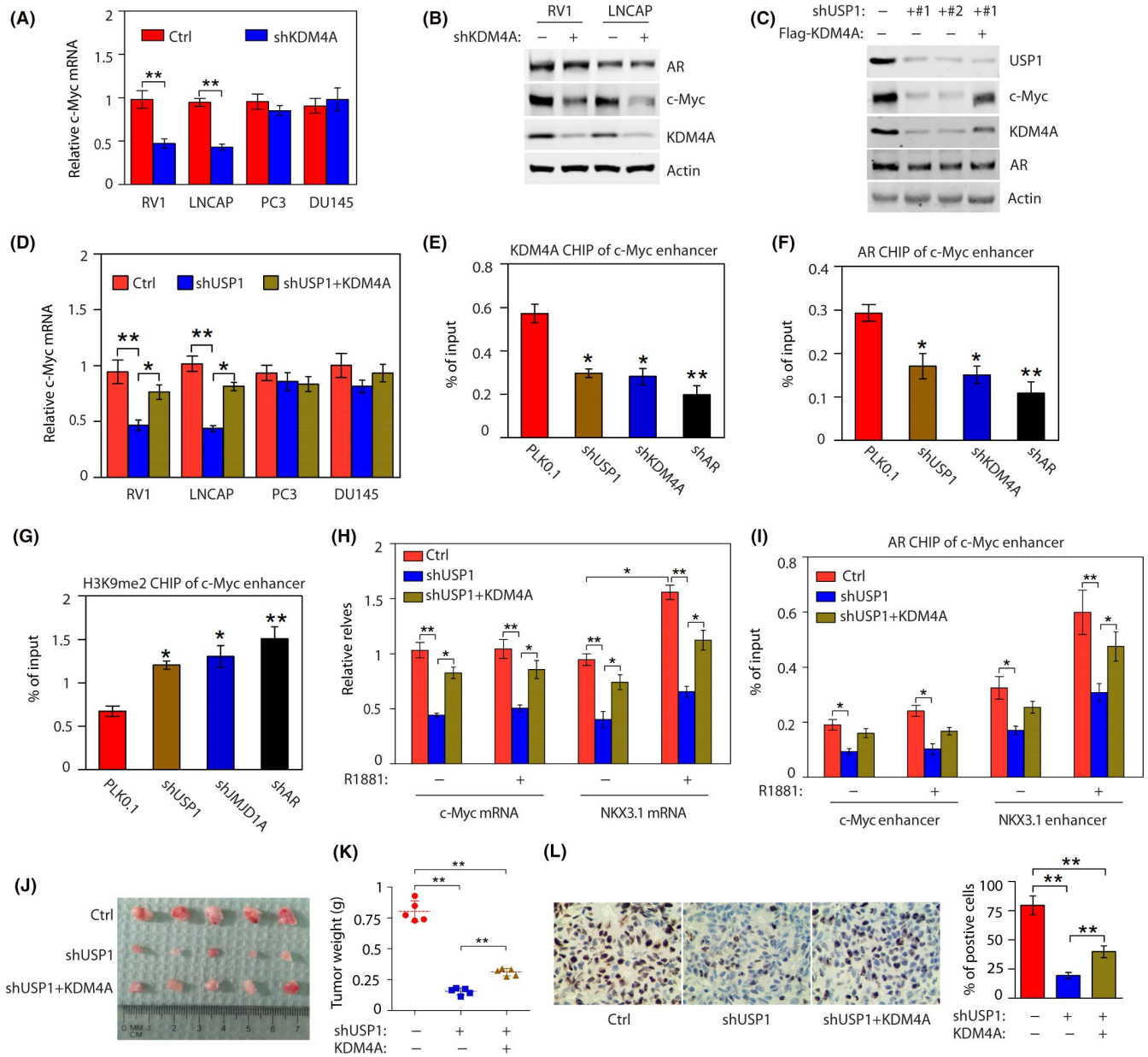


FIGURE 5 c-Myc is a downstream effector of the USP1-KDM4A axis in androgen receptor (AR)-positive cells. A, c-Myc mRNA levels were detected in AR-positive cells (RV1 and LNCAP) and AR-negative cells (PC3 and DU145) stably expressing control (Ctrl) or KDM4A shRNAs (#1 and #2). **P < .01. B, Western blotting of AR-positive cells (RV1 and LNCAP) to examine the indicated proteins. C, RV1 cells stably expressing control (Ctrl) or USP1 shRNAs with or without Flag-KDM4A were analyzed by western blot. D, c-Myc mRNA levels were detected in indicated cells stably expressing Ctrl or USP1 shRNAs with or without Flag-KDM4A. *P < .05; **P < .01. E, RV1 cells were transduced with USP1, KDM4A, or AR shRNAs for 48 h and subjected to ChIP assay using anti-KDM4A Ab. Chromatin was analyzed by quantitative PCR (qPCR) for regions of the c-Myc enhancer showing an androgen-responsive element (ARE). Knockdown (KD) of USP1, KDM4A, or AR decreased KDM4A binding to ARE region. *P < .05; **P < .01. F, Cells as described in (E) were subjected to ChIP assay using anti-AR Ab. Chromatin was analyzed by qPCR for regions of the c-Myc enhancer showing an ARE. KD of USP1, KDM4A, or AR decreased AR binding to ARE region. *P < .05; **P < .01. G, Cells as described in (F) were subjected to ChIP assay using anti-H3K9me2 Ab. KD of USP1, KDM4A, or AR decreased H3K9me2 binding to ARE region. *P < .05; **P < .01. H, RV1 cells stably expressing Ctrl or USP1 shRNAs with or without Flag-KDM4A were maintained in growth media containing 5% charcoal-stripped FBS with or without 1 nmol/L R1881 for 48 h, then c-Myc and NKX3.1 mRNA levels were detected. *P < .05; **P < .01. I, Cells as described in (H) were subjected to ChIP assay by anti-AR Ab. Chromatin was analyzed by qPCR for regions of the c-Myc enhancer and NKX3.1 enhancer showing AREs. J, K, RV1 cells stably expressing Ctrl or shUSP1 RNAs were injected into BALB/c nude mice. Tumor images and weights are shown (mean \pm SD of 5 mice). All statistical analyses were undertaken with ANOVA. *P < .05; **P < .01. L, Representative staining of Ki-67 on tumor sections derived from above. Staining by DAB (brown) and counterstained by hematoxylin (blue). Percentages of Ki-67-positive cells are shown. **P < .01 (ANOVA)

