JARID1B is a histone H3 lysine 4 demethylase up-regulated in prostate cancer

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Histone methylation is a dynamic process that participates in a diverse array of cellular processes and has been found to associate with cancer. Recently, several histone demethylases have been identified that catalyze the removal of methylation from histone H3 lysine residues. Through bioinformatic and biochemical analysis, we identified JARID1B as a H3K4 demethylase. Overexpression of JARID1B resulted in loss of tri-, di-, and monomethyl H3K4 but did not affect other histone lysine methylations. *In vitro* biochemical experiments demonstrated that JARID1B directly catalyzes the demethylation. The enzymatic activity requires the JmjC domain and uses Fe(II) and α-ketoglutarate as cofactors. Furthermore, we found that JARID1B is up-regulated in prostate cancer tissues, compared with benign prostate samples. We also demonstrated that JARID1B associates with androgen receptor and regulates its transcriptional activity. Thus, we identified JARID1B as a demethylase capable of removing three methyl groups from histone H3 lysine 4 and up-regulated in prostate cancer.

demethylation | methylation | transcription | JmjC domain

Histone methylation plays an important role in regulating chromatin dynamics and transcription (1). Methylation can occur on either arginine or lysine residues (2). Each lysine can undergo three distinct stages of methylation, having either one (mono), two (di), or three (tri) methyl groups covalently bonded to the amine group of the lysine side chain, and arginine can be mono- or dimethylated (3). Depending on specific residues, methylation can either activate or repress transcription. In general, lysine methylation at H3K9, H3K27, and H4K20 is associated with transcriptional repression, whereas methylation at H3K4, H3K36, and H3K79 is associated with transcriptional activation. However, recent findings have blurred this generality. For example, methylation at H3K9 can result in transcriptional activation, and methylation at H3K36 can repress transcription (4, 5).

Methylation had long been considered a stable modification, but recent studies have proved otherwise (6–16). The first histone demethylase identified is LSD1, which can remove di- and monomethylation from H3K4 by using an amine oxidase reaction (8). Subsequently, a JmjC domain-containing protein was identified to possess histone demethylase activity, and the JmjC domain was shown as a demethylase signature motif (9). This class of enzymes catalyzes the removal of methylation by using a hydroxylation reaction and required iron and α-ketoglutarate as cofactors. Based on this demethylase signature motif, several proteins were identified to be histone lysine demethylases (6, 7, 10–16).

Prostate cancer is the most common nonskin cancer and the second leading cause of cancer in America. Histone methylation has been suggested to be associated with prostate cancer. For example, it was demonstrated that histone methylations and acetylations can be used to predict the risk of prostate cancer recurrence (17). In addition, EZH2, a H3K27 methyltransferase, is shown to be involved in progression of prostate cancer (18). Furthermore, two of the identified histone demethylases, LSD1 and JHDM2A, were shown to promote androgen receptor (AR)-dependent transcription through removal of H3K9 methylation (11, 19). AR, a member of the nuclear receptor superfamily, plays an important role in prostate cancer development and progression (20).

To identify histone demethylases, we undertook a candidate approach using the JmjC domain as the demethylase signature motif. Toward this end, we have identified JARID1B, also named Plu-1, as a histone demethylase that can catalyze the removal of all three methyl groups from the H3K4 lysine residue. We also demonstrated that JARID1B is up-regulated in prostate cancer and regulates the AR transcriptional function.

Results

Classification of the JmjC Domain-Containing Proteins from Human by Bioinformatic Analysis. To identify histone demethylases, we undertook a bioinformatic approach to analyze human genes containing the JmjC domain because the JmjC domain has been identified as the catalytic domain for histone lysine demethylases (9, 11). Thirty genes from PubMed were obtained, and they were classified into seven major groups (Fig. 1). Excluding the proteins that had been identified to have histone demethylase activity (JHDM1 of group 2, JHDM2 of group 3, and JMJD2 of group 6) [Fig. 1 and supporting information (SI) Table 1], we hypothesized that JARID1 proteins in group 5 are candidates for histone lysine demethylases with different specificity than the identified enzymes. JARID1 is a family of four members (SI Fig. 6), and we were particularly interested in JARID1B because this protein was shown to be up-regulated in breast cancer (21).

