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TSA-Induced JMJD2B Downregulation Is Associated With Cyclin B1-Dependent Survivin Degradation and Apoptosis in LNCap Cells

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ABSTRACT

Histone deacetylase (HDAC) inhibitors are emerging as a novel class of anti-tumor agents and have manifested the ability to induce apoptosis of cancer cells, and a significant number of genes have been identified as potential effectors responsible for HDAC inhibitor-induced apoptosis. However, the mechanistic actions of these HDAC inhibitors in this process remain largely undefined. We here report that the treatment of LNCap prostate cancer cells with HDAC inhibitor trichostatin A (TSA) resulted in downregulation of the Jumonji domain-containing protein 2B (JMJD2B). We also found that the TSA-mediated decrease in survivin expression in LNCap cells was partly attributable to downregulation of JMJD2B expression. This effect was attributable to the promoted degradation of survivin protein through inhibition of Cyclin B1/Cdc2 complex-mediated survivin Thr34 phosphorylation. Consequently, knockdown of JMJD2B enhanced TSA-induced apoptosis by regulating the Cyclin B1-dependent survivin degradation to potentiate the apoptosis pathways. J. Cell. Biochem. 113: 2375–2382, 2012.

KEY WORDS: TSA; JMJD2B; APOPTOSIS; SURVIVIN; CYCLIN B1; PROSTATE CANCER CELL

Histone deacetylase (HDAC) inhibitors, emerging as a new class of anti-cancer agents, were shown to have anti-proliferation, pro-differentiating, as well as pro-apoptotic properties in cancer cells [Kramer et al., 2001; Johnstone, 2002; Richon and O'Brien, 2002], including prostate cancer cells [Gleave et al., 1998; Ellerhorst et al., 1999; Maier et al., 2000; Fronsdal and Saatcioglu, 2005]. The HDAC inhibitors include the classic trichostatin A (TSA), butyrate, sodium butyrate (NaBu), and newer synthetics with more favorable pharmacologic characteristics for therapeutic investigation. Trichostatin A (TSA), a streptomyces product, was originally identified as a fungicidic antibiotic [Tsuji et al., 1976]. It inhibits all class I and II HDACs, and has potent anti-proliferation properties in cancer cells [Yoshida et al., 1990; Furumai et al., 2001]. Differential display analysis revealed that the expression of only 2–5% of the genes in TSA-treated cells was significantly altered, some of which were involved in the inhibition of tumor growth. HDAC inhibitors not only activate gene expression, but also repress a significant number of genes [Van Lint et al., 1996; Kramer et al., 2001]. The basis for this gene selectivity is not yet understood.

The Jumonji proteins, which are characterized by the catalytic Jumonji C (JmjC) 2 domain, impact important processes such as hormone response, stem cell renewal, germ cell development, and cellular proliferation and differentiation. Interestingly, a range of Jumonji proteins are induced in different cancers and have been linked to cell proliferation and suppression of senescence [Whetstine et al., 2006; Yang et al., 2010]. JMJD2B (JmjC domain-containing histone demethylation protein 3A), encoded by the human gene JMJD2B (KIAA0876 gene, mapped to human chromosome 19p13.3), belongs to the JMJD2 family and contains one JmjC domain, one JmjN domain, two PHD-type zinc fingers and two Tudor domains

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[Katoh, 2004; Whetstine et al., 2006]. JMJD2B is highly expressed in ER-positive primary breast cancers and prostate cancer [Yang et al., 2010]. It participates in a variety of biological functions. JMJD2B facilitates transcription of ER responsive genes and functions as a co-factor of estrogen receptor; and knockdown of JMJD2B severely impairs estrogen-induced cell proliferation and the tumor formation capacity of breast cancer cells [Kawazu et al., 2011; Shi et al., 2011]. It has been previously reported that JMJD2B is a transcriptional target of hypoxia-inducible factor HIF [Beyer et al., 2008; Yang et al., 2010]. As a potential novel TP53 target gene, JMJD2B plays a role in mediating the 5-fluorouracil induced DNA damage response in TP53-depleted cancer cells [Adamsen et al., 2007]. Together, these earlier studies highlight that JMJD2B may play important roles in tumorigenesis, cellular growth, and apoptosis.

Based on these data, we hypothesized that the expression of JMJD2B might be associated with HDAC inhibitor-induced apoptosis in human prostate cancer cells. In this study, we showed that the HDAC inhibitors TSA and SAHA led to decreased JMJD2B protein expression in LNCap human prostate cancer cells. We further provided evidence that JMJD2B was functional in TSA-induced apoptosis by regulating the Cyclin B1-dependent degradation of survivin. Our data implicate that adjunctive therapies targeting JMJD2B may potentially sensitize cancer cells to HDAC inhibitors treatment.

MATERIALS AND METHODS

PLASMID, ANTIBODIES, AND REAGENTS

pCMV-HA-hJMJD2B was a gift from Dr. Peter Staller (Biotech Research and Innovation Centre, Copenhagen). Transfections were carried out using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) follow the manufacturer's instructions. Cycloheximide, HDAC inhibitor inhibitors TSA and SAHA were obtained from Sigma–Aldrich (St. Louis, MO) and Cayman Chemical (Ann Arbor, MI) respectively, MG-132 was purchased from Merck Biosciences (Bad Soden, Germany).