3.6 | KDM4A expression positively correlates with USP1 expression in clinical PC samples

Previous studies have reported that KDM4A upregulation has been observed in PC.^{9,27,28} As KDM4A plays a key role in human cancer development, it is possible that in human cancers USP1 promotes the deubiquitination and stabilization of KDM4A. To determine the relevance of regulation of KDM4A/AR-c-Myc signaling by USP1 in patients, we undertook IHC staining of KDM4A and USP1 on PC tissue microarrays. First, we detected the expression of USP1 and KDM4A in PC cell lines and cancer tissue samples. As shown in Figure 6A, USP1 protein levels were positively correlated with KDM4A expression in AR-positive and AR-negative PC cells. Furthermore, western blot analysis showed high USP1 protein levels correlated with increased KDM4A expression in most PC samples (Figure 6B). To determine the relevance of KDM4A regulation by USP1 in patients,

we undertook IHC staining of KDM4A and USP1 in PC tissue microarrays (Figure 6C). Notably, upregulation of KDM4A expression and high USP1 expression were observed in 70% (102 of 146) and 75% (109 of 146) of prostate tumors, whereas only 23% (8 of 35) and 26% (9 of 35) of normal prostate tissues showed high KDM4A expression and upregulation of USP1 expression, respectively (Figure 6D). To our excitement, a significant positive correlation ($R = 0.405$, $P < .001$) between the KDM4A and USP1 protein levels was observed in these prostate tumors: 74.5% (76 of 102) of tumors with high KDM4A expression also displayed upregulation of USP1 expression (Figure 6D). However, it should be noted that 25.5% (26 of 102) of all tumor specimens had high KDM4A expression but low USP1 expression (Figure 6D). Taken together, these results reveal that upregulation of USP1 expression could contribute to KDM4A overexpression in a substantial fraction of human prostate tumors, whereas in other prostate tumors, KDM4A can be activated by other

