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SMYD4 monomethylates PRMT5 and forms a positive feedback loop to promote hepatocellular carcinoma progression

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Abstract

Both lysine and arginine methyltransferases are thought to be promising therapeutic targets for malignant tumors, yet how these methyltransferases function in malignant tumors, especially hepatocellular carcinoma (HCC), has not been fully elucidated. Here, we reported that SMYD4, a lysine methyltransferase, acts as an oncogene in HCC. SMYD4 was highly upregulated in HCC and promoted HCC cell proliferation and metastasis. Mechanistically, PRMT5, a well-known arginine methyltransferase, was identified as a SMYD4-binding protein. SMYD4 monomethylated PRMT5 and enhanced the interaction between PRMT5 and MEP50, thereby promoting the symmetrical dimethylation of H3R2 and H4R3 on the PRMT5 target gene promoter and subsequently activating DVL3 expression and inhibiting expression of E-cadherin, RBL2, and miR-29b-1-5p. Moreover, miR-29b-1-5p was found to inversely regulate SMYD4 expression in HCC cells, thus forming a positive feedback loop. Furthermore, we found that the oncogenic effect of SMYD4 could be effectively suppressed by PRMT5 inhibitor in vitro and in vivo. Clinically, high coexpression of SMYD4 and PRMT5 was associated with poor prognosis of HCC patients. In summary, our study provides a model of crosstalk between lysine and arginine methyltransferases in HCC and highlights the SMYD4-PRMT5 axis as a potential therapeutic target for the treatment of HCC.

Zhenyu Zhou, Zheng Chen, and Qianlei Zhou share co-first authorship.

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KEYWORDS
arginine methyltransferases, lysine methyltransferases, MEP50, miR-29b-1-5p, PRMT5 inhibitor

1 | INTRODUCTION

Systemic therapies, such as immune checkpoint inhibitor and tyrosine kinase inhibitor, are recommended for advanced hepatocellular carcinoma (HCC).[1] Neoadjuvant or adjuvant therapies using tyrosine kinase inhibitors and immune checkpoint inhibitors are also under investigation.[2] However, the efficacy is still far from satisfactory. Novel therapeutic targets are still needed to further improve patient survival.

Protein arginine methyltransferase 5 (PRMT5) is a type II protein arginine methyltransferase that regulates gene expression by symmetrically dimethylating arginine residues in histones (H2AR3me2s, H4R3me2s, H3R2me2s, and H3R8me2s).[3] Among them, H2AR3me2s and H4R3me2s are transcriptional repression markers, whereas H3R2me2s and H3R8me2s are transcriptional activation markers.[3] By regulating target gene expression, PRMT5 elicits potent oncogenic effects in multiple tumor types, including HCC.[4,5] Recently, PRMT5 was also found to affect tumor microenvironment.[6] Based on these preclinical studies, targeting PRMT5 and its pathway has been regarded as a "new avenue" for the treatment of solid and hematological malignancies.[3] Considering the crucial roles of PRMT5 in cancer, it would be interesting and worthwhile to elucidate its regulatory mechanism.

SET and MYND domain-containing proteins (SMYDs), like PRMTs, are a family of methyltransferases, but they catalyze lysine methylation. The SMYDs family consists of five members: SMYD1, SMYD2, SMYD3, SMYD4, and SMYD5.[7] Some of them, such as SMYD2[8,9] and SMYD3,[10,11] have been reported to play crucial roles in malignant tumors. In contrast, the role and molecular mechanism of SMYD4 in malignant tumors are still undefined.

In the present study, we found that SMYD4 was highly upregulated in HCC. SMYD4 promoted HCC cell proliferation and metastasis through interacting with PRMT5. Mechanically, SMYD4 monomethylated PRMT5, and thus regulated the transcription of the PRMT5 downstream targets. Moreover, we found that SMYD4 and PRMT5 formed a positive feedback loop via miR-29b-1-5p. The PRMT5 inhibitor JNJ-64619178 effectively suppressed the oncogenic function of SMYD4. These findings identified SMYD4 as an oncogene in HCC, which may be a novel therapeutic target.

2 | MATERIALS AND METHODS

2.1 | Patients and clinical samples

A tissue microarray containing 243 cases of HCC were acquired from the specimen library of our center, Sun Yat-Sen Memorial Hospital (SYSMH) and were collected between 2010 and 2013. In addition, 6 and 81 pairs of HCC and matched adjacent nontumor samples were collected from SYSMH between 2009 and 2014 for protein and mRNA detection, respectively. All the samples were obtained from patients with HCC who had undergone curative resection and been confirmed by histopathologists. None of the patients underwent preoperative chemotherapy, locoregional therapy, or radiation therapy. The study was conducted in accordance with the Declarations of Helsinki and Istanbul and was approved by the Ethics Committee of Sun Yat-Sen Memorial Hospital (SYSMH). Informed consent was obtained from each patient. The detailed clinical data are listed in Table S1. Cancer clinical staging was based on the AJCC/TNM staging for HCC (8th ed. 2017).

2.2 | Cell culture

Hepatocellular carcinoma (HCC) cell lines (Huh7, Hep3B) were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. HCC cell lines (MHCC-97H, SNU-449) were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology (China). Among these cells, MHCC-97H and SNU-449 were used for SMYD4 knockdown experiments (Table S5), while Huh7 and Hep3B were used for SMYD4 overexpression experiments. All cells were maintained in DMEM (ThermoFisher Scientific) supplemented with 10% FBS (ThermoFisher Scientific) and 1% penicillin/streptomycin (ThermoFisher Scientific) and incubated at 37°C with 5% CO_2. In each experiment, the cells were passaged at least three times after thawing. All cells were authenticated via short tandem repeat (STR) profiling conducted by a diverse company. All cells were confirmed to be free from mycoplasma every 3 months. In some experiments, JNJ-64619178 (300 nmol/L, HY-101564, MedChem Express) was utilized to treat HCC cells.

2.3 | Chromatin immunoprecipitation (ChIP)

EZ-Magna ChIP A/G Kit (Millipore) was utilized for ChIP assay according to the manufacturer's instructions. PRMT5 (Invitrogen, MA1-25470), H4R3me2S (Millipore, 17-10250), or H3R2me2s (ABclonal, A2373) antibodies were used for immunoprecipitation. The immunoprecipitated DNAs together with input DNAs were analyzed using real-time PCR. Primers for CDH1, RBL2, and DVL3 promotors are listed in Table S4.

2.4 | Luciferase reporter assay

The gene promoter sequences of DVL3 and CDH1 were inserted into a luciferase reporter vector and were referred to as pGL4.18-DVL3 and pGL4.18-CDH1. These plasmids were respectively transfected into HCC cells in combination with pGL4.74 (for transfection efficiency control) and the indicated treatments. Dual luciferase reporter assay system (Promega) was used to detect the luciferase activity according to the manufacturer's instructions. To study the regulation of SMYD4 by miR-29b-1-5p, wild-type 3′-UTR sequence of SMYD4 with predicted miR-29b-1-5p target sites or 3′-UTR sequence with the predicted target site mutation was inserted into the psiCHECK-2 (Promega), referred to as wt-SMYD4 3′-UTR and mut-SMYD4 3′-UTR. These plasmids were respectively transfected into HCC cells in combination with miR-control mimics or miR-29b-1-5p mimics. Luciferase activity was detected by a dual-luciferase reporter assay system.

2.5 | Co-immunoprecipitation (Co-IP), silver staining analysis, and liquid chromatography-mass spectrometry (LS-MS) analysis

These experiments were performed as described previously.[11,12] For Co-IP, HCC cell lines including MHCC-97H, Huh7-SMYD4, Huh7 pretransfected with plasmids of Flag-labeled different PRMT5 regions, and HA-labeled SMYD4 were used. The cell protein lysates were mixed with antibodies of PRMT5 (Abcam, ab109451), SMYD4 (Proteintech, 17594-1-AP), HA (Abcam, ab9110), or Flag (Cell Signaling Technology, #14793) according to each experiment. Silver staining was performed using a silver stain kit SilverQuest™ (ThermoFisher Scientific) according to the manufacturer's instructions. LS-MS detection and analysis were performed by BGI Company.