Effect of JARID1B on Histone Lysine Methylation *in Vivo*. To test our hypothesis, cDNA encoding for Myc-tagged JARID1B was transfected into HeLa cells, and the effect on histone lysine methylation was examined by immunocytochemistry using an antibody against H3K4me3. Ectopic expression of Myc-JARID1B resulted in loss of H3K4me3 in contrast to strong stained signals of H3K4me3 in adjacent nontransfected cells (Fig. 2*A Top*). However, overexpression of JARID1B did not affect levels of H3K9me3 (Fig. 2*A Middle*), and H3K36me3 (Fig. 2*A Bottom*). These data indicate that JARID1B is probably a specific H3K4me3 demethylase.

To examine whether JARID1B is able to remove di- and monomethyl groups from H3K4, we also immunostained Myc-JARID1B-transfected cells with antibodies against these histone modifications. Overexpression of JARID1B led to reduction of H3K4me2 (Fig. 2*B Top*) and H3K4me1 (Fig. 2*C Top*) in contrast

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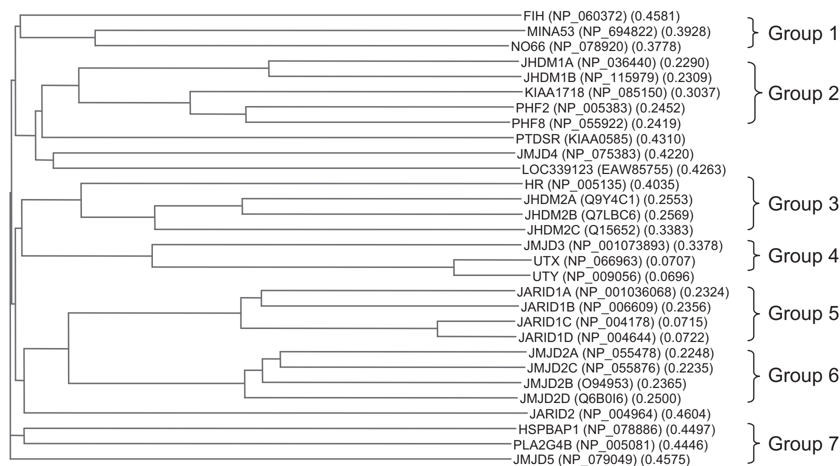

Fig. 1. Alignment of the JmjC domain-containing proteins in human. JmjC domain-containing proteins in human were searched in the PubMed database, and 30 of them were obtained. They were analyzed by the Vector NTI program applying a ClustalW algorithm.

to strong stained signals of H3K4me2 and H3K4me1 in nontransfected cells. The demethylase activities against both di- and monomethyl H3K4 are specific because overexpression of JARID1B had no effect on levels of H3K9me2 (Fig. 2*B Middle*), H3K27me2 (Fig. 2*B Bottom*), and H3K9me1 (Fig. 2*C Middle*), and H3K36me1 (Fig. 2*C Bottom*). Together with the results in Fig. 2*A*, these data indicate that JARID1B is probably a specific demethylase capable of removing tri-, di-, and monomethyl groups from H3K4.

The ability of JARID1B to demethylate tri-, di-, and monomethyl H3K4 was surprising because all identified histone demethylases can remove only one or two methyl groups from a specific lysine residue (SI Table 1). To make sure that the antibodies we used are specific, we performed a dot blot analysis using synthetic peptides with different methylation status. Indeed, each antibody specifically recognized its respective peptide (SI Fig. 7). We also performed immunocytochemistry using a different batch of antibodies and obtained similar results (data not shown), indicating that the antibodies are specific.

Domain Requirement for Enzymatic Activity. JARID1B is a protein of 1,544 aa and consists of several domains implicated in a diverse array of activities in other proteins (Fig. 3*A*). Crystal structure and biochemical study of JMJD2A revealed that JmjN forms an extensive interaction with the catalytic core (7, 22). The JmjC domain contains the catalytic pocket for demethylation (22). The PHD domain binds H3K4me3 with a high affinity (23–26). The Bright/Arid domain and zinc-finger-like domain are for protein–DNA and protein–protein interactions. To determine which domains are required for demethylase activity, we first examined whether the N-terminal fragment (ΔC) containing JmjN, Arid, PHD1, JmjC, and zinc-finger-like domains is sufficient for enzymatic activity. Overexpression of this fragment results in reduction of tri-, di-, and monomethyl H3K4 (Fig. 3 *B* and *C*) but had no effect on levels of H3K27 and H3K36 methylation (SI Fig. 8), indicating that the specific enzymatic activity is located at the N terminus of the protein.