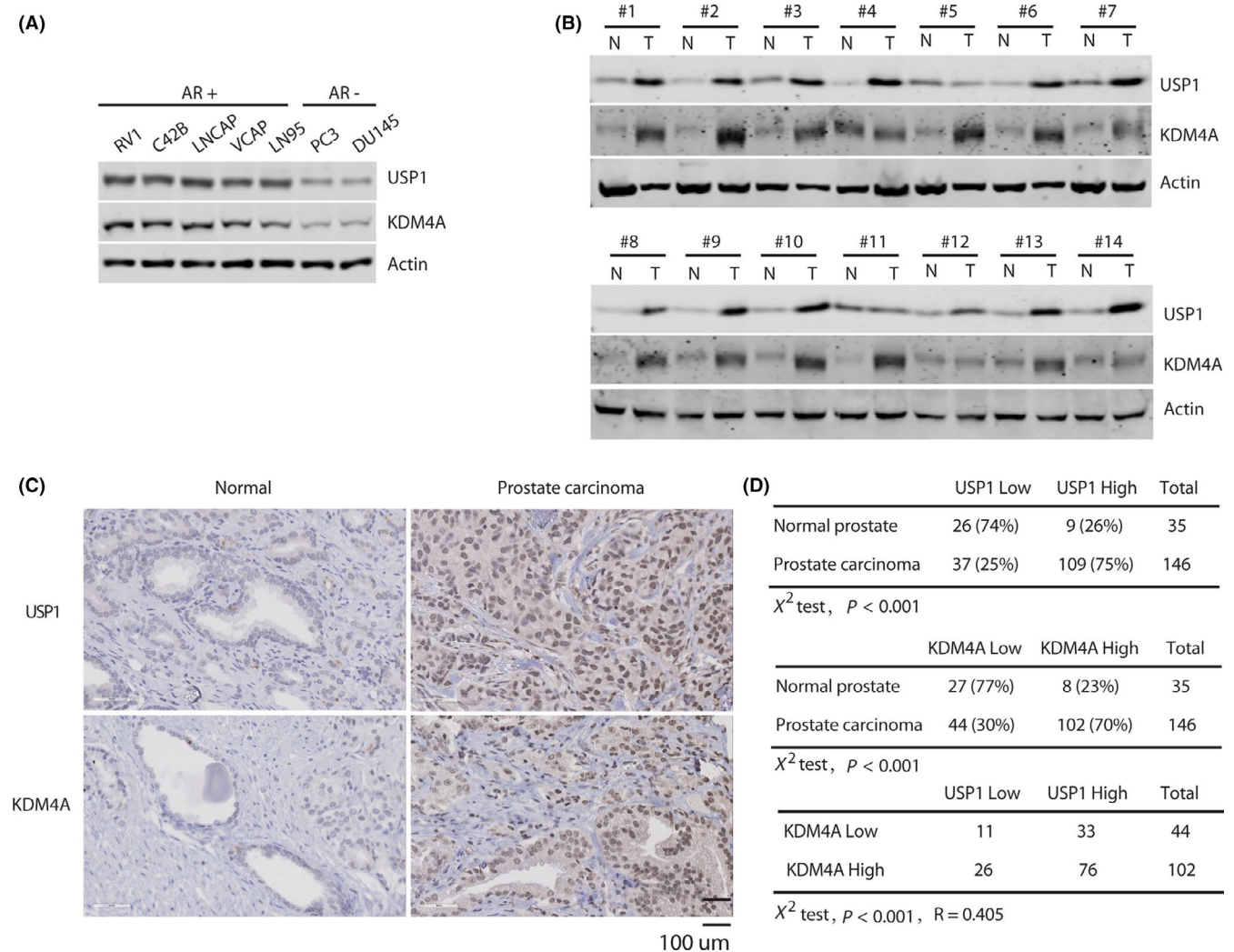


FIGURE 6 KDM4A expression positively correlates with USP1 expression in clinical prostate cancer (PC) samples. A, Expression of USP1 and KDM4A in androgen receptor (AR)-positive (RV1, C42B, LNCAP, VCAP, and LN95) and AR-negative cells (PC3 and DU145) was analyzed by western blot. B, A subset of prostate tumor (T) and normal tissues (N) were subjected to western blotting, to examine USP1 and KDM4A protein levels. C, Representative staining of USP1 and KDM4A in PC and normal prostate tissues. D, Quantification of USP1 and KDM4A protein levels in normal and PC tissues, and the correlation study of USP1 and KDM4A expression level in prostate carcinoma. Statistical analyses were undertaken with the χ^2 test, $P < .001$. R , Pearson's correlation coefficient