CELL LINES AND CULTURE CONDITIONS CELL CULTURE

The PC-3, LNCap, and DU145 cells were purchased from the Institute of Cell Biology (Shanghai, China). Cells were cultured in IMDM, 1640 and F12 medium, respectively, supplemented with 10% FBS, 100 mg/ml penicillin and 100 mg/ml streptomycin, and kept in a humidified atmosphere containing 5% CO_2 at 37°C.

RNA EXTRACTION AND RT-PCR

Total RNA was extracted from the cancer cells according to the Promega Total RNA Isolation System manual. RNA (1 μg per sample) was reverse transcribed to cDNA in a total volume of 20 μl using a reverse transcriptase reaction kit (Promega, Madison, WI). Quantitative real-time PCR reactions were performed with an ABI Prism 7,000 sequence detection system (Applied Biosystems, Foster City, CA) using a SYBR® Green Realtime PCR Master Mix (Toyobo, Osaka, Japan). The PCR primer sequences were as follows.

Survivin: 5′-TCAAGGACCACCGCATCTCTA-3′ (sense) and 5′-TGAAGCAGAAGAAACACTGGGC-3′ (antisense) [Wu et al., 2007]; β-actin: 5′-TCGTGCGTGACATTAAGGAG-3′ (sense) and 5′-ATGCCAGGGTACATGGTGGT-3′ (antisense) [Kramer et al., 2001]; Cdc2: 5′-GGGGATTCAGAAATTGATCA-3′ (sense) and 5′-TGTCAGAAAGCTACATCTTC-3′ (antisense) [Furukawa et al., 1996]; Cyclin B1: 5′-AAGAGCTTTAAACTTTGGTCTGGG-3′ (sense) and 5′-CTTTGTAAGTCCTTGATTTACCATG-3′ (antisense) [Sawa et al., 2001].

RNA INTERFERENCE (RNAi)

Human JMJD2B siRNA against KDM4B gene (JMJD2B siRNA1#, catalog no. SI00449785; JMJD2B siRNA2#, catalog no. SI03067421), and the negative control siRNA (Allstars control negative siRNA, catalog no. 1027281) were purchased from Qiagen (Hilden, Germany). RNAiFect transfection reagent (Qiagen) was used to deliver the siRNAs following the manufacturer's instructions. Twenty-four hours after transfection, the medium was replaced with the fresh medium containing 5% serum.

CELLULAR IMMUNOFLUORESCENCE

Cells were fixed in 1% formaldehyde in culture medium for 10 min at 37°C, and then permeabilized with 0.2% Triton X-100 in PBS for 10 min at 4°C. Endogenous JMJD2B was detected with rabbit anti-JMJD2B (Santa Cruz Biotechnology, Santa Cruz, CA, sc-67192) antibody, and visualized with an FITC-conjugated anti-rabbit IgG secondary antibody. DAPI (4′,6-diamidino-2-phenylindole) was used to stain the nucleus. Photographs were taken under a confocal fluorescence microscope.

CO-IMMUNOPRECIPITATION (CoIP) ASSAY

Cells were collected and lysed with the buffer (50 mM Tris–HCl; 150 mM NaCl; 1% Nonidet P-40; 1 mM EDTA, pH 7.5); and protease inhibitor cocktail (Roche) at 4°C for 30 min. After centrifugation, the supernatants were pre-cleared with salmon sperm DNA/Protein A-agarose beads (Upstate) for 1 h. The antibodies were added for immunoprecipitation at 4°C for 3 h. Protein G/A-Sepharose beads were then added to the mixture and were rotated overnight at 4°C. The samples were washed with Buffer A (20 mM Tris–HCl; 10 mM NaCl; 0.5% Nonidet P-40; 1 mM EDTA) and boiled in SDS-sample loading buffer and analyzed by Western blotting. Anti-Cyclin B1 (sc-595), anti-JMJD2B (sc-67192) were purchased from Santa Cruz Biotechnology. Anti-HA antibody (#2367) was purchased from Cell Signaling Technology (Danvers, MA). Anti-Cdc2 (BS1820) was purchased from Bioworld Technology, Inc. (St. Louis, MO).

CELL PROLIFERATION ASSAY

Cell proliferation was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay. Cells were seeded on 96-well plates at a density of 2×10^3 cells/well. After transfection or treatment, cells were incubated with 5 mg/ml MTT solution for 4 h. The medium was aspirated, and the formazan product was solubilized with 100 μl of DMSO. Viability was assessed by measuring the absorbance at 492 nm with a microplate reader.

STATISTICAL ANALYSIS

Immunoblotting and immunofluorescence data were from at least three independent experiments. Quantification of data was done based on averaging of at least three independent experiments and

the error bars denote the standard deviations. The Student's *t*-test was used to calculate the statistical significance of data. The significance level was set as $^{*}P < 0.05$ and $^{**}P < 0.01$.