2.6 | Statistical analysis

All data analyses were performed using SPSS version 25.0. Continuous data are reported as mean ± SD. The significance of differences was analyzed using the chi-square test, Fisher's exact test, Student's *t*-test, and one-way ANOVA test, as appropriate. Pearson's correlation test was used to analyze the correlation between SMYD4 and PRMT5 expression in HCC cases. Kaplan–Meier analysis and log-rank tests were used for survival and recurrence rate analyses. The Cox proportional hazards regression model was used to verify independent risk factors based on the results of the univariate analyses. Statistical significance was set at $p < 0.05$.

Additional methodological details are described in Appendix S1.

3 | RESULTS

3.1 | SMYD4 expression is upregulated in human HCC

To understand the role of SMYD4 in HCC, we first assessed SMYD4 expression in HCC samples using TCGA and GEO datasets. We found that SMYD4 expression was significantly higher in HCC samples than in adjacent nontumor samples and normal liver samples (Figure 1A,B). Moreover, SMYD4 expression tended to increase as the degree of tumor differentiation decreased (Figure 1C). The HCC cohort from our center containing 81 pairs of HCC and adjacent nontumor samples further confirmed the high mRNA expression of SMYD4 in HCC tissues (Figure 1D). Additionally, SMYD4 expression in undifferentiated cases was significantly higher than in differentiated cases (Figure 1D). We then detected SMYD4 protein expression in the tissue microarray of our center. The results showed that 61.3% (149 of 243) of HCC cases displayed high SMYD4 expression, and SMYD4 was predominantly observed in the cytoplasm (Figure 1E). Additionally, we also confirmed the high protein expression of SMYD4 in another six pairs of HCC tissues using Western blot (Figure 1F). High SMYD4 expression was significantly associated with large tumor size, microvascular invasion (MVI), and advanced TNM stage (Table S1). Moreover, HCC patients with high SMYD4 expression were more likely to have shorter overall and disease-free survival (Figure 1G). Cox regression analysis indicated that SMYD4 expression was an independent predictor of survival and recurrence in patients with HCC after curative resection (Table S2). Thus, data from both public databases and our center suggested that SMYD4 may act as an oncogene in HCC.

3.2 | SMYD4 promotes HCC cell proliferation and metastasis

We then explored the oncogenic function of SMYD4 in HCC. Huh7 and Hep3B cell lines were used to establish SMYD4 stable overexpression cells (Figure S1A,B). SNU-449 and MHCC-97H cell lines were used to establish SMYD4 stable knockdown cells (Figure S1A,C). As we observed above that high SMYD4 expression was associated with large tumor size and MVI in HCC samples, we investigated whether SMYD4 affected HCC cell proliferation, migration, and invasion. The results showed that SMYD4 overexpression significantly promoted HCC cell migration, invasion, and proliferation (Figure 2A and Figure S2A), whereas SMYD4 knockdown yielded opposite results (Figure 2B and Figure S2B). Results from subcutaneous xenograft, lung metastasis, and liver orthotopic xenograft models showed that SMYD4 overexpression not

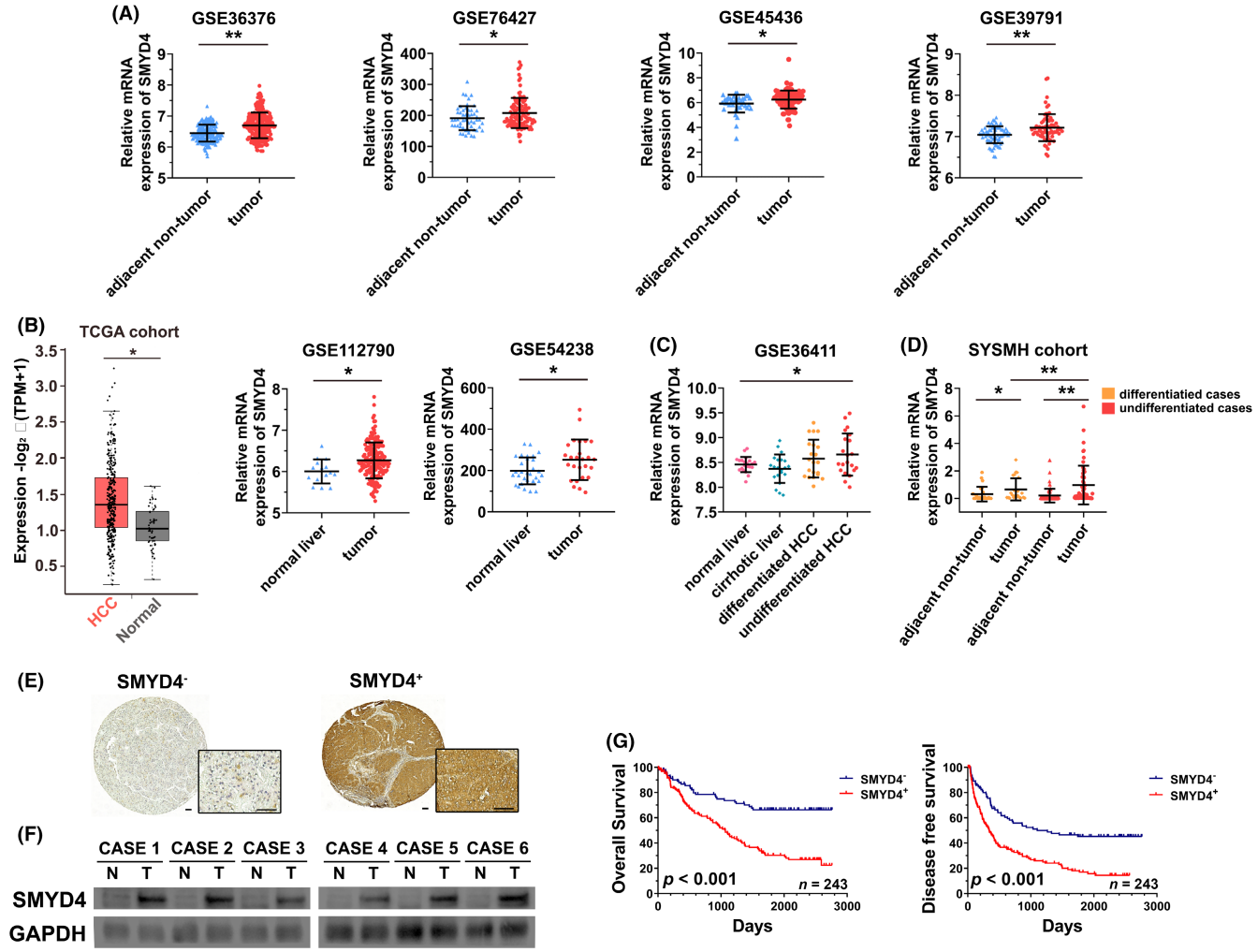

FIGURE 1 SMYD4 expression is elevated in HCC and associated with poor clinical outcome. (A, B) Analysis of GEO and TCGA-LIHC datasets showed an increase of SMYD4 mRNA in hepatocellular carcinoma (HCC) tissues compared with adjacent nontumor tissues and normal liver tissues. (C) Analysis of GSE36411 showed that SMYD4 expression was upregulated in HCC and more increased in undifferentiated cases. (D) The high expression of SMYD4 in HCC especially undifferentiated cases were confirmed in our center cohort. (E) The expression of SMYD4 in 243 HCC samples was detected by immunohistochemistry. (F) The expression of SMYD4 in six pairs of HCC tissues was detected by western blot. (G) The clinical significance of SMYD4 expression in overall survival and disease-free survival was confirmed in our center cohort by Kaplan–Meier survival analysis ($n = 243$). $^{*}p < 0.05$, $^{**}p < 0.01$, scale bar: 50 μm.

only promoted subcutaneous xenograft tumor growth (Figure 2C) and increased the incidence of lung metastasis (Figure 2D) but also promoted orthotopic xenograft tumor growth in the liver of mice (Figure 2E). Ki67 staining confirmed that SMYD4 overexpression enhanced the proliferation capacity of HCC cells in vivo (Figure 2F). In contrast, knockdown of SMYD4 inhibited subcutaneous xenograft tumor growth (Figure S3A–D). Together, these results suggest that SMYD4 may exert its oncogenic function in HCC by promoting cell proliferation, migration, and invasion.