FIGURE 7 Targeting USP1 promotes prostate cancer cells' response to therapeutic agent enzalutamide. A, RV1 (left panel) or LNCAP (right panel) cells were treated with the indicated concentrations of ML323 for 48 hours and analyzed by western blotting for the indicated proteins. B, RV1 or LNCAP cells were treated with 5 μ mol/L ML323 for 48 h and mRNA levels of the indicated genes were detected (mean \pm SEM of 3 independent experiments). * $P < .05$; ** $P < .01$. C, RV1 cells were seeded at low density on the plate, and maintained for 2 wk. Numbers of colonies were counted in 5 high-power fields (mean \pm SEM of 3 independent experiments). ** $P < .01$. D, RV1 cells were stably expressed with or without Flag-KDM4A constructs and treated with either vehicle or USP1 inhibitor ML323 for 24 h. Cells were subjected to western blot to examine the indicated protein levels. E, Cells as described in (D) were treated with the indicated concentrations of enzalutamide and cell survival was determined (mean \pm SD [$n = 3$]). F, RV1 cells stably expressing control (Ctrl) or USP1 shRNA with or without Flag-KDM4A were subjected to western blotting to detect the indicated protein levels. G, Cells as described in (F) were treated with the indicated concentrations of enzalutamide and cell survival was determined (mean \pm SD [$n = 3$]). H, Schematic representation of how USP1 regulates androgen receptor (AR) and c-Myc activities through KDM4A

posttranslational regulation, eg downregulation of microRNA-10 was correlated with high expression levels of KDM4A in human prostate tumors.²⁹

3.7 | Targeting USP1 promotes PC cell response to therapeutic agent enzalutamide

Our above findings suggest that targeting the USP1-KDM4A axis could antagonize the growth of PC cells. Although specific KDM4A inhibitors are not available, a special selective USP1 inhibitor, ML323, has been developed.²⁵ We asked whether ML323 could be used to target the KDM4A stability. We found that ML323 treatment of 2 AR-positive cell lines (RV1 and LNCAP) and 1 AR-negative cell line (PC3) led to the downregulation of KDM4A protein levels in a dose-dependent manner and the reduced KDM4A levels were accompanied by reduced levels of the KDM4A downstream target c-Myc (Figures 7A and S5A). Moreover, qPCR data analysis showed that treatment of RV1 or LNCAP cells with 5 μ M ML323 reduced c-Myc mRNA levels, but not others (Figures 7B and S5B); c-Myc mRNA levels were not decreased in AR-negative PC3 cells (Figure S5B). These results indicated that USP1 inhibitor ML323 can destabilize KDM4A protein. To test the effect of ML323 on PC cell growth, we treated RV1 and LNCAP cells with different doses of ML323 and undertook colony formation assays. As shown in Figures 7C and S5C, inhibition of USP1 led to decreased colony formation.

KDM4A regulates AR activity as an AR coactivator⁶ and the second new-generation AR pathway inhibitor enzalutamide extends patient life by only a few months.^{30,31} Thus, we tested whether inhibition of USP1 by its pharmacological inhibitor or specific shRNA affects cell response to enzalutamide. RV1 and LNCAP cells were treated with vehicle or 5 μ mol/L USP1 inhibitor and then responses to enzalutamide were subsequently measured. We observed that the USP1 inhibitor promoted KDM4A degradation (Figure 7D). ML323 sensitized cells to enzalutamide treatment, whereas overexpression of KDM4A in the cells was significantly resistant to enzalutamide treatment even in the presence of ML323 (Figures 7E and S5D). Similarly, USP1 knockdown in RV1 cells using 2 specific shRNAs significantly decreased KDM4A protein levels (Figure 7F) and increased cellular sensitivity to enzalutamide treatment, whereas overexpression of KDM4A in depleted cells with USP1 reversed hypersensitivity to enzalutamide treatment (Figures 7G and S5E). These results

