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Original Article

Upregulation of PSMD10 caused by the JMJD2A histone demethylase

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Abstract: PSMD10, also known as gankyrin, is associated with the proteasome and has been shown to be an oncoprotein in the liver. Here, we report that PSMD10 expression is stimulated by the histone demethylase JMJD2A/KDM4A and its interaction partner, the ETV1 transcription factor, in LNCaP prostate cancer cells. Global analysis of expression patterns revealed that PSMD10 mRNA levels are positively correlated with those of both JMJD2A and ETV1. In human prostate tumors, PSMD10 is highly overexpressed at the protein level and correlates with JMJD2A overexpression; further, PSMD10 expression is enhanced in the prostates of transgenic JMJD2A mice. Moreover, PSMD10 is particularly overexpressed in high Gleason score prostate tumors. Downregulation of PSMD10 in LNCaP prostate cancer cells impaired their growth, indicating that PSMD10 may exert a pro-oncogenic function in the prostate. Lastly, we observed that PSMD10 expression is correlated to YAP1, a component of the Hippo signaling pathway and whose gene promoter is regulated by JMJD2A, and that PSMD10 can cooperate with YAP1 in stimulating LNCaP cell growth. Altogether, these data indicate that PSMD10 is a novel downstream effector of JMJD2A and suggest that inhibition of the JMJD2A histone demethylase by small molecule drugs may be effective to curtail the oncogenic activity of PSMD10 in various PSMD10-overexpressing tumors.

Keywords: ETV1, gankyrin, JMJD2A, histone demethylase, KDM4A, prostate cancer, PSMD10

Introduction

Many epigenetic regulators have been identified during the last decade that function to suppress or to enhance tumor formation [1, 2]. One of the most versatile epigenetic marks is the modification of various histone lysine residues by methylation, which is controlled by the antagonism between histone methyltransferases and demethylases [3]. Over 20 different histone demethylases are currently known and the vast majority of them belongs to the JmjC domain-containing (JMJD) protein family [4]. Amongst the JMJD proteins, also called KDM (lysine demethylase) proteins, the JMJD2 subfamily is the largest one with 6 respective genes in the human genome [5-7]. The first JMJD2 protein characterized was JMJD2A/KDM4A that was reported to act as a transcriptional corepressor [8, 9]. However, the subsequent characterization of JMJD2A as an enzyme capable of removing the repressive H3K9me3 and H1.4K26me3 marks revealed that it can also function as a transcriptional coactivator [10-13].

Bioinformatical analyses as well as immunohistochemistry have shown that JMJD2A is overexpressed in prostate tumors and that high JMJD2A expression correlates with increased Gleason score and metastasis [14, 15]. Furthermore, transgenic mice that prostate-specifically overexpress JMJD2A develop prostatic intraepithelial neoplasia, demonstrating that JMJD2A overexpression is an underlying cause for the initiation of prostate cancer [15]. Mechanistically, JMJD2A can interact with two prostate cancer relevant proteins, the androgen receptor and ETV1 (ETS variant 1), and affect their transcriptional potential [15, 16]. The androgen receptor is abnormally activated or overexpressed in prostatic malignancies and

a prime target for their therapy [17]. Like the androgen receptor, ETV1 is a DNA-binding transcription factor highly regulated through post-translational modification [18-23] and overexpressed in about 5-10% of all human prostate tumors [24, 25]. Furthermore, ETV1 transgenic mice develop prostatic intraepithelial neoplasia and combining ETV1 overexpression with complete loss - but not with inactivation of just one allele - of the PTEN tumor suppressor leads to adenocarcinomas [26-28]. In addition, joint overexpression of ETV1 and JMJD2A combined with PTEN haplo insufficiency results in the progression of prostatic intraepithelial neoplasia to the carcinoma stage, indicating that the JMJD2A-ETV1 complex has oncogenic properties [15].

PSMD10 (proteasome 26S subunit, non-ATPase, 10), also called gankyrin, was originally cloned as a component of the 26S proteasome [29, 30]. In particular, PSMD10 binds to the S6 ATPase of the proteasome, yet this interaction seems to be not stable and suggests that PSMD10 may interact with other protein complexes aside from the proteasome [31]. The yeast ortholog of PSMD10 is not required for cell growth and viability, implying that mammalian PSMD10 might not be an essential protein [30, 31]. Notably, PSMD10 is an oncoprotein and appears to play an important role in liver cancer. It is overexpressed in hepatocellular carcinomas, predicts a poor outcome and is required for efficient liver cancer cell growth and invasion in vitro as well as tumorigenicity and metastasis in vivo [32-34]. However, the role of PSMD10 in prostate cancer has remained unexplored. In this report, we describe that PSMD10 is one downstream effector of the histone demethylase JMJD2A, thereby implicating PSMD10 as a potential promoter of prostate tumorigenesis.