RESULTS

HDAC INHIBITORS DOWNREGULATED THE EXPRESSION OF JMJD2B IN LNCap PROSTATE CANCER CELLS

We first determined the JMJD2B expression profile in different prostate cancer cell lines, and we found that LNCap cells expressed a higher level of JMJD2B in comparison with PC-3 and DU145 cells (Fig. S1A). We then explored whether JMJD2B was involved in the HDAC inhibitor-mediated growth arrest in LNCap cells. As can be seen from the Western blots in Figure 1A, treatment of LNCap cells with TSA resulted in downregulation of JMJD2B protein in a dose-dependent and time-dependent manner. Also, a reduced expression of JMJD2B was detected by immunofluorescence after treatment with 300 nM TSA (Fig. 1B). Meanwhile, real-time PCR verified that JMJD2B mRNA level was significantly downregulated by TSA (Fig. 1C). Similarly, SAHA, a clinically relevant HDAC inhibitor [Butler et al., 2000], had an equivalent effect on downregulating JMJD2B protein level in a dose-dependent manner in LNCap cells (Fig. 1D). Thus, these results support our assumption that suppression of JMJD2B expression in LNCap cells may participate in HDAC inhibitor-induced inhibition of cell proliferation.

THE TSA-MEDIATED DECREASE IN SURVIVIN EXPRESSION WAS JMJD2B-DEPENDENT IN LNCap CELLS

Survivin is a member of the inhibitor of apoptosis (IAP) gene family. Survivin has been implicated to play a role in drug resistance, and is downregulated by HDAC inhibitors [Zhang et al., 2008; Chowdhury et al., 2011; Hsu et al., 2011]. We detected that treatment of LNCap cells with TSA resulted in a decreased survivin protein expression, and this was accompanied by an increase in the level of cleaved caspase-3 (Fig. 2A). These data raised the possibility that TSA-mediated survivin decrease may be JMJD2B-dependent in LNCap cells. To test this assumption, we used the RNAi approach to knockdown the endogenous JMJD2B expression, and the suppression effect of specific JMJD2B siRNAs was confirmed by Western blotting (Fig. 2B *left*). As shown in Figure 2B *right*, knockdown of

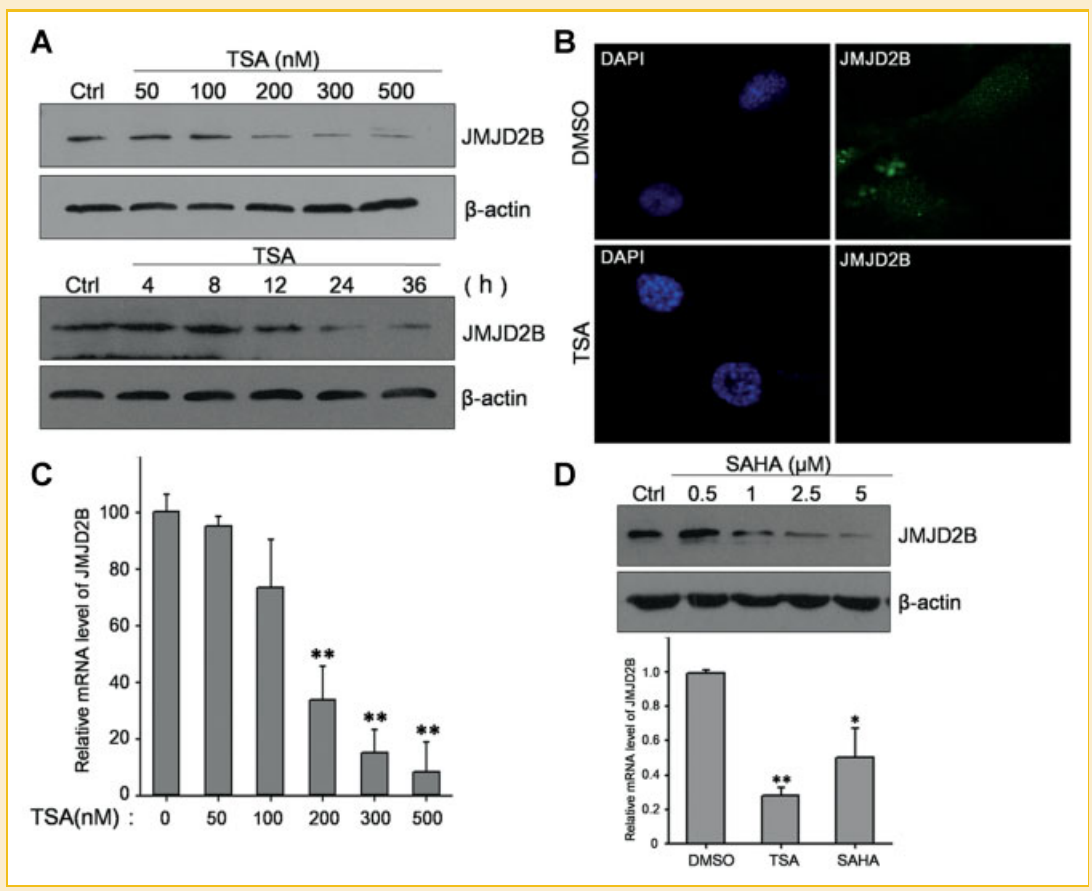

Fig. 1. Treatment of LNCap prostate cancer cells with HDAC inhibitors downregulated the JMJD2B expression. A: Effect of TSA on endogenous JMJD2B protein level. LNCap cells were treated with indicated concentrations of TSA for 48 h (top) or with 300 nM TSA for the indicated time (bottom), and subjected to Western blotting. B: Immunofluorescence detection of JMJD2B protein after TSA treatment. Cells were treated with TSA at 300 nM for 48 h. Immunofluorescence was performed with anti-JMJD2B antibody. Images were obtained under an Olympus confocal laser microscope. C: Effect of TSA on JMJD2B mRNA level. D: Effects of SAHA on JMJD2B mRNA and protein levels. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