To further locate which domains are required for demethylase activity, we generated mutants of JARID1B with deletions of predicted functional domains and examined their activity *in vivo*. Deletion of the PHD domain in the N terminus did not affect demethylase activity (Fig. 3 *D* and *C*). However, deletion of JmjN, Arid, JmjC, or zinc-finger-like domain abolished enzymatic activity (Fig. 3*D* and SI Fig. 9). These data indicate that the enzymatic activity of JARID1B not only requires JmjN and JmjC, similar to other identified JmjC domain-containing dem-

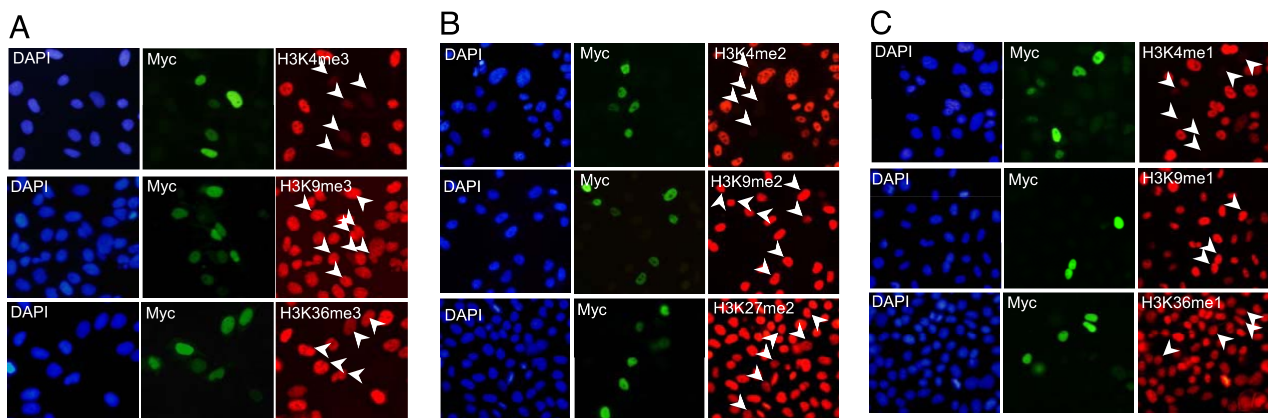

Fig. 2. JARID1B removed H3K4 methylation *in vivo*. HeLa cells transfected with Myc-JARID1B were immunostained with specific antibodies against distinctly methylated lysine residues. (*A–C Left*) DAPI staining. (*A–C Center*) Myc staining. (*A–C Right*) Methylated lysine staining. (*A Top*) H3K4me3. (*A Middle*) H3K9me3. (*A Bottom*) H3K36me3. (*B Top*) H3K4me2. (*B Middle*) H3K9me2. (*B Bottom*) H3K27me2. (*C Top*) H3K4me1. (*C Middle*) H3K9me1. (*C Bottom*) H3K36me1. Arrowheads indicate Myc-JARID1B-expressed cells.