Materials and methods

Knockdown experiments

All shRNAs were cloned into pSIREN-RetroQ (Clontech) and targeted the following sequences: PSMD10 #1, 5'-TCGAATAACTGTTGAGATT-3'; PSMD10 #2, 5'-GAGAATGGTGGAAGGTTAA-3'; YAP1, 5'-AGTAATAGTTGGTTGTGAA-3'; JMJD2A #3, 5'-GTTGAGGATGGTCTTACCT-3'; JMJD2A #5, 5'-GGACTTAGCTTCATAACTA-3'. ETV1 shRNA #1 and shRNA #5 were described before [35]. The resulting retroviral vectors were cotransfected with two packaging plasmids encoding VSVG and Gag-Pol into human 293T cells, which were grown in DMEM media supplemented with 10% fetal bovine serum [36], by the calcium phosphate coprecipitation method [37, 38]. Resulting virus was harvested as described before [39]. Then, LNCaP cells were infected with retrovirus for two or three times and selected by incubation with 1 μg/ml puromycin [40].

RT-PCR

Total RNA was isolated from LNCaP cells with Trizol (Invitrogen) and reverse transcribed utilizing $pd(N)_6$ random primers. Then, PSMD10 mRNA levels were quantitated using iQ SYBR Green Supermix (BioRad) and employing a real-time PCR machine. PSMD10 mRNA levels were normalized to those of GAPDH by the comparative cycle time method [41]. Primers specific for PSMD10 were 5'-GGCCGATAAATCCCTGGCTA-3' and 5'-CAGGCTAAGTGTAGAGGAGTG-3' (yielding a 459 bp product), while primers for amplifying a 226 bp GAPDH cDNA fragment were 5'-GAGCCACATCGCTCAGACACC-3' and 5'-TGACAAGCTTCCCGTTCTCAGC-3'.

Western blotting

Protein extracts were run on SDS polyacrylamide gels [42], proteins transferred to PVDF membrane (Millipore) and then challenged with antibodies [43]. Next, secondary antibodies coupled to horseradish peroxidase were employed for incubation of the membranes [44] and signals detected with enhanced chemiluminescence and exposure to film [45].

Immunohistochemistry

A human tissue microarray encompassing 31 matching normal and cancerous prostate tissue cores (AccuMax A302IV, slide #139) was treated for 20 min with Bond Epitope Retrieval Solution I and then stained with a Leica Bond-III apparatus. Mouse monoclonal PSMD10 antibody was from Santa Cruz Biotechnology (gankyrin 3A6C2, sc-101498) and employed at a dilution of 1:100. Staining intensity was scored on a scale of 0-3, while a score of 1-4 was employed to determine the percentage of stained cells. The final staining index was calculated as the product of these two scores. Since the tissue microarray contained two cores for