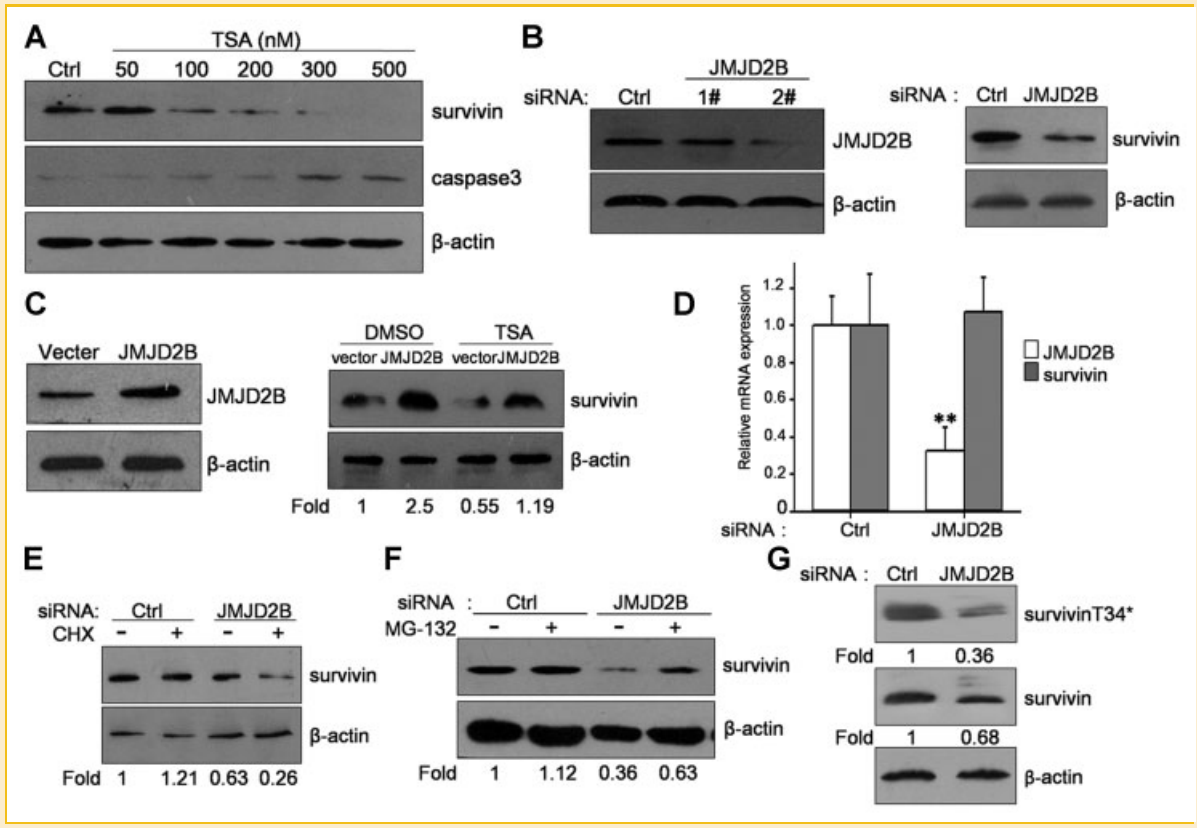

Fig. 2. TSA-mediated decrease in survivin expression was JMJD2B-dependent in LNCap cells. A: Effect of TSA on survivin protein level. B: LNCap cells were transfected with JMJD2B or control siRNA for 48 h. Western blotting confirmation of siRNA-mediated JMJD2B knockdown. JMJD2B siRNA 2# was used in this study. JMJD2B knockdown decreased survivin protein level. C: LNCap cells were transfected with empty vector or with the plasmid encoding human JMJD2B protein for 24 h, and cells were then treated with DMSO or 300 nM TSA. D: Knockdown of JMJD2B did not inhibit the survivin mRNA expression. E: LNCap cells were transfected with siRNA or siRNA plus 50 μg/ml Cycloheximide (CHX) for additional 3 h. Survivin and β-actin protein levels were assessed by Western blotting. F: MG-132 blocked JMJD2B siRNA-induced survivin downregulation. LNCap cells were transfected with siRNA with or without 5 μmol/L MG-132 treatment for additional 24 h. G: Knockdown of JMJD2B inhibited survivin Thr34 phosphorylation. The level of survivin phosphorylation on Thr34 was assessed by Western blotting with a Thr34 phospho-specific antibody (anti-survivin T34*).

JMJD2B by siRNA resulted in a significant decrease in survivin protein level. Alternatively, we ectopically overexpressed JMJD2B protein in LNCap cells by transfecting the JMJD2B expression plasmid (Fig. 2C *left*), and simultaneously treated the cells with 300 nM TSA to inhibit the survivin expression. As can be seen from Figure 2C *right*, overexpression of JMJD2B in LNCap cells treated with TSA did not result in reduction of survivin protein, whereas an increased level of survivin was induced by JMJD2B overexpression in cells without TSA treatment. This indicated that TSA-mediated decrease in survivin expression was dependent on JMJD2B.