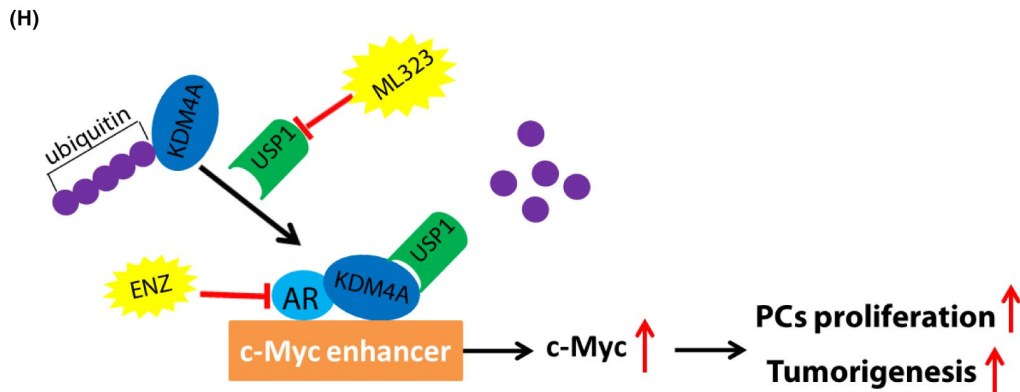
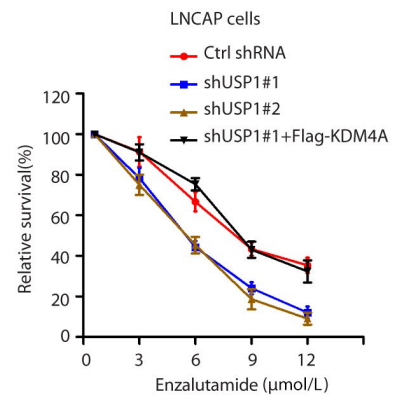
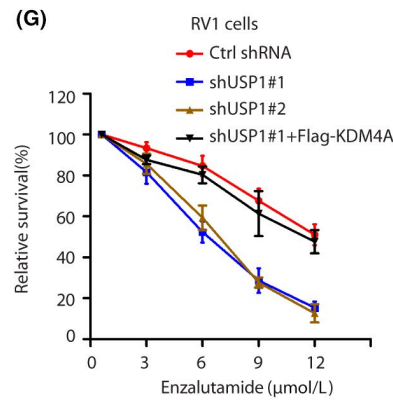
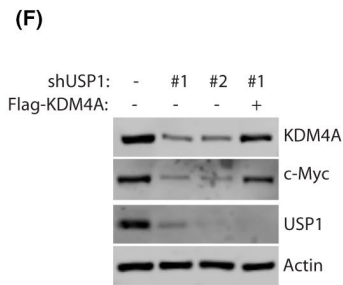
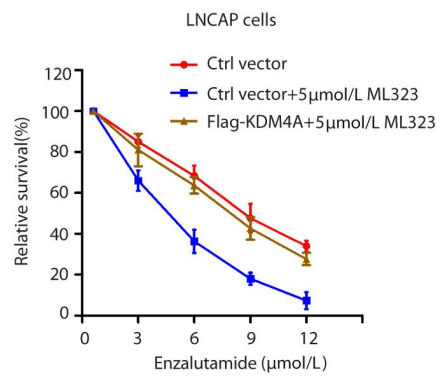
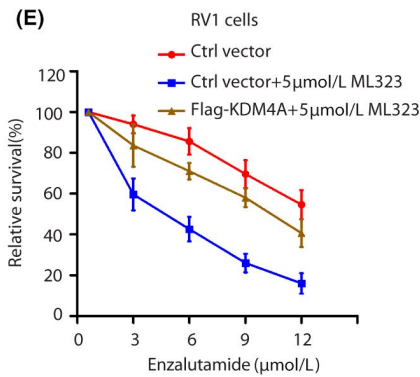
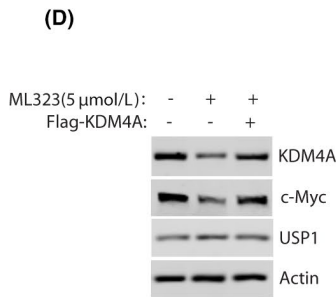
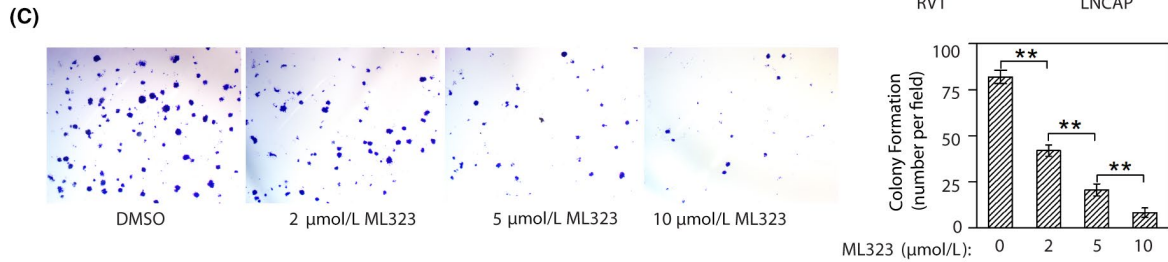
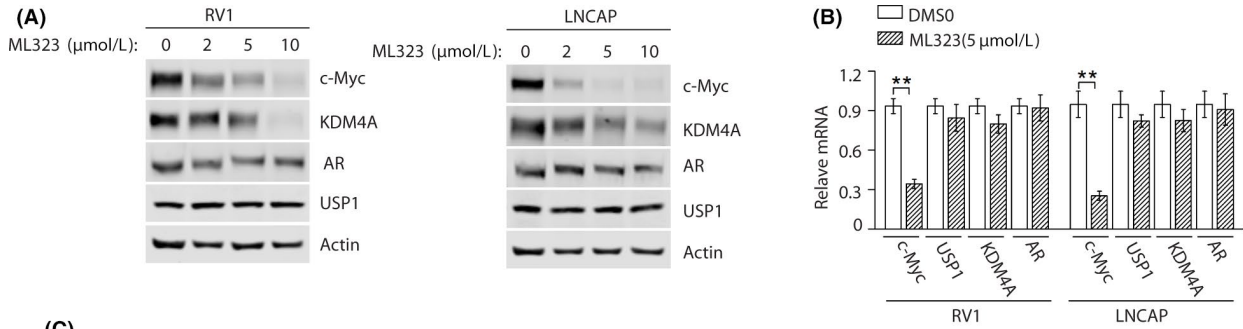
reveal that targeting USP1 promotes PC cell response to therapeutic agent enzalutamide. In future, the combination of USP1 inhibitor and enzalutamide should be tested in vivo. Collectively, we showed that USP1 interacts with KDM4A and promotes its deubiquitination, ultimately leading to KDM4A stabilization, and inhibition of USP1 promotes the response of PC cells to therapeutic agent enzalutamide (Figure 7H).