3.3 | SMYD4 interacts with and monomethylates PRMT5

Next, we sought to elucidate the underlying mechanism responsible for the tumor-promoting function of SMYD4 in HCC. Immunofluorescence (IF) staining and subcellular fractionation analysis showed that the SMYD4 protein was mainly localized in the cytoplasm (Figure 3A). Substrate binding and subsequent methylation are major characteristics through which cytoplasmic SMYDs perform their functions.[7,13] We speculated that SMYD4, like other SMYDs, might function in the cytoplasm of HCC cells by binding to and methylating specific substrates. We performed mass spectrometry analysis using the proteins immunoprecipitated with the SMYD4 antibody and found that PRMT5, a well-known protein arginine methyltransferase, was a potential interaction partner of SMYD4 (Figure 3B and Table S3). Results from Co-IP and GST pull-down assays verified the interaction between SMYD4 and PRMT5 (Figure 3C,D). IF staining indicated that SMYD4 and PRMT5 were colocalized in the cytoplasm (Figure 3E). Recent studies have indicated that PRMT5 consists of a triosephosphate isomerase (TIM) barrel on the N-terminus, a middle Rossmann-fold, and a β-barrel on

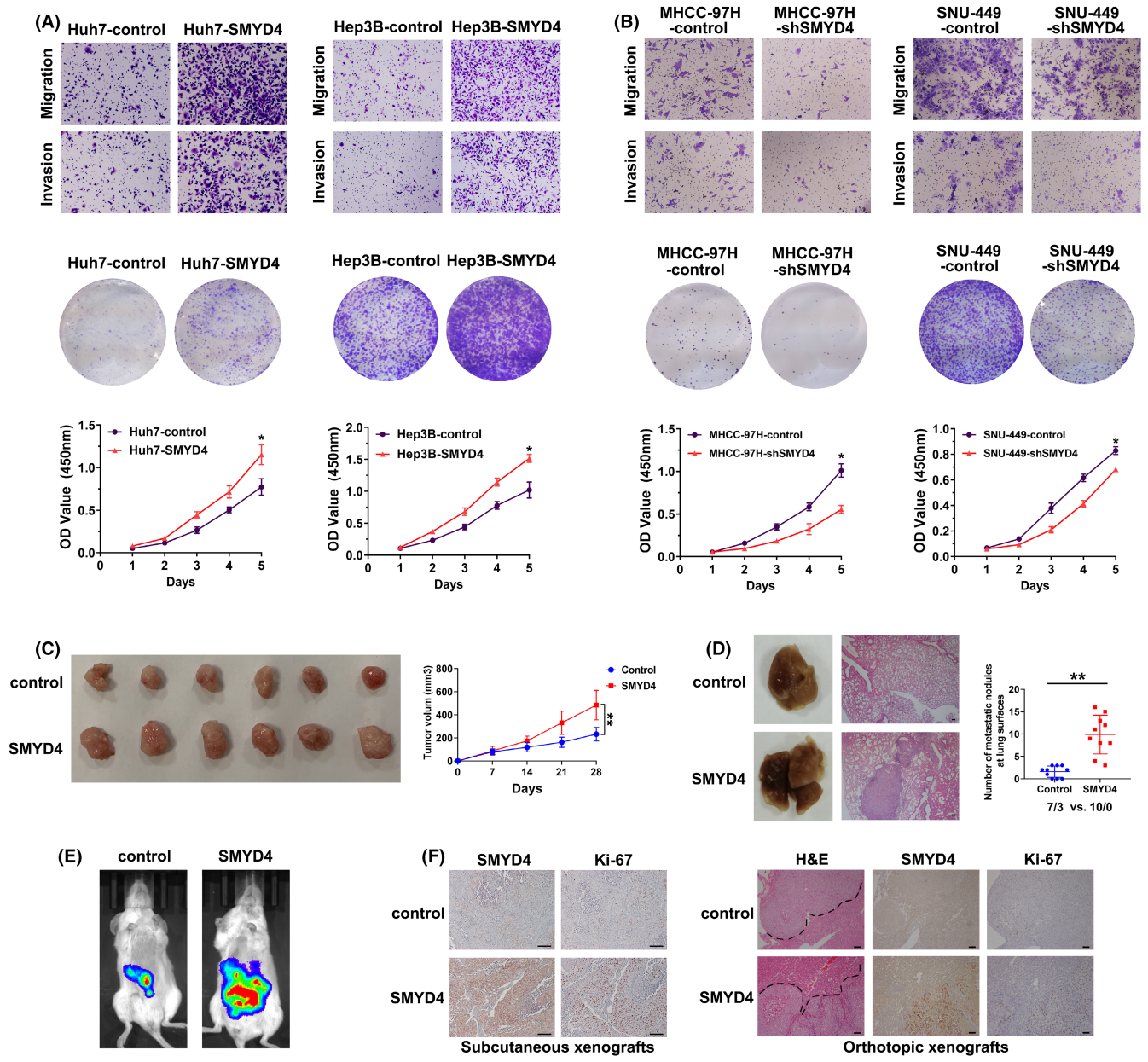

FIGURE 2 SMYD4 promotes hepatocellular carcinoma (HCC) cell proliferation and metastasis. (A, B) Migration, invasion, colony formation, and CCK-8 assays were performed using SMYD4 overexpression or knockdown cells. (C) Representative images of tumors derived from nude mice subcutaneously implanted with huh7-SMYD4 or its control cells ($n=6$). The tumor volumes were measured every week and indicated by curves. (D) Lungs derived from nude mice with tail intravenous injection of huh7-SMYD4 or its control cells and the corresponding H&E staining images ($n=10$). Number of metastatic foci in the lungs of mice from each group was counted and indicated. (E) Orthotopic xenograft model using huh7-SMYD4 or its control cells ($n=4$). (F) H&E staining or SMYD4 and Ki67 staining were conducted in serial sections of the subcutaneous xenograft tumors or orthotopic xenograft tumors from both groups. $^{*}p<0.05$, $^{**}p<0.01$, scale bar: 100 μm.

the C-terminal[14] (Figure 3F). We performed IP assays to investigate whether SMYD4 interacted with one of these regions. The results showed that SMYD4 interacted with the TIM barrel and Rossmann fold of PRMT5 (Figure 3F). Furthermore, we found that SMYD4 overexpression increased, while knockdown decreased, the lysine monomethylation of PRMT5 (Figure 3G and Figure S4). Our results suggested that SMYD4 interacts with and catalyzes PRMT5 lysine monomethylation in HCC. SMYD4 may function through PRMT5 in HCC.