We showed above that JMJD2B significantly affected the survivin protein level (Fig. 2B,C); however, it had little effect on survivin mRNA (Fig. 2D), suggesting that JMJD2B may be able to influence the stability of survivin protein in LNCap cells. To assess this effect, we treated LNCap cells with Cycloheximide to block protein synthesis, and the degradation of survivin was determined by Western blotting. As can be seen from Figure 2E, knockdown of JMJD2B promoted survivin degradation in LNCap cells. Next, we determined whether proteasome-dependent degradation was involved. After transfection with JMJD2B siRNA or control siRNA, the proteasome inhibitor MG-132 blocked the degradation of survivin protein in LNCap cells (Fig. 2F). Since the stability of survivin protein is maintained by phosphorylation at Thr34 of the protein, and Thr34 phosphorylation is important for its biological activity and function [O'Connor et al., 2000, 2002; Wall et al., 2003]. We then showed that survivin Thr34 phosphorylation was decreased after the suppression of JMJD2B, which in turn contributed to the reduction of survivin stability (Fig. 2G). Thus, knockdown of JMJD2B caused survivin degradation through a mechanism involving decreased survivin Thr34 phosphorylation.

KNOCKDOWN OF JMJD2B REDUCED THE STABILITY OF CYCLIN B1 PROTEIN

Because survivin can be phosphorylated at Thr34 by the Cyclin B1-Cdc2 complex, the above observations prompted us to explore the effect of JMJD2B downregulation on the production of Cyclin B1and Cdc2. We detected no significant difference in both protein and mRNA expression of Cdc2 upon knockdown of JMJD2B. In contrast, Cyclin B1 protein level, but not its mRNA level, was decreased after JMJD2B siRNA transfection (Fig. 3A), demonstrating that silencing of JMJD2B expression led to loss of Cyclin B1, probably by destabilizing the protein. Additionally, we discovered that proteasome inhibitor MG-132 reduced the rate of Cyclin B1 degradation upon JMJD2B knockdown (Fig. 3B), supporting the previous notion

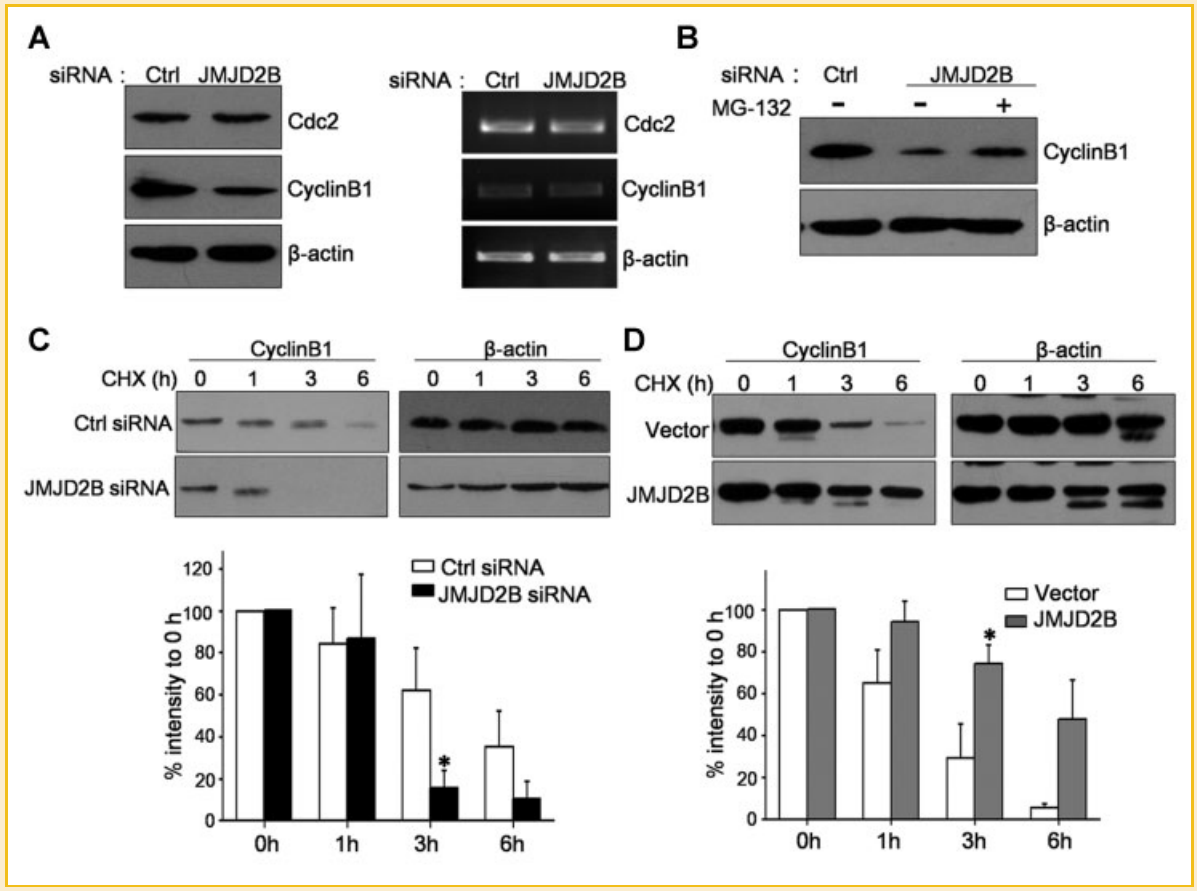

Fig. 3. JMJD2B affected the stability of Cyclin B1. A: Knockdown of JMJD2B decreased Cyclin B1 protein level, but not the Cyclin B1 mRNA level. LNCap cells were transfected with JMJD2B siRNA or control siRNA for 48 h. Cyclin B1, Cdc2 and β-actin protein levels were determined by Western blotting. The mRNA transcription levels were analyzed by RT-PCR. B: MG-132 blocked the JMJD2B-mediated Cyclin B1 downregulation. LNCap cells were treated under the same conditions as in Figure 2F. C: Knockdown of JMJD2B promoted Cyclin B1 degradation. LNCap cells transfected with siRNA were treated with Cycloheximide (CHX) for indicated time. Cyclin B1 protein level was examined by Western blotting and the band intensity was quantified by densitometry as ratio to time 0 h. D: JMJD2B blocked Cyclin B1 degradation. LNCap cells transiently transfected with pCMV-HA-hJMJD2B or control vector were treated with CHX.