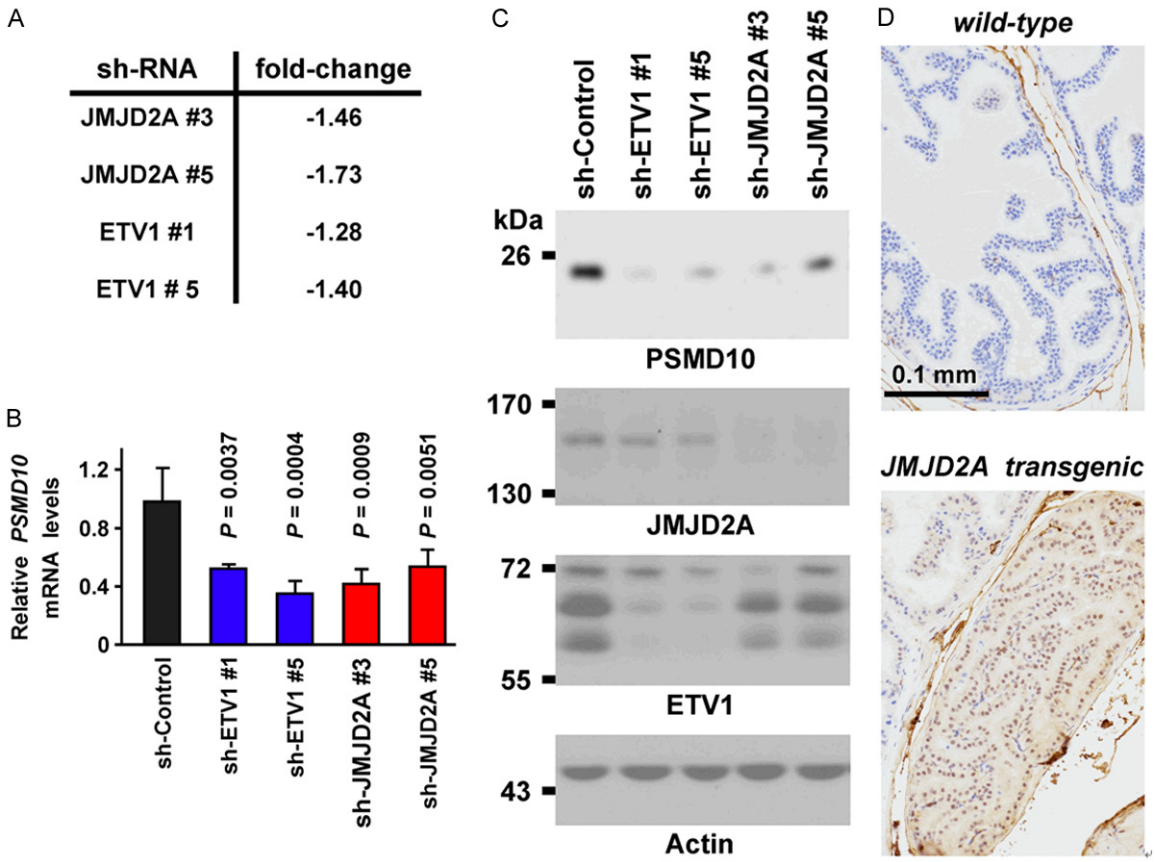

Figure 1. PSMD10 as a target of JMJD2A. A: Analysis of published microarray experiments (Gene Expression Omnibus GSE47750) with LNCaP prostate cancer cells. Shown are changes (compared to control shRNA) of PSMD10 mRNA levels upon downregulation of JMJD2A or ETV1. B: Quantitative RT-PCR results for PSMD10 mRNA after downregulation of JMJD2A or ETV1 in LNCaP cells. Shown are averages (n = 3) with standard deviations. Statistical significance was determined by one-way ANOVA with Bonferroni correction. C: Corresponding western blots. D: Immunohistochemical staining for PSMD10 in the prostate from a wild-type or JMJD2A transgenic mouse.

tumor tissue, but only one core for normal tissue, the staining index for tumor tissue was defined as the average from each pair of tumor cores. For staining of mouse tissue derived from syngeneic wild-type and JMJD2A transgenic mice [15], 40 min of treatment with Bond Epitope Retrieval Solution I was followed by staining with rabbit polyclonal PSMD10 antibodies (Santa Cruz Biotechnology, gankyrin H-231, sc-8991) at a 1:100 dilution.

Cell growth assay

LNCaP cells stably expressing shRNA or HA-tagged PSMD10 (or empty vector pQCXIH) were seeded into 96-wells at a density of 3000 cells per well [46]. Then, cells were grown for the indicated number of days, after which growth was determined with the PrestoBlue cell viability kit (Invitrogen) by measuring fluorescence at 590 nm.

Statistical analysis

Statistical tests are described in the respective figure legends. R values are Pearson correlation coefficients. A *P* value of less than 0.05 was considered statistically significant.

Results

Stimulation of PSMD10 expression by JMJD2A and ETV1

To understand the physiological role of the histone demethylase JMJD2A and its interaction partner ETV1, we previously performed mRNA microarray experiments with LNCaP prostate cancer cells in order to detect genes commonly regulated by JMJD2A and ETV1 [15]. Analysis of these microarray data revealed that among the 256 genes becoming downregulated by > 1.4-fold upon JMJD2A shRNA expression was

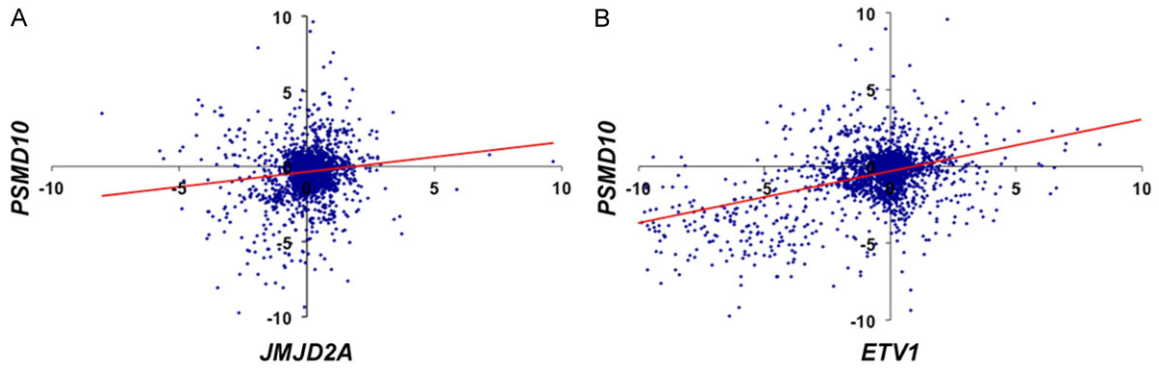

Figure 2. Global correlation between PSMD10 and either JMJD2A or ETV1 mRNA expression. A: PSMD10 versus JMJD2A mRNA levels. R = 0.12, P = 2.3×10^{-9}. Trendline is indicated in red color. B: Likewise for PSMD10 and ETV1. R = 0.42, P = 10^{-110}.