3.4 | SMYD4 regulates the transcriptional activity of PRMT5

We then explored the biological effects of PRMT5 after methylation by SMYD4. We found that SMYD4 knockdown did not influence PRMT5 expression (Figure 4A), suggesting monomethylation by SMYD4 may influence the function of PRMT5. We selected E-cadherin, RBL2, and DVL3 for further verification, the expression of which has been demonstrated to be transcriptionally regulated

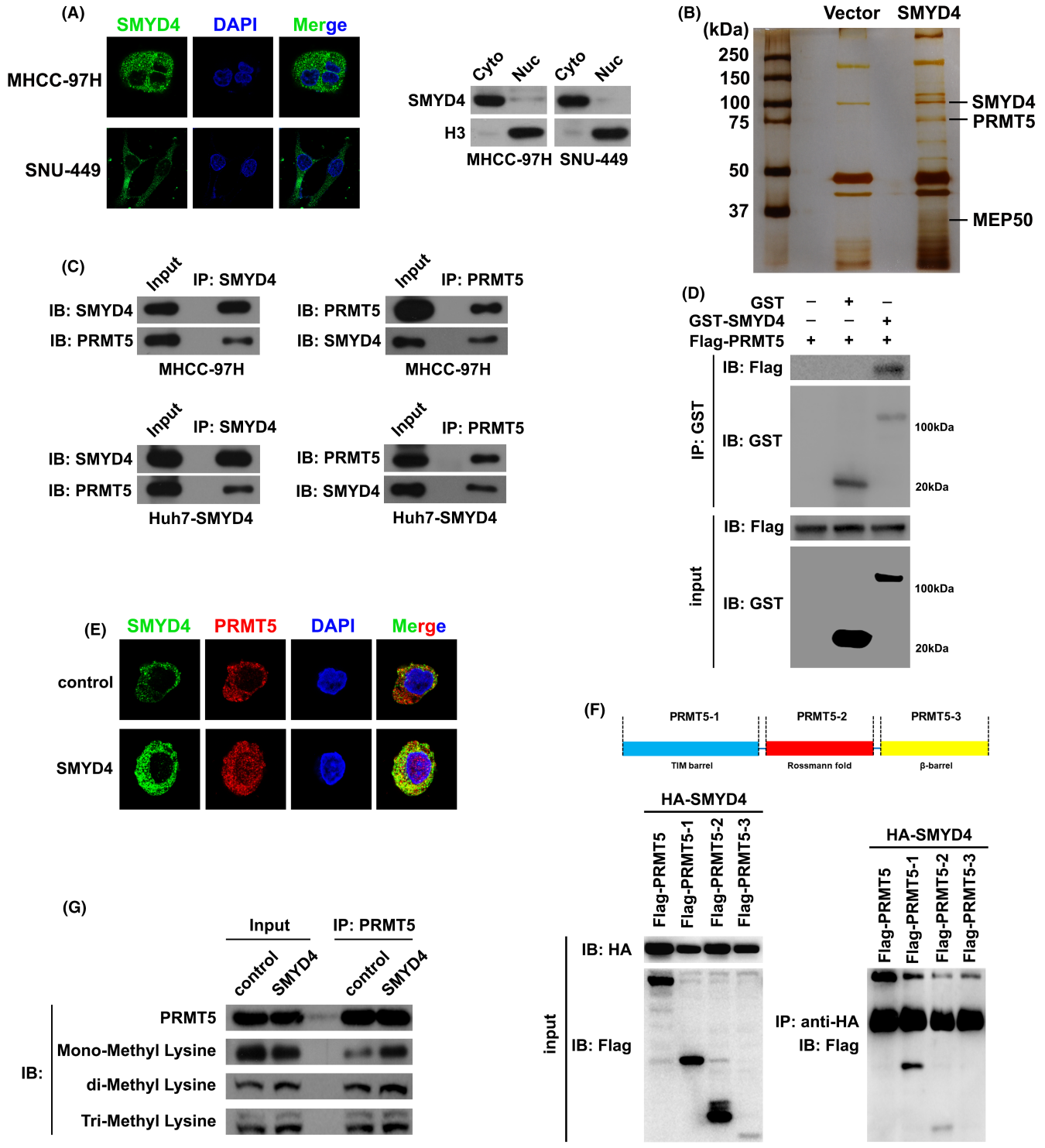

FIGURE 3 SMYD4 interacts with and monomethylates PRMT5. (A) IF showed the subcellular localization of SMYD4 (left panel). Subcellular fractions were isolated to analyze the expression of SMYD4 in cytoplasm and nucleus using Western blotting (right panel). (B) Silver staining of proteins immunoprecipitated with SMYD4 antibody in huh7-SMYD4 and its control cells. The black lines indicate the potential bands of PRMT5 and MEP50. (C) Lysates of MHCC-97H and Huh7-SMYD4 cells were immunoprecipitated for SMYD4 (endogenous and exogenous) or PRMT5 and immunoblotted for PRMT5 and SMYD4, respectively. (D) GST pull-down assay was used to confirm the protein interaction between SMYD4 and PRMT5. (E) IF showed the colocalization of SMYD4 and PRMT5 in MHCC-97H and Huh7-SMYD4 (transient transfection) cells; green: SMYD4, red: PRMT5, blue: DAPI. Merge shows the overlapped images. (F) Schematic illustration of the three regions of PRMT5 (upper panel). Flag-tagged full-length PRMT5 and a different region of PRMT5 were respectively cotransfected with HA-tagged SMYD4 into Huh7 cells. Cell lysates were immunoprecipitated with HA antibody and immunoblotted with Flag antibody. (G) Lysates of Huh7-SMYD4 and its control cells were immunoprecipitated with PRMT5 antibody. Membranes between 60 to 80 kDa (PRMT5 location) were immunoblotted with antibodies of PRMT5, monomethylated lysine, dimethylated lysine, and trimethylated lysine.