4 | DISCUSSION

Our study found that KDM4A is required to support increased cellular proliferation and cell survival in PTEN prostate tissue. USP1 is a deubiquitinase that regulates KDM4A K48-linked deubiquitin and stability. In addition, we found that c-Myc serves as a key downstream effector of the USP1-KDM4A/AR axis in driving PC cell proliferation as well as survival. These results suggest that KDM4A might function as a potential prognostic marker and USP1 could be an anticancer therapeutic target in PC.

KDM4A is upregulated in various cancers including lung, breast, colon, and PC and its expression is vital for cell survival.^{10-11,32-36} Here, we found that KDM4A is overexpressed in PTEN KO mouse prostate tissue and human prostate tumors, which supports that KDM4A drives PC progression. Moreover, we showed that KDM4A knockdown decreased cell proliferation in vitro and in vivo. These results provide supportive evidence for KDM4A as a potential therapeutic target in PC. KDM4A reportedly serves as an AR coactivator and regulates gene expression through H3K9 and H3K36 demethylation.⁶⁻⁸ Consistent with previous findings that KDM4A is an AR coactivator and AR upregulates c-Myc mRNA independent of its ligand in PC cells, we found that KDM4A can recruit AR to the c-Myc enhancer and demethylate H3K9me2 at that locus, whereas androgen is not required for the KDM4A-dependent binding of AR to c-Myc enhancer.