PSMD10, yet PSMD10 did not meet the criterion of > 1.4-fold downregulation with ETV1 shRNA (**Figure 1A**). However, when we performed quantitative RT-PCR to validate the microarray data, we found that both ETV1 and JMJD2A shRNAs led to more than 1.8-fold downregulation of PSMD10 mRNA levels (**Figure 1B**), indicating that the microarray data underestimated the degree of PSMD10 regulation by JMJD2A and ETV1. Altogether, these data suggest that PSMD10 transcription is regulated by both JMJD2A and ETV1.

Since mRNA levels are not always indicative of protein levels, we explored if PSMD10 protein levels were also reduced upon either JMJD2A or ETV1 downregulation in LNCaP cells. We indeed observed that this was the case (**Figure 1C**). In addition, we performed immunohistochemistry on prostates from transgenic mice that prostate-specifically overexpressed JMJD2A [15] and found robustly enhanced PSMD10 protein expression in JMJD2A transgenic compared to syngeneic wild-type mice (**Figure 1D**). This supports the notion that JMJD2A overexpression results in a concomitant increase of PSMD10 protein levels.

We then interrogated PSMD10 expression in a collection of 3949 mRNA microarrays representing a multitude of different tissues and experimental conditions [47]. In this huge dataset, we observed a strong positive correlation between JMJD2A and PSMD10 mRNA expression levels (**Figure 2A**). An even stronger correlation existed between ETV1 and PSMD10 (**Figure 2B**). Collectively, these data indicate that PSMD10 gene transcription is regulated by JMJD2A and ETV1 not only in the prostate but rather globally.

PSMD10 overexpression in human prostate tumors

Since both JMJD2A and ETV1 are highly implicated in prostate tumorigenesis, we next analyzed the expression of PSMD10 in human prostate tumors. First, we employed published microarray data [48] and found that PSMD10 mRNA levels correlated with those of JMJD2A in human prostate tumors (**Figure 3A**), further supporting the notion that JMJD2A stimulates PSMD10 expression. Possibly due to the fact that ETV1 is only overexpressed in 5-10% of prostate tumors [24, 25], we did not detect any significant correlation between ETV1 and PSMD10 in this microarray data set because the number of samples (n = 85) might be too low. Analysis of other published microarray data [49, 50] indicated that PSMD10 mRNA levels correlate with the Gleason score (**Figure 3B** and **3C**); please note that a Gleason score of 6 or less implies a good prognosis, while Gleason scores above 6 are associated with progressively poorer outcome. This suggests that PSMD10 expression increases with the aggressiveness of prostate cancer and may thus contribute to the transition from indolent to advanced disease.

To complement the bioinformatics analysis, we also stained a human tissue microarray containing matching normal and cancerous prostate tissues. We found that PSMD10 protein expression was highly overexpressed in the vast majority of human prostate tumors (**Figure 4A**). Moreover, since we previously stained the same tissues with JMJD2A antibodies [15], we were able to correlate PSMD10 with JMJD2A staining. A highly significant correlation between JMJD2A and PSMD10 protein levels