that Cyclin B1 was degraded through the proteasome pathway. To further validate this finding, cells transfected with JMJD2B siRNA or control siRNA were treated with CHX to block protein synthesis. We showed that in the presence of CHX, degradation of Cyclin B1 occurred at a faster rate in cells transfected with JMJD2B siRNA in contrast to the cells transfected with the control siRNA (Fig. 3C). In LNCap cells transiently transfected with pCMV-HA-JMJD2B, overexpression of JMJD2B also reduced the rate of Cyclin B1 degradation (Fig. 3D). Statistical analysis of the quantitative data from three independent experiments was carried out and the results were displayed below the representative images in Figure 3C,D. These results indicated that the stability of Cyclin B1 was affected by the presence of JMJD2B.

JMJD2B WAS CO-IMMUNOPRECIPITATED WITH CYCLIN B1 AND KNOCKDOWN OF JMJD2B SUPPRESSED THE BINDING OF CYCLIN B1 TO Cdc2

We next studied if downregulation of JMJD2B affected the interaction between Cyclin B1 and Cdc2. Immunoprecipitation with an anti-Cyclin B1 antibody revealed a much fainter Cdc2 band in cells transfected with JMJD2B siRNA (Fig. 4A), suggesting that binding of Cyclin B1 to Cdc2 was inhibited by JMJD2B knockdown. Conversely, overexpression of JMJD2B increased the binding of Cyclin B1 to Cdc2 (Fig. 4B *upper right*). In addition, after immunoprecipitation with an anti-Cyclin B1 antibody, JMJD2B was detected, and an intensified band appeared in the JMJD2B overexpressing cells (Fig. 4B *upper right*), suggesting that JMJD2B formed a complex with Cyclin B1. Moreover, after immunoprecipitation with an anti-HA antibody, the exogenous JMJD2B-HA was co-immunoprecipitated with Cyclin B1 but not Cdc2 in the LNCap cells transfected pCMV-HA-JMJD2B (Fig. 4B *lower right*). Also, in LNCap cells JMJD2B was co-immunoprecipitated with Cyclin B1, further implicating an interaction between endogenous JMJD2B and Cyclin B1 (Fig. 4C *left*). However, no interaction between endogenous JMJD2B and Cdc2 was discerned (Fig. 4C *right*). These results indicate that JMJD2B knockdown may promote Cyclin B1 degradation, possibly through formation of a complex with Cyclin B1 to suppress the binding of Cyclin B1 to Cdc2, thereby inhibit the activity of Cyclin B1–Cdc2 kinase.

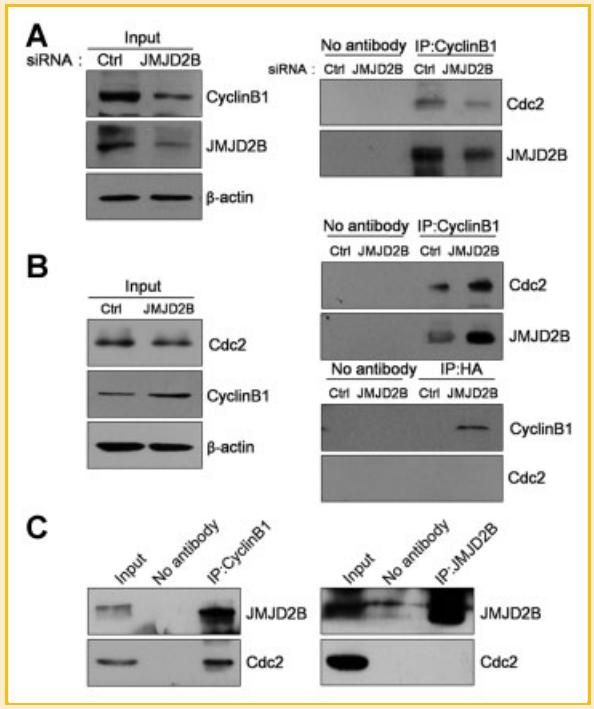

Fig. 4. JMJD2B was co-immunoprecipitated with Cyclin B1, and JMJD2B knockdown suppressed the binding of Cyclin B1 to Cdc2. A: JMJD2B knockdown suppressed the binding of Cyclin B1 to Cdc2. LNCap cells were transfected with JMJD2B siRNA or control siRNA, and immunoprecipitation was performed with an anti-Cyclin B1 antibody and immunoblotting was carried out with anti-Cdc2 antibody. B: JMJD2B was co-immunoprecipitated with Cyclin B1 and increased the binding of Cyclin B1 to Cdc2. LNCap cells were transfected with pCMV-HA-hJMJD2B or control vector. *Upper right*: immunoprecipitation was performed with an anti-Cyclin B1 antibody and immunoblotting was carried out with antibodies against Cdc2 and JMJD2B. *Lower right*: exogenous JMJD2B-HA was co-immunoprecipitated with Cyclin B1 but not with Cdc2. Immunoprecipitation was performed with an anti-HA antibody. C: The endogenous JMJD2B was co-immunoprecipitated with Cyclin B1 but not with Cdc2 in LNCap cells.