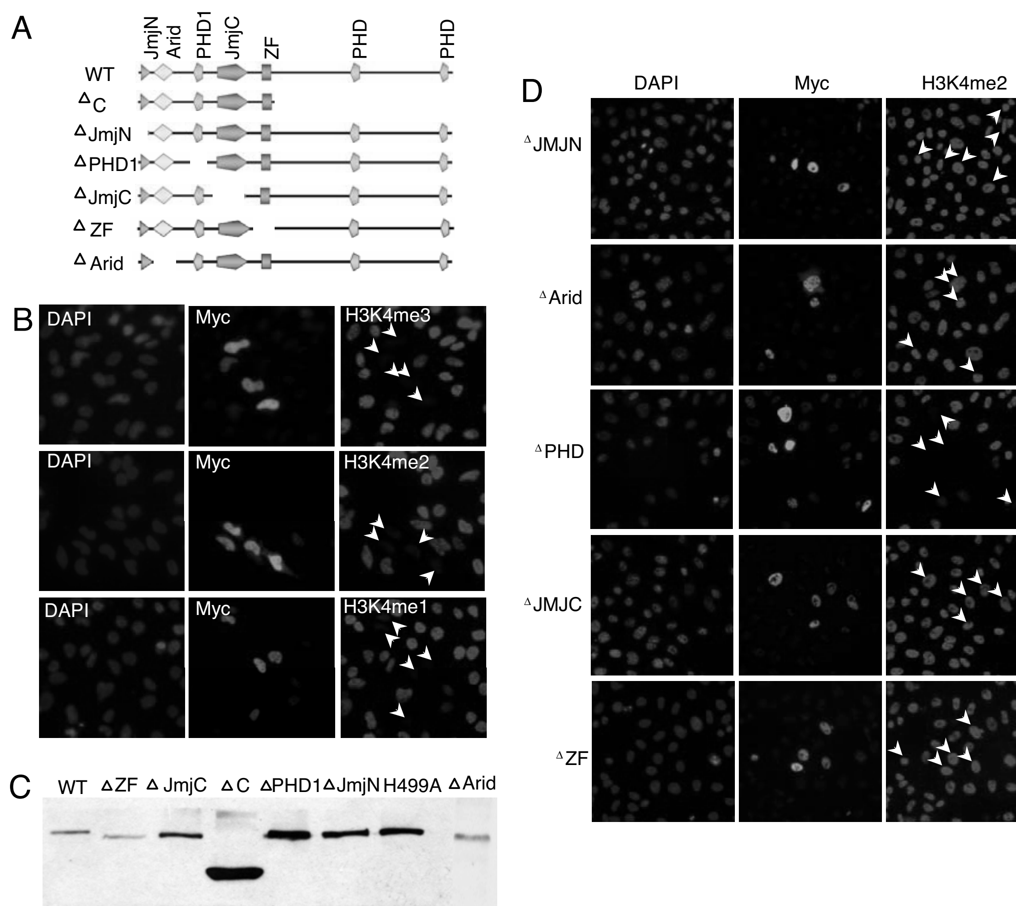

Fig. 3. JmjN, Arid, JmjC, and zinc-finger like domains are required for demethylase activity *in vivo*. (*A*) Schematic representation of the mutants with deletions of predicted functional domains. (*B* and *D*) HeLa cells transfected with the C-terminal truncated ΔC fragment (*B*) or each individual domain-deleted mutant (*D*) were immunostained with antibodies against specific methylated H3K4. (*Left*) DAPI staining. (*Center*) Myc staining. (*Right*) Methylated H3K4 staining. (*B Top*) H3K4me3. (*B Middle*) H3K4me2. (*B Bottom*) H3K4me1. (*D Right*) H3K4me2). Arrowheads indicate cells expressing deletion mutants. (*C*) Western blot analysis of the expression of different mutants.

ethylases (22), but also is regulated by other domains such as Arid and zinc-finger-like domains.

JARID1B Is an Iron- and α-Ketoglutarate-Dependent Dioxygenase. To determine the chemical mechanism for enzymatic activity, we carried out *in vitro* biochemical experiments using the ΔC fragment because this fragment maintained the specific enzymatic activity of the enzyme (Fig. 3*B* and SI Fig. 8). The purified ΔC fragment (SI Fig. 10) was incubated with histones, and the effect on lysine methylation was determined by Western blot analysis. In the presence of various cofactors, the ΔC fragment was able to reduce the levels of tri-, di-, and monomethyl H3K4 but had no effect on trimethyl H3K9 (Fig. 4*A*), indicating that JARID1B directly catalyzes demethylation, and the ΔC fragment maintains the specific enzymatic activity. To test whether JARID1B is a dioxygenase belonging to the cupin superfamily, the catalytic reaction was carried out in the absence of a cofactor each time. The enzymatic activity was significantly diminished in the absence of ascorbic acid, α-ketoglutarate, or Fe(II) or in the presence of the iron chelating agent EDTA (Fig. 4 *B* and *C*). When *N*-oxalylglycine, an α-ketoglutarate analogue that presumably replaces α-ketoglutarate from the enzyme, was added to the reaction, the demethylase activity was inhibited and the effect of *N*-oxalylglycine was dose-dependent (Fig. 4 *D* and *E*). These data suggest that JARID1B is an iron- and α-ketoglutarate-dependent dioxygenase.

The crystal structure of the JmjC domain of JMJD2A indicates that the catalytic domain is a jellyroll-like structure with eight β-sheets (22). Three residues for chelating iron are conserved and required for demethylase activity. In JARID1B, these three residues are His-499, Glu-501, and His-587. To determine whether this structure is required for enzymatic activity, we mutated His-499 to Alanine (H499A). Transfection of H499A resulted in loss of enzymatic activity, because the levels of H3K4me3, H3K4me2, and H3K4me1 did not change in the transfected and nontransfected cells (Fig. 4*F*). These data further confirm that JARID1B is an iron-dependent dioxygenase.