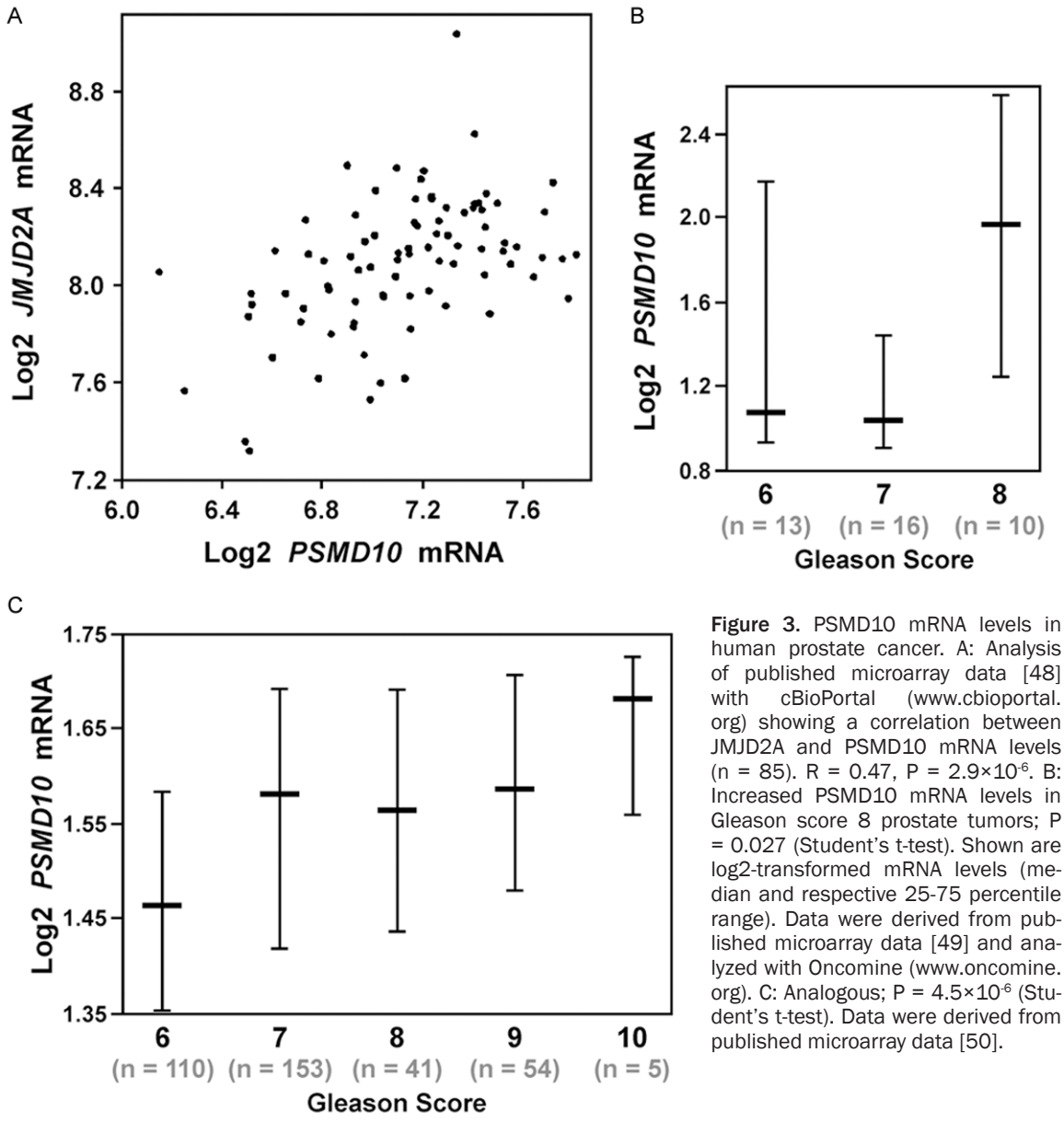

Figure 3. PSMD10 mRNA levels in human prostate cancer. A: Analysis of published microarray data [48] with cBioPortal (www.cbioportal.org) showing a correlation between JMJD2A and PSMD10 mRNA levels (n = 85). R = 0.47, P = 2.9×10^{-6}. B: Increased PSMD10 mRNA levels in Gleason score 8 prostate tumors; P = 0.027 (Student's t-test). Shown are log2-transformed mRNA levels (median and respective 25-75 percentile range). Data were derived from published microarray data [49] and analyzed with Oncomine (www.oncomine.org). C: Analogous; P = 4.5×10^{-6} (Student's t-test). Data were derived from published microarray data [50].

was thereby uncovered (**Figure 4B**), which further corroborates that PSMD10 expression is stimulated by JMJD2A. Unfortunately, we could not find a suitable ETV1 antibody for immunohistochemistry, therefore not allowing us to assess if likewise ETV1 and PSMD10 protein levels might be correlated in prostate tissue.

PSMD10 as a cell growth regulator

To determine if and how PSMD10 affects prostate cancer cells, we downregulated PSMD10 with two different shRNAs in LNCaP cells. Growth of these cells was then assessed and found to be significantly reduced after six days (**Figure 5A**). We also overexpressed PSMD10 in LNCaP cells, yet this did not result into any change of cell growth (**Figure 5B**), possibly because endogenous PSMD10 amounts were already at saturating levels. Regardless, our experiments demonstrate that PSMD10 is a positive regulator of growth in LNCaP prostate cancer cells, which is consistent with its original identification as an oncoprotein [32].

Cooperation between PSMD10 and YAP1

We previously identified the YAP1 gene, which encodes for a transcriptional cofactor in the Hippo signaling pathway [51], as a target of the

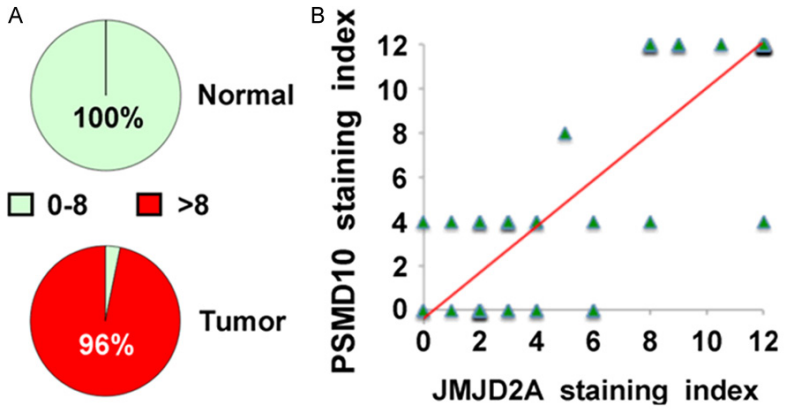

Figure 4. Overexpression of PSMD10 in human prostate tumors. A: Nuclear PSMD10 staining in 31 matching normal prostates and tumors was graded on a scale of 0-12. Staining was discriminated between grades 0-8 and > 8; P = 1.4×10^{-16} (two-tailed Fisher's exact probability test). B: Correlation between nuclear JMJD2A and PSMD10 staining across the 31 matching normal and cancerous prostate specimens. R = 0.87, P = 5.1×10^{-21}. Trendline is indicated in red color.