KNOCKDOWN OF JMJD2B POTENTIATED THE TSA-INDUCED APOPTOSIS IN LNCap CELLS

Our flow cytometric analysis revealed a sharp rise in apoptosis rate in LNCap cells in the presence of 300 nM TSA (Fig. 5A). Since we showed that HDAC inhibitors downregulated JMJD2B expression, and knockdown of JMJD2B caused degradation of survivin, we next tested whether knockdown of JMJD2B in TSA-treated LNCap cells has any effect on the apoptosis of the cells. As shown in Figure 5B, MTT assays revealed that JMJD2B knockdown exacerbated the reduction of cell viability caused by TSA treatment in LNCap cells. Flow cytometric analysis showed that, although knockdown of JMJD2B or 100 nM TSA alone did not significantly induce apoptosis, knockdown of JMJD2B plus TSA treatment increased the number of apoptotic cells (Fig. 5C). Furthermore, we showed in Figure 5D that the levels of cleaved caspase-3 and caspases-7 were very high in the cells treated with a combination of JMJD2B siRNA and TSA, whereas they were only weakly visible in cells treated 100 nM TSA or transfected JMJD2B siRNA alone. Therefore, the above data implicated a novel role for JMJD2B in TSA-induced apoptosis.

DISCUSSION

Conceptually, hyperacetylation of histones following inhibition of HDAC activity would be predicted to promote gene expression. However, treatment of cells with HDAC inhibitors like TSA does not lead to a general increase in gene expression, but changes the activity of only a limited number of genes [Van Lint et al., 1996; Kramer et al., 2001]. Genes with upregulated activity upon HDAC inhibitor treatment comprise the cell cycle inhibitors as well as the pro-apoptotic genes [Cameron et al., 1999; Kim et al., 2000]. In addition, a smaller number of genes have been shown to be downregulated by HDAC inhibitors; these include bcl-2, HIF-1, and VEGF [Eickhoff et al., 2000; Kim et al., 2001]. The causes and basis for such selectivity are not yet fully understood. We identified in this study that JMJD2B is a novel target gene that is downregulated by HDAC inhibitors TSA and SAHA in LNCap prostate cancer cells. To confirm that TSA can work as a HDAC inhibitor, we determined its action in acetylation of histone H3 using anti-acetylated H3 in Western blotting (Fig. S1A). TSA has been shown to exhibit significant anti-proliferative and apoptosis induction properties in various cancer cells [Kim et al., 2000; Wu et al., 2007; Hsu et al., 2011], include the prostate cancer cells [Fronsdal and Saatcioglu, 2005; Rokhlin et al., 2006]. The LNCap cells contain higher level of JMJD2B protein compared to PC-3 and DU145 cells (Fig. S1B). Moreover, we showed that TSA treatment resulted in down-regulation of JMJD2B at both mRNA and protein levels in a dose-dependent and time-dependent manner (Fig. 1). To our knowledge, this is the first report providing evidence that HDAC inhibitors downregulate the expression of JMJD2B in human cancer cells.

Survivin is highly expressed in most tumor cell types but is absent in normal cells, making it a potential target for cancer therapy. Our observation that treatment of LNCap cells with TSA resulted in a decrease in protein level of survivin, is consistent with the results from Zhang et al. [Wu et al., 2007; Zhang et al., 2008]. Meanwhile, we detected that TSA-mediated decrease in survivin level in LNCap cells was dependent on the suppression of JMJD2B; and this decrease was implemented via the proteasomal degradation (Fig. 2). Previous reports have shown that degradation of survivin is associated with a strong inhibition of Cyclin B1–Cdc2 complex-mediated phosphorylation of Thr34 in survivin protein. We investigated the effect of TSA on expression of Cyclin B1 and Cdc2, and the phosphorylation of survivin, and we found that they were downregulated in a dose-dependent manner during apoptosis induced by TSA (Fig. S1C). Our results also demonstrated that knockdown of JMJD2B was able to cause a decrease in survivin phosphorylation through degradation of Cyclin B1 (Fig. 3). Moreover, our results revealed that JMJD2B bound directly or indirectly to Cyclin B1 and promoted the binding of Cyclin B1 to Cdc2 (Fig. 4). Anaphase-promoting complex/cyclosome (APC/C) and its substrates are known to be associated with proteasomal destruction of Cyclin B1 [Aulia and Tang, 2006; Buschhorn and Peters, 2006]. We speculate that JMJD2B may interact with Cyclin