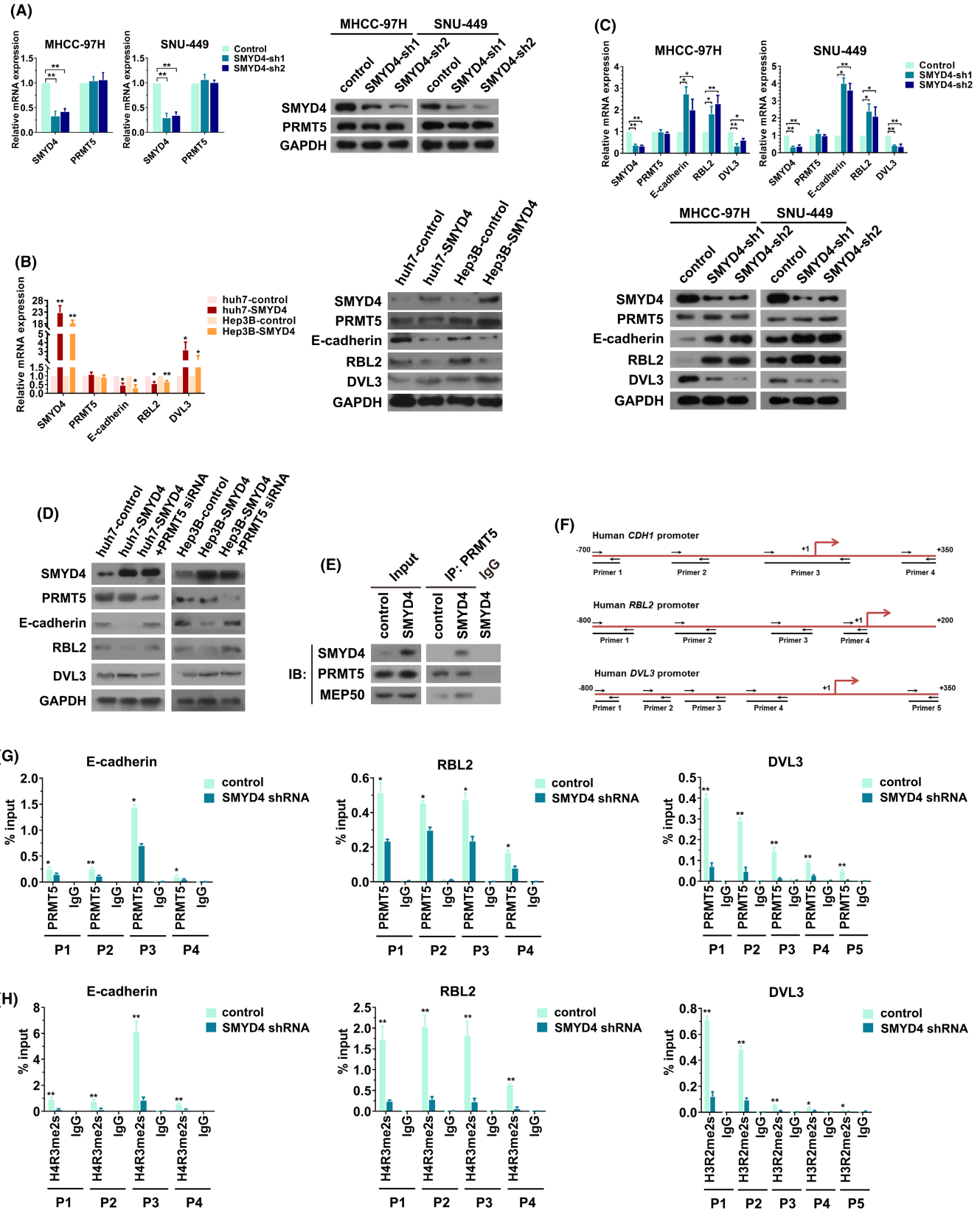

FIGURE 4 SMYD4 regulates the transcriptional activity of PRMT5. (A) mRNA and protein expression of PRMT5 in hepatocellular carcinoma (HCC) cells with SMYD4 knockdown. (B, C) mRNA and protein expression of PRMT5, E-cadherin, RBL2, and DVL3 in HCC cells with SMYD4 overexpression or knockdown. (D) HCC cells overexpressing SMYD4 were transfected with PRMT5 siRNA and then detected for protein expression of PRMT5, E-cadherin, RBL2, and DVL3. (E) Lysates of Huh7-SMYD4 and its control cells were immunoprecipitated for PRMT5 and immunoblotted for SMYD4, PRMT5, and MEP50. (F) Schematic illustrations of the promoter region of *CDH1* (E-cadherin), *RBL2*, and *DVL3* as well as location of the detected primers of each promoter. (G, H) ChIP assays were performed in MHCC-97H-SMYD4 shRNA and its control cells using antibody against PRMT5, H4R3me2s, or H3R2me2s as indicated. Immunoprecipitated DNA was measured by real-time PCR using primers indicated in (F). $^{*}p<0.05$, $^{**}p<0.01$.

by PRMT5.[15–17] Results from real-time PCR and Western blotting showed that SMYD4 overexpression suppressed E-cadherin and RBL2 expression and promoted DVL3 expression (Figure 4B). In contrast, SMYD4 knockdown increased E-cadherin and RBL2 expression and suppressed DVL3 expression (Figure 4C). Furthermore, PRMT5 knockdown significantly attenuated the SMYD4-mediated downregulation of E-cadherin and RBL2 and upregulation of DVL3 (Figure 4D and Figure S5A). These results suggest that the interaction between

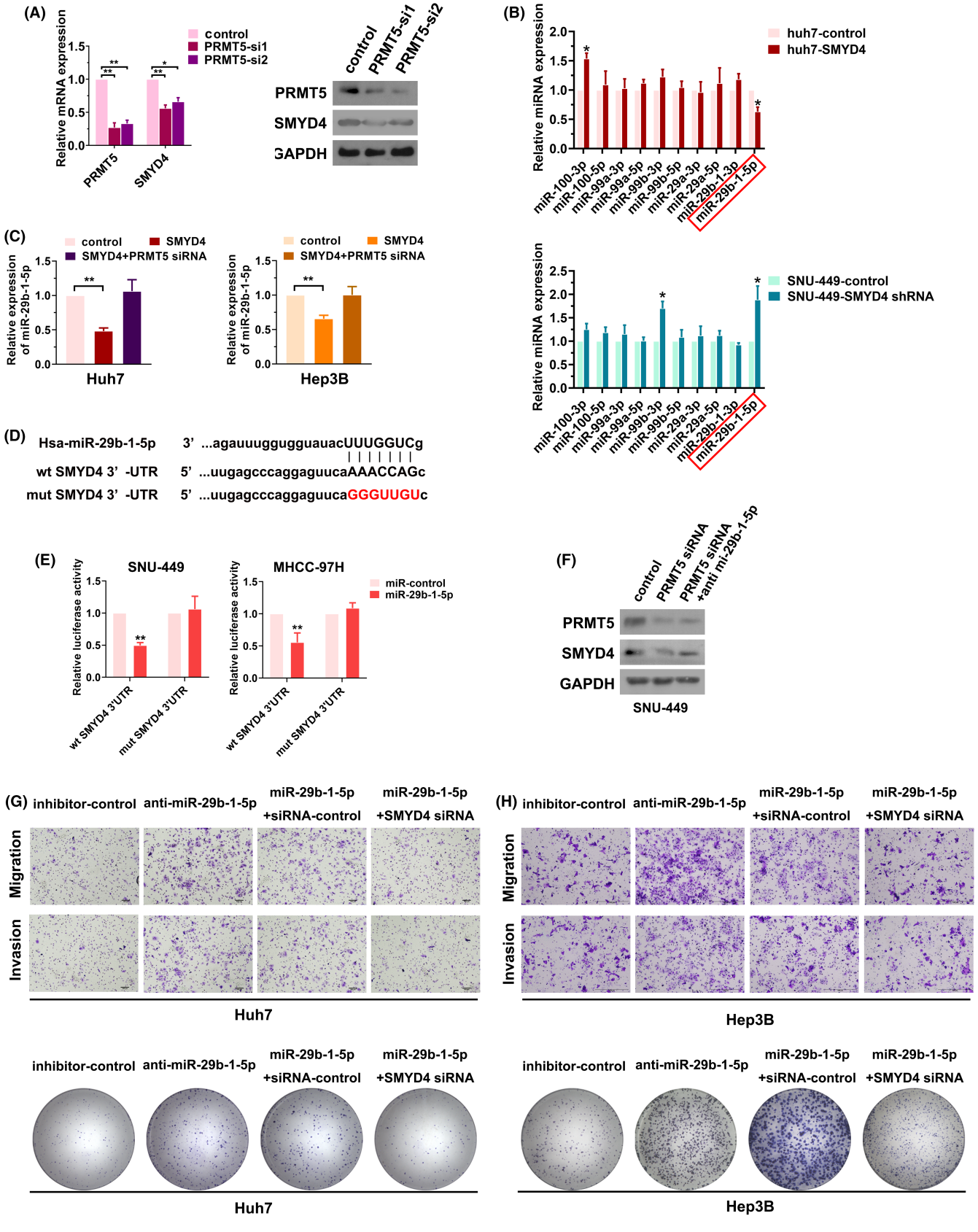

FIGURE 5 PRMT5 positively regulates SMYD4 expression through miR-29b-1-5p. (A) The mRNA and protein expression of SMYD4 in hepatocellular carcinoma (HCC) cells with or without PRMT5 knockdown. (B) The expression of PRMT5-targeted miRNAs in HCC cells with SMYD4 overexpression or knockdown. (C) The expression of miR-29b-1-5p in SMYD4-overexpressing HCC cells with or without PRMT5 knockdown. (D) The sequence of wild-type (wt) 3′-UTR and mutant (mut) 3′-UTR of SMYD4 responding to the seed region of miR-29b-1-5p. (E) Luciferase reporter assay in HCC cells cotransfected with the indicated luciferase reporter (wt or mut vector) and miR-29b-1-5p or control mimics. (F) The protein expression of SMYD4 in PRMT5-knockdown HCC cells with or without miR-29b-1-5p knockdown. (G, H) Migration, invasion, and colony formation assays were performed using miR-29b-1-5p-knockdown HCC cells with or without SMYD4 knockdown. $^{*}p < 0.05$, $^{**}p < 0.01$.

SMYD4 and PRMT5 may affect the methyltransferase activity of PRMT5.