JARID1B Is Up-Regulated in Prostate Cancer and Regulates Androgen Receptor Function. JARID1B, also known as PLU-1, was shown to have highly restricted expression in normal adult tissue but is up-regulated in breast cancer (21). Because we are interested in prostate cancer, we wished to examine whether JARID1B is also up-regulated in prostate cancer. To test this, we searched the Oncomine database, a cancer-profiling database containing >10,000 microarrays (27). Analysis of one set of data (28) in Oncomine revealed that the expression of JARID1B is limited in benign prostate but is up-regulated in prostate cancer (Fig. 5*A*). Its expression is even higher in metastatic prostate cancer. We also examined the expression level of JARID1B in a limited number of frozen prostate tissues and detected high levels of JARID1B expression in prostate cancer tissue samples in con-

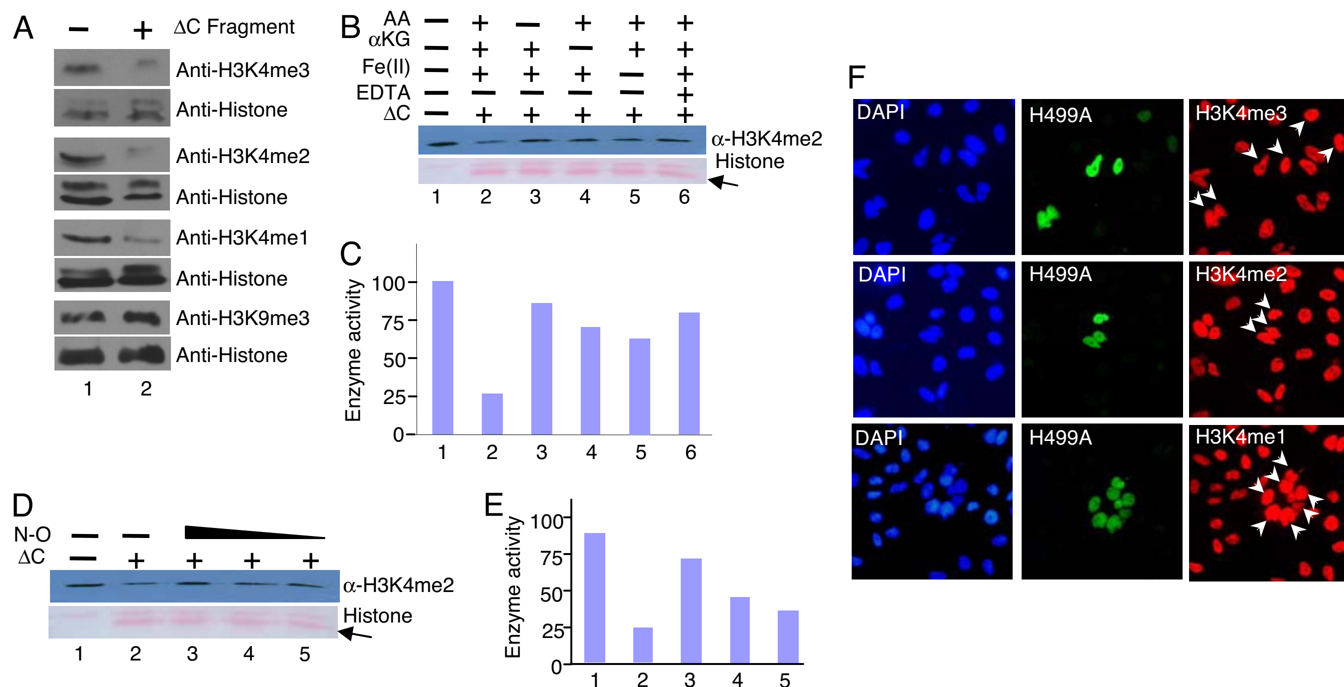

Fig. 4. JARID1B has demethylase activity *in vitro* and is a Fe(II)- and α-ketoglutarate-dependent dioxygenase. (*A*) Histones were reacted with (lane 2) or without (lane 1) the purified ΔC fragment in the presence of all cofactors, and Western blot analysis was performed by using antibodies against tri-, di-, and monomethyl H3K4 or H3K9me3. (*B*) Histones were reacted with (lanes 2–6) or without (lane 1) the purified ΔC fragment in the presence of all cofactors (lane 2), with the addition of EDTA (lane 6), or in the absence of each cofactor (lanes 3–5), and Western blot analysis was performed by using an antibody against H3K4me2. Ponceau S staining of histones was used as the loading control. The arrow indicates a nonspecific protein copurified with the ΔC fragment (lower band). (*C*) Quantification of the data in *B*. (*D*) Histones were reacted with (lanes 2–5) or without (lane 1) the purified ΔC fragment in the presence of all cofactors and decreasing amounts of *N*-Oxalylglycine (N-O; 10, 1, 0.1 mM, lanes 3–5), and Western blot analysis was performed by using an antibody against H3K4me2. Ponceau S staining of histones was used as the loading control. The arrow indicates a nonspecific protein copurified with the ΔC fragment (lower band). (*E*) Quantification of the data in *D*. (*F*) HeLa cells transfected with the H499A mutant were immunostained with antibodies against distinctly methylated H3K4. (*Left*) DAPI staining. (*Center*) Myc staining. (*Right*) Methylated H3K4 staining. (*Top*) H3K4me3. (*Middle*) H3K4me2. (*Bottom*) H3K4me1. Arrowheads indicate cells expressing H499A mutant.

trast to no detectable expression in tissues of benign prostate hyperplasia (Fig. 5*B*). This is consistent with a report that JARID1B was not detectable in normal prostate tissue (21).

Androgen receptor (AR) plays an important role in prostate cancer development and progression (20). To test whether JARID1B regulates AR transcriptional activity, we performed luciferase reporter assays. Indeed, transfection of JARID1B enhanced AR transcriptional activity, and the effect is dose-dependent (Fig. 5*C*). The enhancement of the AR transcriptional activity by JARID1B required enzymatic activity because deletion of the JmjC domain abolished the stimulation, as did H499A mutant (Fig. 5*E*).