JMJD2A-ETV1 complex [15]. Interestingly, Ingenuity pathway analysis of our published microarray data [15] revealed that PSMD10 and YAP1 participate in a common network of JMJD2A-regulated genes in LNCaP prostate cancer cells (**Figure 6A**). This prompted us to study if PSMD10 and YAP1 are coregulated on a global scale. And indeed, there was a significant correlation between PSMD10 and YAP1 mRNA levels across 3949 microarray data sets (**Figure 6B**).

We then asked the question if this coregulation of PSMD10 and YAP1 might be biologically relevant. To this end, we downregulated either PSMD10 or YAP1 alone in LNCaP prostate cancer cells, or both together. Efficient reduction of PSMD10 and/or YAP1 protein levels was obtained with our shRNAs (**Figure 6C**). Then, we examined how this would affect LNCaP cell growth. As expected from published data for YAP1 [15, 52] or from the results for PSMD10 shown in **Figure 5A**, individual depletion of YAP1 or PSMD10 reduced prostate cancer cell growth (**Figure 6D**). Excitingly, joint downregulation of YAP1 and PSMD10 resulted in a significant further reduction of cell growth, indicating that YAP1 and PSMD10 cooperate in facilitating LNCaP cell growth.

Discussion

In this report, we provide evidence that PSMD10 expression is regulated by the JMJD2A histone demethylase, which likely does so in conjunction with the ETV1 transcription factor. Furthermore, we show for the first time that PSMD10 is overexpressed in prostate tumors and its expression appears to correlate with the severity of the disease. Lastly, our data have uncovered that PSMD10 is a growth promoting protein in prostate cancer cells and may cooperate with YAP1 in this regard.

Although our findings demonstrate that JMJD2A and ETV1 can stimulate the expression of PSMD10 in LNCaP prostate cancer cells, it is unclear whether or not this involves the direct binding of these two transcription factors to the PSMD10 gene promoter and its subsequent stimulation. For instance, we cannot exclude an indirect mechanism by which JMJD2A and ETV1 upregulate expression of another transcription factor that then binds to and stimulates the PSMD10 promoter. Regardless, our discovery that JMJD2A and ETV1 expression levels are globally correlated with those of PSMD10 suggests that PSMD10 is not only an effector of JMJD2A and ETV1 in prostate cancer cells, but potentially in many other cancers. In particular, this may be relevant in breast cancer, since overexpression or dysregulation of JMJD2A and ETV1 has been reported for breast tumors [53-57] and overexpressed PSMD10 appears to promote especially the metastasis of breast cancer cells [58, 59].

Interestingly, PSMD10 is also overexpressed in human cholangiocarcinomas and a predictor for overall survival [60]. Likewise, YAP1, which is a versatile transcriptional regulator [61, 62], was reported to be overexpressed in this cancer and phenocopied PSMD10 with regard to its ability to promote tumorigenesis and metastasis [63]. Similar to our data showing a correlation between PSMD10 and YAP1 mRNA levels, there was significant coexpression of YAP1 and PSMD10 at the protein level in cholangiocarcinomas. Further, YAP1 and PSMD10 were each