MEP50 is a canonical cofactor of PRMT5, which is important for the methyltransferase activity of PRMT5.[18] Our results showed that SMYD4 overexpression enhanced the interaction between PRMT5 and MEP50 (Figure 4E), while SMYD4 knockdown decreased the PRMT5–MEP50 interaction (Figure S5B), suggesting the interaction between SMYD4 and PRMT5 may enhance the methyltransferase activity of PRMT5. We thus further investigated the mechanism of SMYD4-mediated transcription of E-cadherin, RBL2, and DVL3. PRMT5 suppresses *CDH1* (E-cadherin) and *RBL2* gene transcription by symmetrically dimethylating H4R3 at the promoter[15,16] and activates *DVL3* gene transcription by symmetrically dimethylating H3R2.[17] Our ChIP data with antibodies for PRMT5 revealed a significant enrichment of PRMT5 at the *CDH1*, *RBL2*, and *DVL3* gene promoters, but the recruitment of PRMT5 was significantly reduced when SMYD4 was knocked down (Figure 4F,G). Consistently, in the PRMT5-binding regions, there was significant enrichment of H4R3me2s and H3R2me2s, which also decreased when SMYD4 was knocked down (Figure 4F,H). We then performed luciferase reporter assays to confirm the functional regulation. The results showed that knockdown of SMYD4, consistent with PRMT5 siRNA treatment, suppressed *DVL3* promoter activity and enhanced *CDH1* promoter activity (Figure S5C), whereas overexpression of SMYD4 had opposite results (Figure S5D). More importantly, PRMT5 knockdown significantly reversed the effect of SMYD4 on *DVL3* and *CDH1* promoter activity (Figure S5D). In addition, we found that PRMT5 could not upregulate DVL3 expression and downregulate the expression of E-cadherin and RBL2 when SMYD4 was knocked down (Figure S5E,F). Together, these results suggest that SMYD4 is a vital regulator of PRMT5 in HCC and is responsible for the transcriptional activity of PRMT5.

3.5 | PRMT5 positively regulates SMYD4 expression through miR-29b-1-5p

We analyzed the correlation between SMYD4 and PRMT5 expression in HCC tissues. The results showed that SMYD4 was positively correlated with PRMT5 in both TCGA-LIHC and our center cohorts (Figure S6). However, our above results indicate that SMYD4 did not affect PRMT5 expression. We hypothesized that PRMT5 could regulate SMYD4 expression in HCC. The results showed that knockdown of PRMT5 inhibited SMYD4 expression at both mRNA and protein levels (Figure 5A), suggesting there may be a positive feedback loop between SMYD4 and PRMT5 in HCC.

Studies have indicated that PRMT5 also transcriptionally regulates the expression of a series of miRNAs.[19] We examined the miR-99 family (miR-99a-5p, miR-99b-5p, and miR-100-5p) and miR-29b-1-3p, which have been reported to be PRMT5-target miRNAs.[20,21] Additionally, miRNAs sharing the same promoter were also examined. We found that only miR-29b-1-5p was significantly regulated by SMYD4 among the ten miRNAs (Figure 5B). We also confirmed that PRMT5 knockdown significantly increased miR-29b-1-5p expression (Figure S7A) as well as reversed SMYD4-mediated downregulation of miR-29b-1-5p (Figure 5C). Functional experiments showed that miR-29b-1-5p knockdown promoted HCC cell migration, invasion, and proliferation (Figure S7B). Thus miR-29b-1-5p was selected for further studies.

Interestingly, we found SMYD4 was a potential target of miR-29b-1-5p predicted by two miRNA databases (TargetScan and miRanda) (Figure 5D). Further studies showed that miR-29b-1-5p overexpression remarkably downregulated the expression of SMYD4 and DVL3 and upregulated the expression of RBL2 and E-cadherin at both mRNA and protein levels (Figure S7C,D), whereas miR-29b-1-5p knockdown showed opposite results (Figure S7C,D). Results from luciferase reporter assay showed that miR-29b-1-5p overexpression significantly decreased the luciferase activity of cells transfected with the wild-type 3′-UTR of SMYD4 but not the cells transfected with a mutant 3′-UTR vector (Figure 5D,E; Figure S7E). Furthermore, both real-time PCR and Western blotting showed that miR-29b-1-5p knockdown significantly abolished the downregulation of SMYD4 induced by PRMT5 siRNA treatment (Figure 5F and Figure S7F). Finally, we determined whether SMYD4 was a functional mediator of miR-29b-1-5p. Transwell and colony formation assays showed that SMYD4 knockdown significantly reversed the tumor-promoting effect induced by the miR-29b-1-5p inhibitor (Figure 5G,H; Figure S8). Together, these results suggested that PRMT5 and SMYD4 form a positive feedback loop in HCC cells via miR-29b-1-5p.

3.6 | PRMT5 inhibitor suppresses the oncogenic effect of SMYD4

Considering the positive feedback loop formed by SMYD4 and PRMT5, we next investigated whether PRMT5 inhibitor could suppress the oncogenic effect of SMYD4 in HCC. Our results showed that PRMT5 inhibitor, JNJ-64619178, significantly rescued the SMYD4-induced dysregulation of E-cadherin, RBL2, DVL3, and miR-29b-1-5p (Figure 6A,B). Moreover, JNJ-64619178 treatment not only decreased the SMYD4-promoted enrichment of H4R3me2s