To determine whether AR is associated with JARID1B, we carried out coimmunoprecipitation experiments. Precipitation of AR was able to pull down JARID1B when both proteins were coexpressed (Fig. 5*D*, lane 4). To determine whether the association is important for the potentiation of the AR transcriptional activity, we examined the effect of the C-terminal-deleted mutant (ΔC) on AR-dependent transcription. Potentiation was diminished when the C terminus was deleted (Fig. 5*E*). Because we did not observe interactions between AR and the C-terminal-deleted JARID1B (data not shown), and because the C-terminal domain is responsible for the interaction between JARID1A, a highly conserved homolog of JARID1B, and the nuclear receptors (29), we concluded that the association between AR and JARID1B is important for potentiation of AR transcriptional activity.

Discussion

Using the JmjC domain as the demethylase signature motif, we have identified JARID1B as a histone demethylase that can catalyze the removal of all three methyl groups from the H3K4 lysine residue. Together with recent identification of the other members of the JARID1 family as H3K4 demethylases (12–16), our finding greatly expands the family of histone lysine demethylases. Because LSD1 can also remove H3K4 methylations, these findings indicate that the removal of methylation at a specific lysine residue may be catalyzed by more than one histone demethyase. This is similar to the situation where several histone methyltransferases are involved in the addition of methylation to a single lysine residue. However, LSD1 removes di- and monomethylation, whereas members of the JARID1 family can remove tri-, di-, and monomethylation from H3K4. Our findings also support the concept that the JmjC domain-containing demethylases have broader substrate specificity than LSD1 and that more JmjC domain containing proteins may function as histone lysine demethylases.

The JmjC domain-containing demethylases can remove methylation from different histone lysine residues (JHDM1 demethylates H3K36, JHDM2 demethylates H3K9, JHDM3 demethylates H3K9 and H3K36, and JARID1 demethylates H3K4) or different states of methylation in the same lysine residue (tri-, di-, or monomethylation). How is the substrate specificity selected by different JmjC domain-containing demethylases? It is possible that the specificity is determined by the amino acid sequences within the JmjC domain. This possibility is supported by a crystal structure study indicating that substrate specificity is determined by the JmjC domain (30). Our structural and functional studies showing that deletion of the PHD domains did not change the substrate specificity (Fig. 3*B* and SI Fig. 8) also support the possibility.