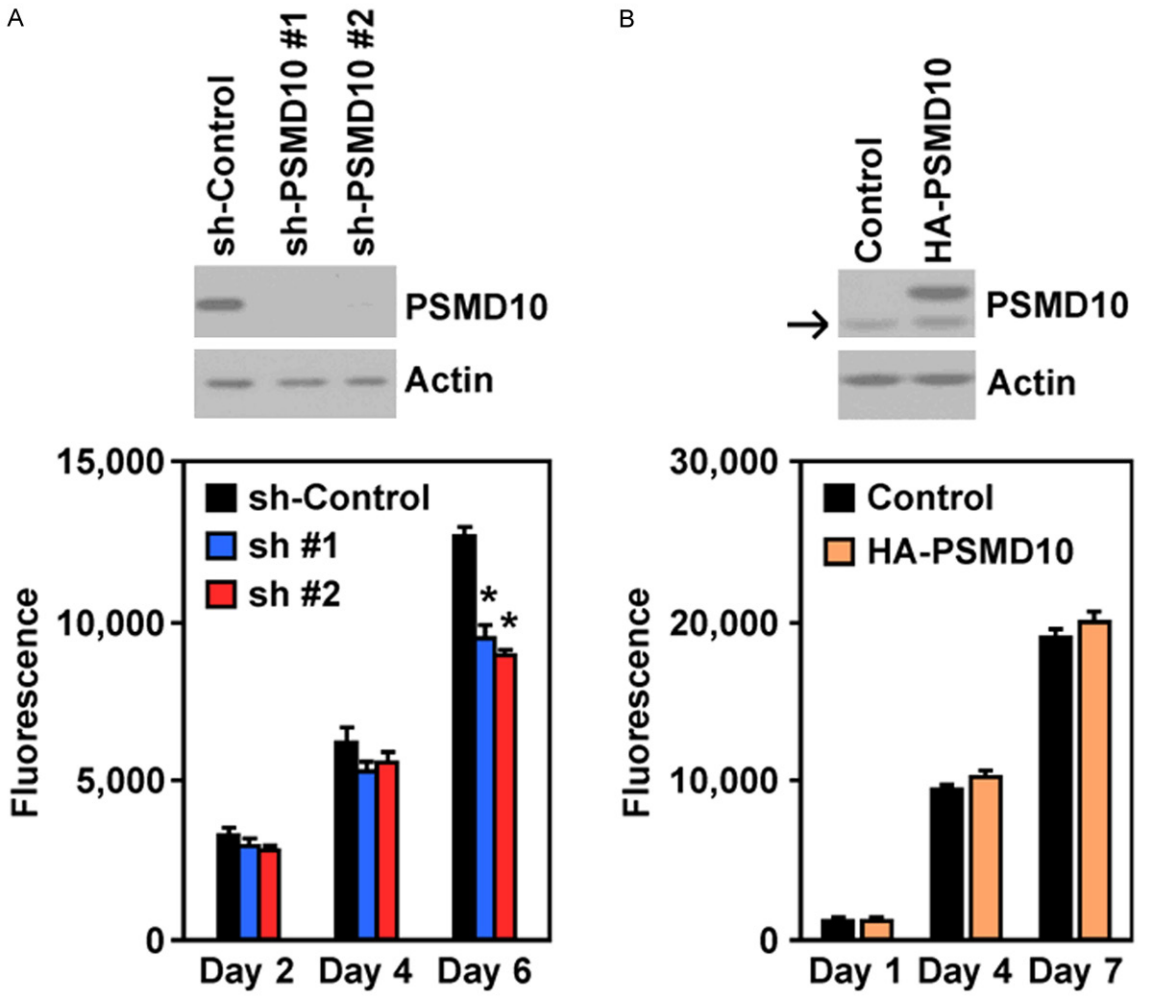

Figure 5. Role of PSMD10 in cell proliferation. A: Downregulation of PSMD10 with two different shRNAs in LNCaP prostate cancer cells reduces their growth. Shown are averages (n = 3) with standard deviations. *, $P < 0.0001$ (one-way ANOVA with Bonferroni correction). Corresponding western blots are shown on the top. B: Analogous, overexpression of HA-tagged PSMD10 in LNCaP cells. Arrow points at the endogenous PSMD10 protein, which runs at a slightly lower apparent molecular weight than the ectopic HA-tagged PSMD10.

able to stimulate the expression of the other, which seems to occur through indirect transcriptional mechanisms [63]. However, this is different from our results in LNCaP cells, since there was no impact on the expression of the respective other protein when YAP1 or PSMD10 were downregulated (see **Figure 6C**). Hence, it may be that there are cell type-specific differences in how transcription of the YAP1 and PSMD10 genes are regulated.

Several mechanisms by which PSMD10 contributes to liver oncogenesis have been described. These include the activation of the CDK4 protein kinase, possibly by blocking the interaction of the cell cycle inhibitor p16 with CDK4, which leads to enhanced phosphorylation of the retinoblastoma tumor suppressor and its subsequent degradation [31, 32, 64]. Likewise, the stability of the p53 tumor suppressor is compromised by PSMD10, since PSMD10 binds to the MDM2 ubiquitin ligase and thereby increases MDM2-mediated ubiquitylation of p53 [65]. Further, PSMD10 facilitates the degradation of the transcription factor C/EBP when it is phosphorylated on S193, which promotes carcinogen-induced liver tumorigenesis [66]. On the other hand, PSMD10 protects OCT4, a transcription factor playing important roles in stem cell maintenance, from degradation by sequestering WWP2, an E3 ligase normally targeting OCT4 for proteasomal