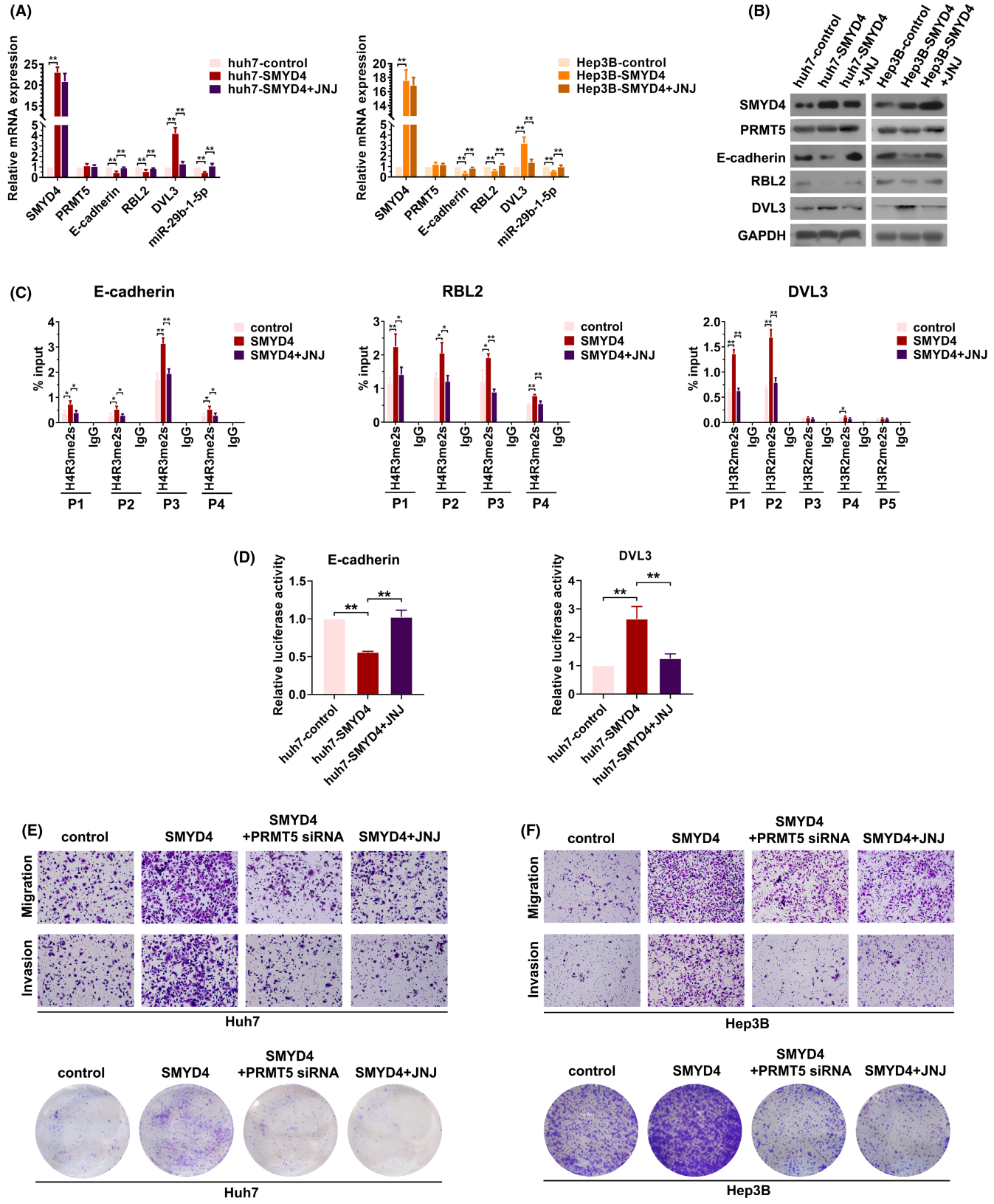

FIGURE 6 PRMT5 inhibitor suppresses the oncogenic effect of SMYD4 in vitro. (A, B) hepatocellular carcinoma (HCC) cells overexpressing SMYD4 were treated with PRMT5 inhibitor JNJ-64619178 or not and then detected for the mRNA and protein expression of PRMT5, E-cadherin, RBL2, DVL3, and miR-29b-1-5p. (C) ChIP assays were performed in huh7-SMYD4 and its control cells with or without JNJ-64619178 treatment using antibody against H4R3me2s or H3R2me2s antibodies as indicated. Immunoprecipitated DNA was measured by real-time PCR using primers indicated in Figure 4F. (D) Luciferase reporter assay in Huh7-SMYD4 cells transfected with the indicated luciferase reporter together with or without JNJ-64619178 treatment. (E, F) Migration, invasion, and colony formation assays were performed using SMYD4-overexpressing cells with PRMT5 knockdown or inhibition. $^{*}p < 0.05$, $^{**}p < 0.01$. JNJ, JNJ-64619178.

and H3R2me2s in the PRMT5-binding region of the target gene promoters (Figure 6C) but also rescued the effect of SMYD4 on *CDH1* and *DVL3* promoter activity (Figure 6D). Functionally, consistent with PRMT5 siRNA treatment, JNJ-64619178 treatment significantly suppressed SMYD4-enhanced cell migration, invasion, and proliferation (Figure 6E,F; Figure S9).

Subcutaneous xenograft, lung metastasis, and liver orthotopic xenograft models were used to further assess the effect of JNJ-64619178 on the function of SMYD4 in vivo. In each experiment, 7 days after tumor cell injection, the mice were treated with JNJ-64619178 (10 mg/kg, once a day) for 21 days (Figure 7A). We found that JNJ-64619178 treatment rescued the SMYD4-enhanced tumor growth and lung metastasis incidence (Figure 7B–F). Ki67 staining further confirmed the inhibitory effect of JNJ-64619178 on SMYD4-mediated tumor growth (Figure 7F,G). In addition, JNJ-64619178 treatment restored the SMYD4-mediated upregulation of

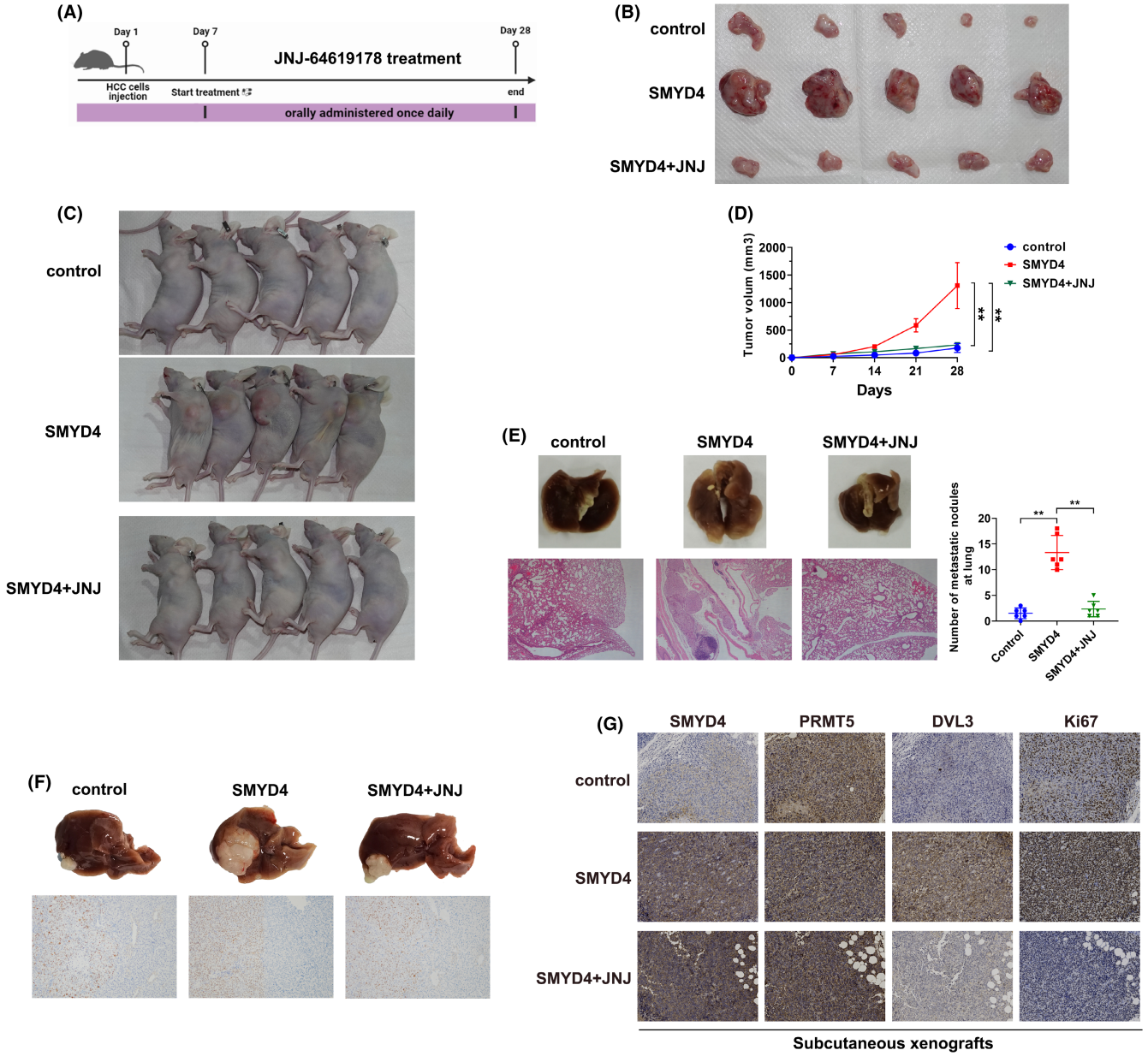

FIGURE 7 PRMT5 inhibitor suppresses the oncogenic effect of SMYD4 in vivo. (A) Schematic illustration of the in vivo experiments. (B, C) Nude mice were subcutaneously injected with Hep3B-SMYD4 or its control cells, with JNJ-64619178 treatment or not. The nude mice and its tumors in each group are presented ($n = 5$). (D) The tumor volumes of each group in subcutaneous xenograft experiments were measured every week and indicated by curves. (E) Lungs derived from nude mice with tail intravenous injection of Hep3B-SMYD4 or its control cells, with JNJ-64619178 treatment or not, and the corresponding H&E staining images ($n = 6$). Number of metastatic foci in the lungs of mice from each group was counted and indicated. (F) Orthotopic xenograft model using Hep3B-SMYD4 or its control cells, with JNJ-64619178 treatment or not ($n = 4$). Ki67 staining was conducted in sections of tumors from the three groups. (G) SMYD4, PRMT5, DVL3, and Ki67 stainings were conducted in serial sections of the subcutaneous xenograft tumors from the three groups. $^{**}p < 0.01$, scale bar: 100 μm. JNJ, JNJ-64619178.

DVL3 in xenografts (Figure 7G). Collectively, these data indicated JNJ-64619178 may be a choice for SMYD4-targeted therapy in HCC.