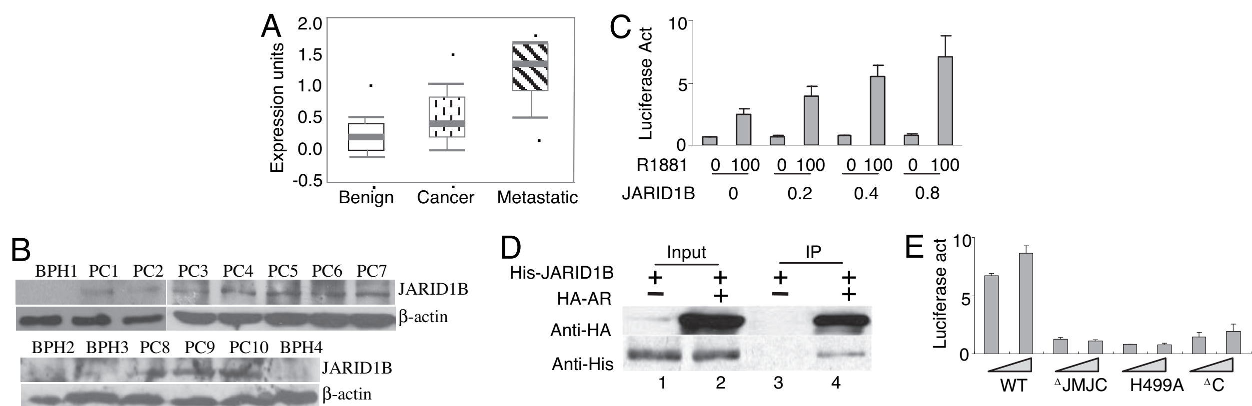

Fig. 5. JARID1B is up-regulated in prostate cancer and regulates the AR transcriptional activity. (*A*) Gene expression data in Oncomine was analyzed. The thick bars in the boxes are average expression levels and the boxes represent 95% of the samples. The error bars are above or below the boxes, and the range of expression levels is enclosed by two dots. The patient numbers for each category are: $n = 23$ for benign prostate, $n = 64$ for prostate carcinoma, and $n = 25$ for metastatic prostate. ($P = 1.8e\text{-}10$, and correlation = 0.561). (*B*) Western blot analysis of prostate tissue samples. BPH, benign prostate hyperplasia; PC, prostate cancer. (*C*) Escalated amounts of JARID1B were cotransfected into LNCaP cells with 100 ng of AR and 100 ng of PSA-luciferase in the presence or absence of 100 pM of R1881. Luciferase activity was measured 2 days later. (*D*) His-JARID1B was transfected into 293T cells with (lanes 2 and 4) or without (lanes 1 and 3) HA-AR, and immunoprecipitation was conducted with anti-HA antibody. Input (lanes 1 and 2) and the precipitate (lanes 3 and 4) were immunoblotted with anti-HA (*Upper*) and anti-His antibodies (*Lower*). (*E*) Escalated amounts (100 and 200 ng) of wild-type or mutant JARID1B were cotransfected into LNCaP cells with 100 ng of AR and 100 ng of PSA-luciferase in the presence of 100 pM of R1881. Luciferase activity was measured 2 days later.

Our structural and functional studies demonstrate that the enzymatic activity of JARID1B requires not only the JmjC domain but also the Arid domain and zinc-finger-like domain because deletion of each of these two domains abolished enzymatic activity (Fig. 3*D*). The JmjC domain is required because it is the catalytic center of the demethylases. Why are both the Arid domain and zinc-finger-like domain required for the demethylase activity? The Arid domain was shown to be a DNA-binding domain and the Arid domain of JARID1B bound DNA with little or no sequence specificity (31). JARID1B contains a C5HC2 zinc-finger-like domain, which may be involved in interaction with DNA or proteins as are other zinc-finger-containing proteins (32). It is conceivable that these domains may provide anchoring sites for the histone demethylase to dock onto the chromatin or to slide in the chromatin fiber. That the binding and catalytic domains locate in different regions of a protein is a common phenomenon. For example, a transcriptional factor can have a DNA-binding domain and an activation domain in different regions of the protein.