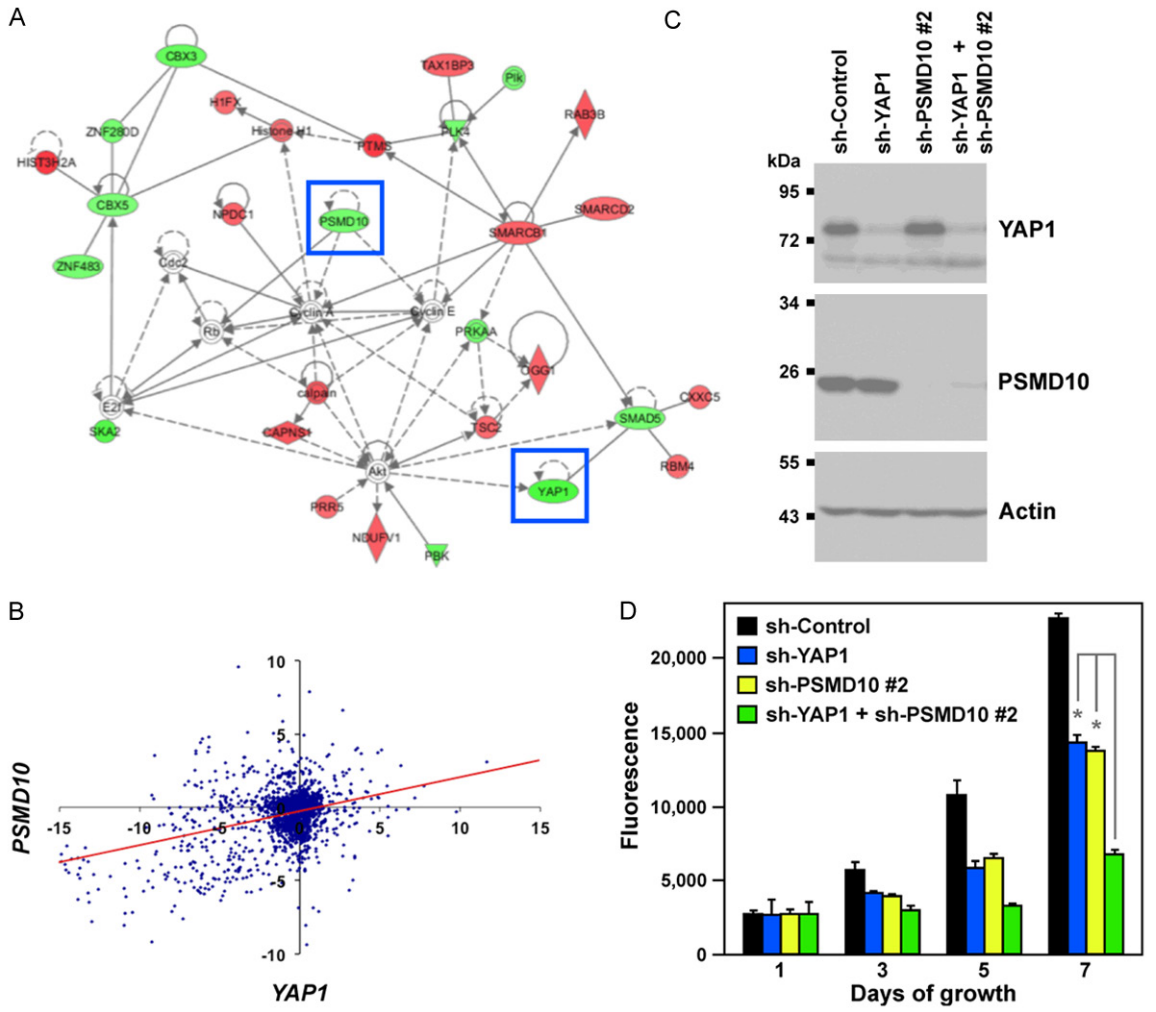

Figure 6. Relationship between PSMD10 and YAP1. A: Ingenuity pathway analysis revealing a gene network containing both YAP1 and PSMD10 upon JMJD2A downregulation in LNCaP cells. B: Global coexpression of PSMD10 and YAP1 mRNA. R = 0.44, P = 4.6×10^{-114}. Trendline is indicated in red color. C: Joint downregulation of YAP1 and PSMD10 in LNCaP prostate cancer cells. Shown are indicated western blots. D: Corresponding cell growth assay. Averages (n = 3) and standard deviations are depicted. *, P < 0.0001 (one-way ANOVA with Bonferroni correction).

destruction. The consequence is an expansion of tumor-initiating cells in the liver, which is predicted to aggravate hepatocarcinogenesis [67]. It remains to be studied if these or other mechanisms pertain to PSMD10's function as a growth promoter in prostate cancer cells.

Despite the fact that PSMD10 is an established oncoprotein, there are currently no drugs available to inhibit its activity. Our study implicates that alternative routes of reducing PSMD10 activity should be considered, namely the inhibition of either JMJD2A or ETV1 since that would reduce PSMD10 expression. In fact, multiple small molecule drugs have been developed to inhibit the enzymatic activity of the histone demethylase JMJD2A, but their utility has not yet been tested in the clinic [68-75]. Similarly, some progress has been made to develop inhibitors of the ETV1 transcription factors [76, 77]. Another possibility of counteracting PSMD10 overexpression may entail targeting the YAP1 protein, since YAP1 might be stimulating PSMD10 transcription in some cell types and/or the co-overexpressed YAP1 protein could aggravate the oncogenic impact of PSMD10. Similar to JMJD2A and ETV1, potential inhibitors of YAP1 were discovered, but have not yet been clinically tested for cancer therapy [78, 79].

In conclusion, we have identified PSMD10 as a downstream effector of the histone demethylase JMJD2A and the DNA-binding ETV1 protein.

Hence, together with YAP1, which is likewise regulated by these two oncogenic transcription factors, PSMD10 overexpression may contribute to tumor formation in the prostate and other organs.

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Disclosure of conflict of interest

None.

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