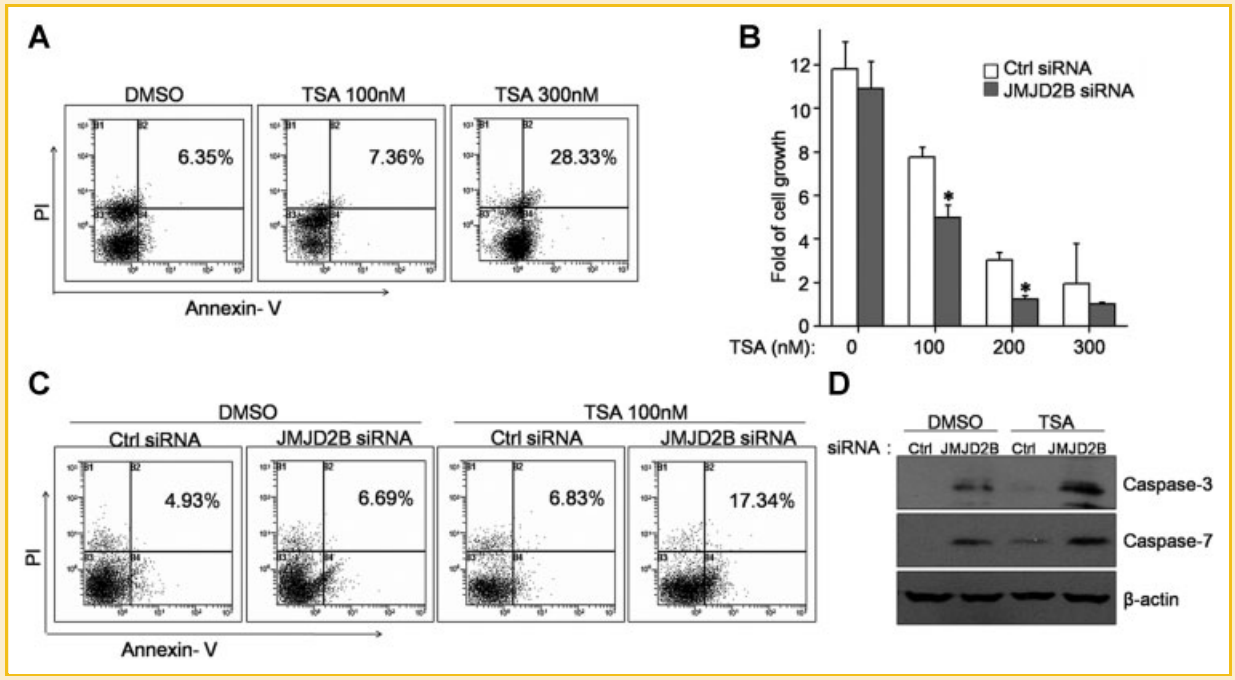

Fig. 5. Knockdown of JMJD2B promoted TSA-induced apoptosis in LNCap cells. A: Exposure of LNCap cells to TSA increase the rate of apoptosis. LNCap cells were treated with 100 and 300 nM TSA for 48 h, respectively, and apoptosis was determined by flow cytometry using Annexin V-FITC. B: Knockdown of JMJD2B intensified the proliferation of LNCap cells inhibited by TSA. Cells transfected with JMJD2B or control siRNA were treated with TSA for additional 48 h and cell proliferation was tested by MTT assay. C: Knockdown of JMJD2B enhanced TSA-induced apoptosis in LNCap cells. Cells transfected with JMJD2B siRNA and treated with 100 nM TSA for additional 48 h. The cell apoptosis was detected by flow cytometry. D: Knockdown of JMJD2B increased the level of cleaved caspase-3 and caspases-7 in response to TSA.

B1 and block its binding to the APC/C. Apparently, further mechanistic studies are needed to gain more in-depth insight into the details of how JMJD2B regulates the proteasomal destruction of Cyclin B1.

The JMJD2 family members exert diverse functions in many biological contexts, such as chromatin-mediated gene regulation, epigenetics-based memory systems, generation of primordial germ cells, cell apoptosis, tumorigenesis and differentiation [Fodor et al., 2006; Yang et al., 2010; Kawazu et al., 2011; Shi et al., 2011]. In this study, we showed that knockdown of JMJD2B by specific siRNA enhanced TSA-induced apoptosis (Fig. 5). While overexpression of JMJD2B was not sufficient to rescue TSA-induced apoptosis, it is reasonable to speculate that JMJD2B may function as a part of TSA-induced apoptosis machinery only when it is suppressed. However, further studies are necessary to establish this hypothesis. In addition, we showed that TSA-mediated downregulation of JMJD2B in LNCap cells contributed to the degradation of survivin. Given that downregulation of both Cyclin B1 and survivin could inhibit tumor cells growth and cause cell cycle arrest, we investigated the influence of JMJD2B on cell cycle and proliferation control in LNCap prostate cancer cells. We demonstrated that suppression of JMJD2B by siRNA for 48 h weakly arrested LNCap cells at the G2/M phase (Fig. S1D). Moreover, knockdown of JMJD2B by JMJD2B shRNA and neomycin selected for 14 days resulted in significant inhibition of cell proliferation (Fig. S1E).

To conclude, we established in this study that treatment of LNCap prostate cancer cells with HDAC inhibitors resulted in downregulation of JMJD2B, which promoted the degradation of survivin protein, indicating that JMJD2B downregulation contributed to the TSA-induced apoptosis through a mechanism involving Cyclin B1-dependent survivin degradation. Significantly, these results hinted the value of inhibiting survivin in prostate cancer therapy. Moreover, our data also suggest that JMJD2B may play a role in response to drugs in prostate cancer chemotherapeutics and inhibition of JMJD2B with novel molecular genetic approaches in combination with the use of chemotherapeutic drugs may improve the current regimen.

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