JARID1B is one of the four members of the JARID1 protein family. All four members of this family have recently been shown to possess H3K4 demethylase activity (12–16). Although functional redundancy may exist, each member may participate in different biological processes through spatial and temporal expression or through recruitment to different chromosomal regions. For example, JARID1A, also named RBP-2, was identified as a binding protein for the retinoblastoma gene product pRB and regulated cellular differentiation (33). JARID1A is ubiquitously expressed (34). However, JARID1B, also named Plu-1, has a restricted expression pattern with low levels of expression in all tissues other than the testis (21). JARID1B was shown to be up-regulated in breast cancer and probably involved in breast cancer development (15, 21). JARID1C, also named SMCX, was shown to be involved in X-linked mental retardation (35).

H3K4 methylation is a critical histone modification regulating transcription. High levels of H3K4me3 and H3K4me2 are associated with the 5′ regions of virtually all active genes and are in positive correlation with transcription rates, active polymerase II occupancy, and histone acetylation (36). This would predict that demethylases for H3K4me3 and H3K4me2 should have a repressive effect on transcription. Contrary to this, we discovered that the demethylase for H3K4 potentiates the transcription activity of the androgen receptor. This is consistent with a recent discovery that H3K4 trimethylation is associated with active gene repression (25) and suggests that H3K4 methylation may have a spatial and temporal effect on transcription. The context-dependent effect was also shown for JARID1A, which can function as a transcriptional activator or a repressor depending on the specific genes analyzed (29, 33). In addition, LSD1 can function in both gene-activation and -repression programs by recruiting distinct coactivator or corepressor complexes (37).

Although JARID1B has low levels of expression in almost all tissues examined, this protein was shown to be up-regulated in breast cancer (21). Furthermore, JARID1B was demonstrated to promote growth of breast cancer cells *in vitro* and *in vivo* (15). Through gene expression and Western blot analysis, we demonstrated that JARID1B is up-regulated in prostate cancer (Fig. 5 *A* and *B*). We also demonstrated that JARID1B regulated AR transcriptional activity (Fig. 5 *C* and *E*). It remains to be determine whether JARID1B is involved in prostate cancer and to understand the role of JARID1B in prostate cancer development and progression.

Experimental Procedures

Materials and Reagents. Antibodies for different histone methylations were purchased from Abcam and Upstate Biotechnology and secondary antibodies from Jackson ImmunoResearch and Molecular Probes (see *SI Text* for details). The chemicals are mainly from Sigma.

Plasmid Construction. Deletion and point mutations were generated by ligating PCR products into pcDNA3.1. Two-way or three-way ligations were used in the plasmid construction (see *SI Text* for details).

Transfection and Western Blotting. HeLa, LNCaP, and 293T cells were grown in Iscove's DMEM containing 10% FBS. Transfection was carried out by using Lipofectamine 2000 (Invitrogen). Tissues were obtained from the radical prostatectomy performed at Sixth Peoples Hospital of Shanghai with prior approval from the Institutional Review Board.

Immunocytochemistry. HeLa cells were plated on glass coverslips and transfected the next day. Seventy-two hours later, the cells

were fixed in 4% paraformaldehyde and permeabilized with cold PBS containing 0.2% Triton X-100. After blocking, the cells were incubated with primary antibodies, washed with secondary antibodies, stained with DAPI, and mounted before viewing.

Expression and Purification of the ΔC Fragment of JARID1B. pET28a (+)-ΔC-JARID1B in Rosetta *Escherichia coli* was cultured at 37°C at 250 rpm. Protein expression was induced by IPTG O/N at 16°C when the OD reached 0.5–0.6. Bacteria were collected, resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and sonicated 25 times at 200 W (sonicated for 2 s and paused for 15 s). Supernatant was loaded into equilibrated a Ni-NTA column and washed with buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), buffer 2 (50 mM NaH_2PO_4, 300 mM NaCl, 40 mM imidazole, pH 8.0), and buffer 3 (50 mM NaH_2PO_4, 300 mM NaCl, 60 mM imidazole, pH 8.0). The recombinant proteins were eluted by 250 mM imidazole.

Demethylation Assay. Bulk histones were incubated with the purified His-tagged ΔC fragment in demethylation buffer (20 mM Tris·HCl pH 7.3, 150 mM NaCl, 50 μM $(NH_4)_2Fe(SO_4)_2 + 6(H_2O)$, 1 mM α-ketoglutarate, and 2 mM ascorbic acid) for 3 h at 37°C. A total of 5–10 μg of the fragment and 2.5–5 μg of bulk histones were reacted in a total volume of 100 μl of reaction. The reaction was stopped with SDS loading buffer, and Western blot analysis was performed.

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