Whether the KDM4A/AR-c-Myc pathway can be regulated in PC remains an unanswered question. Identifying new upstream effectors of this pathway could contribute to improved understanding of the molecular events involved in PC progression and clarify new potential therapeutic targets in PC. Herein, by performing tandem affinity purification, we identified that the KDM4A binding protein, USP1, functions as a deubiquitinase and stabilizes KDM4A, which in turn facilitates AR recruitment to the c-Myc enhancer.



Thus, our study identified a new player in the AR signaling pathway. Furthermore, we found that USP1 knockdown reduced tumorigenesis and promoted PC cell response to therapeutic agent enzalutamide in a KDM4A-dependent manner. The supportive evidence is as follows: (i) USP1 knockdown decreased PC cell proliferation in vitro and tumorigenesis in vivo, and promoted the response to therapeutic agent enzalutamide; and (ii) inhibition of USP1 sensitized cancer cells to therapeutic agent enzalutamide, whereas this effect was blunted by overexpression of KDM4A. Collectively, our study indicates that USP1 is a new upstream effector of the KDM4A/AR-c-Myc pathway in PC. To our interest, USP1 reportedly destroys K63-linked polyubiquitin chains on histones and then promotes to recruit 53BP1 at DNA damage sites; another previous report found that KDM4A regulates DNA repair by controlling the recruitment of 53BP1 at DNA damage sites and RNF8-dependent degradation of KDM4A regulates DNA repair by controlling the recruitment of 53BP1 at DNA damage sites.^{37,38} Here, we found USP1 promoted K48-linked deubiquitination of KDM4A and stability in PC cells. These findings suggest that USP1 might promote K48-linked deubiquitination of KDM4A, which abrogate the E3 ubiquitin ligases RNF8 ubiquitin KDM4A following DNA damage. Further study will be needed to understand how RNF8 and USP1 coordinate KDM4A expression, which could explain why the activity of KDM4A is tightly regulated under physiological conditions. In addition, in our study, USP1 directly binds and stabilizes KDM4A but does not affect its transcription, suggesting deubiquitination is the major regulation of KDM4A by USP1. We then propose that KDM4A is upregulated in PC at the posttranscriptional level. Upregulation of USP1 could be a potential mechanism. In fact, we observed that high expression of KDM4A was positively correlated with high USP1 expression in most human prostate tumors (Figure 6D). This evidence strongly supports our hypothesis and clarifies a physiological relationship between KDM4A and USP1 in PC.

Androgen receptor normally activated by androgens is vital for PC cells.³⁹ Blockade of the AR signal pathway was shown to be an effective PC therapeutic strategy.^{30,40} It was recently reported that enzalutamide, one of the most effective AR-directed therapies, could only increase overall survival by approximately 2.5-5 months and the survival benefits were achieved in only approximately 50% of PC patients treated.^{30,31} In addition, many patients eventually develop enzalutamide resistance, which results in shorter survival.^{41,42} To our excitement, several studies have reported that the deubiquitinating enzyme USP1 inhibitor ML323 could specially inhibit the deubiquitinating activity of USP1^{25,43,44} and we further showed that the USP1 inhibitor ML323 inhibits the USP1-KDM4A/AR-c-Myc pathway, therefore decreasing PC cell survival. Notably, we found that combined treatment with enzalutamide and USP1 inhibitor achieved further improvement in PC cell response. In future, we will test whether inhibition of USP1 in enzalutamide-resistant cells could promote the PC cell response to enzalutamide. Although c-Myc has been considered an undruggable target, potential c-Myc small molecule inhibitors were recently developed.⁴⁵ Our findings suggest it would be better to test the possibility of c-Myc inhibitor and USP1 inhibitor for the treatment of PC.

In summary, this study identifies USP1 as a critical deubiquitinase for stabilizing KDM4A, thereby promoting PC growth and tumorigenesis. Anticancer effects of USP1 inhibition could be partially reversed by overexpression of KDM4A in vitro and in vivo. Interestingly, USP1 was positively correlated with KDM4A protein expression in prostate human tumors. Combination treatment of enzalutamide and targeting KDM4A stabilization through pharmacological inhibition of USP1 by ML323 could thus open an avenue for therapeutic intervention in PC patients.

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DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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