3.7 | SMYD4-PRMT5 coexpression is associated with HCC patient outcomes

Finally, we explore the clinical significance of the SMYD4-PRMT5 axis in patients with HCC using our tissue microarray. PRMT5 expression was positively correlated with SMYD4 expression in HCC clinical samples (Figure 8A,B). High DVL3 expression and low E-cadherin expression were more frequently observed in SMYD4 and PRMT5 high coexpression cases (Figure 8A,C). Kaplan–Meier analysis revealed that patients with simultaneously high expression of SMYD4 and PRMT5 had the shortest overall and disease-free survival, whereas SMYD4 and PRMT5 double-low-expression cases had the best prognosis (Figure 8D). These results provide clinical evidence that the SMYD4-PRMT5 positive-feedback axis is associated with the prognosis of HCC patients.

4 | DISCUSSION

SMYDs are involved in a wide range of biological processes, including the development and progression of many cancer types.[7] Among the five members, SMYD2 and SMYD3 are the best studied.[13] As for SMYD3, our previous study showed that it promotes HCC cell epithelial mesenchymal transition and metastasis through trimethylating H3K4 on the *SLUG* promoter, recruiting reader protein to H3K4me3, and then upregulating Slug expression.[11] We also found that SMYD3, together with HBx, promotes HCC progression by upregulating lncIHS, a lncRNA that we identified as an oncogene in HCC.[22] To further investigate the SMYDs family in HCC, we focused on SMYD4, the role of which has not yet been well defined in malignant tumors. In breast cancer, SMYD4 is reported as a tumor

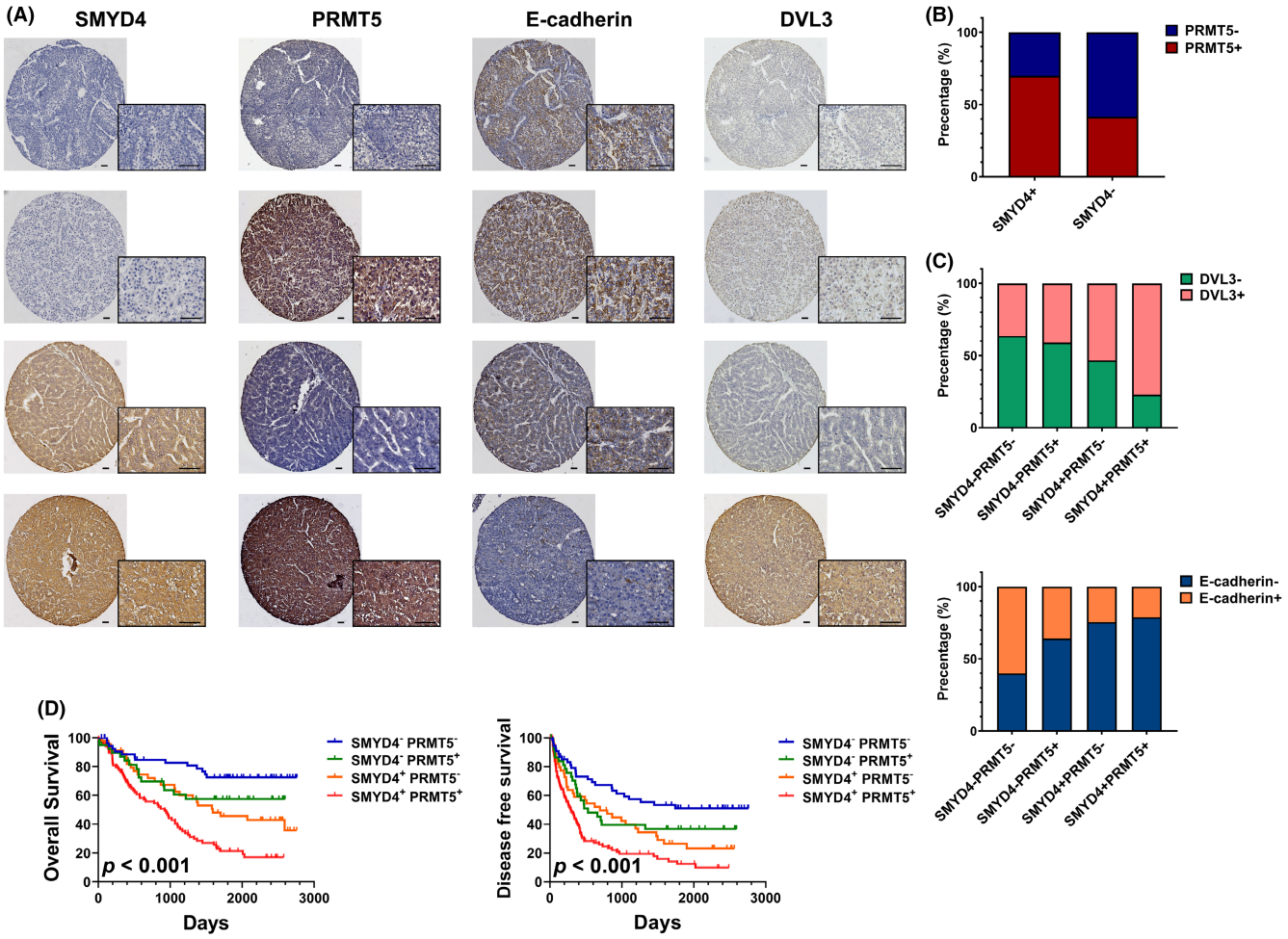

FIGURE 8 SMYD4-PRMT5 correlate with hepatocellular carcinoma (HCC) patient outcomes. (A) SMYD4, PRMT5, E-cadherin, and DVL3 stainings were conducted in serial sections of the tissue microarray of 243 HCC samples. Scale bar: 50 μm. (B) PRMT5 expression was positively correlated with SMYD4 expression in HCC clinical samples. (C) High DVL3 expression and low E-cadherin expression were more frequently observed in SMYD4 and PRMT5 high-coexpression cases. (D) Kaplan–Meier analysis of the overall survival or disease-free survival in HCC patients according to the coexpression level of SMYD4 and PRMT5.

suppressor.[23,24] It was found that SMYD4 was markedly decreased in breast cancer tissues and that disruption of SMYD4 expression promoted the transformation of breast epithelial cells.[23,24] In contrast, recently a research suggested that SMYD4 may act as an oncogene by binding to the promoter of *Nanog* (a stem cell transcription factor) and activate its expression.[25] In the present study (Figure 9), we found that SMYD4 is upregulated in HCC tissues in both public datasets and our center cohort. High SMYD4 expression correlates with poor patient survival. Moreover, our data showed that SMYD4 promotes HCC proliferation and metastasis in both in vitro and in vivo models. Mechanistically, SMYD4 functions in HCC by interacting with PRMT5, a well-known oncogene. Thus, we provide more comprehensive evidence to demonstrate that SMYD4 acts as an oncogene in HCC.

We and others have previously demonstrated that PRMT5 plays an essential role in HCC tumor growth and metastasis.[4,26–29] However, the regulation of PRMT5 in HCC remains little explored. One recent study reported that LINC01138 interacts with PRMT5 and blocks its ubiquitination and degradation in HCC.[29] In this study, we found that SMYD4 interacts with the TIM barrel and Rossmann fold of PRMT5 and leads to lysine monomethylation of PRMT5. The TIM barrel domain is the binding region for MEP50.[18,30] MEP50 is a canonical cofactor of PRMT5 which functions to stabilize the PRMT5 complex and enhances its methyltransferase activity.[18] Our results suggested that lysine monomethylation promotes the interaction between PRMT5 and MEP50, thereby enhancing the methyltransferase activity of PRMT5. Actually, studies on other malignant tumors have shown that PRMT5 can be regulated by post-translational modification.[31–33] Fan et al. found that, in myeloproliferative neoplasms, PRMT5 is phosphorylated by JAK2V617F at the N-terminal region.[31] The phosphorylation disrupts the association between PRMT5 and MEP50 and inhibits its methyltransferase activity.[31] On the other hand, PRMT5 was found to be asymmetrically dimethylated by CARM1 (PRMT4) at Arg-505, which is essential for PRMT5

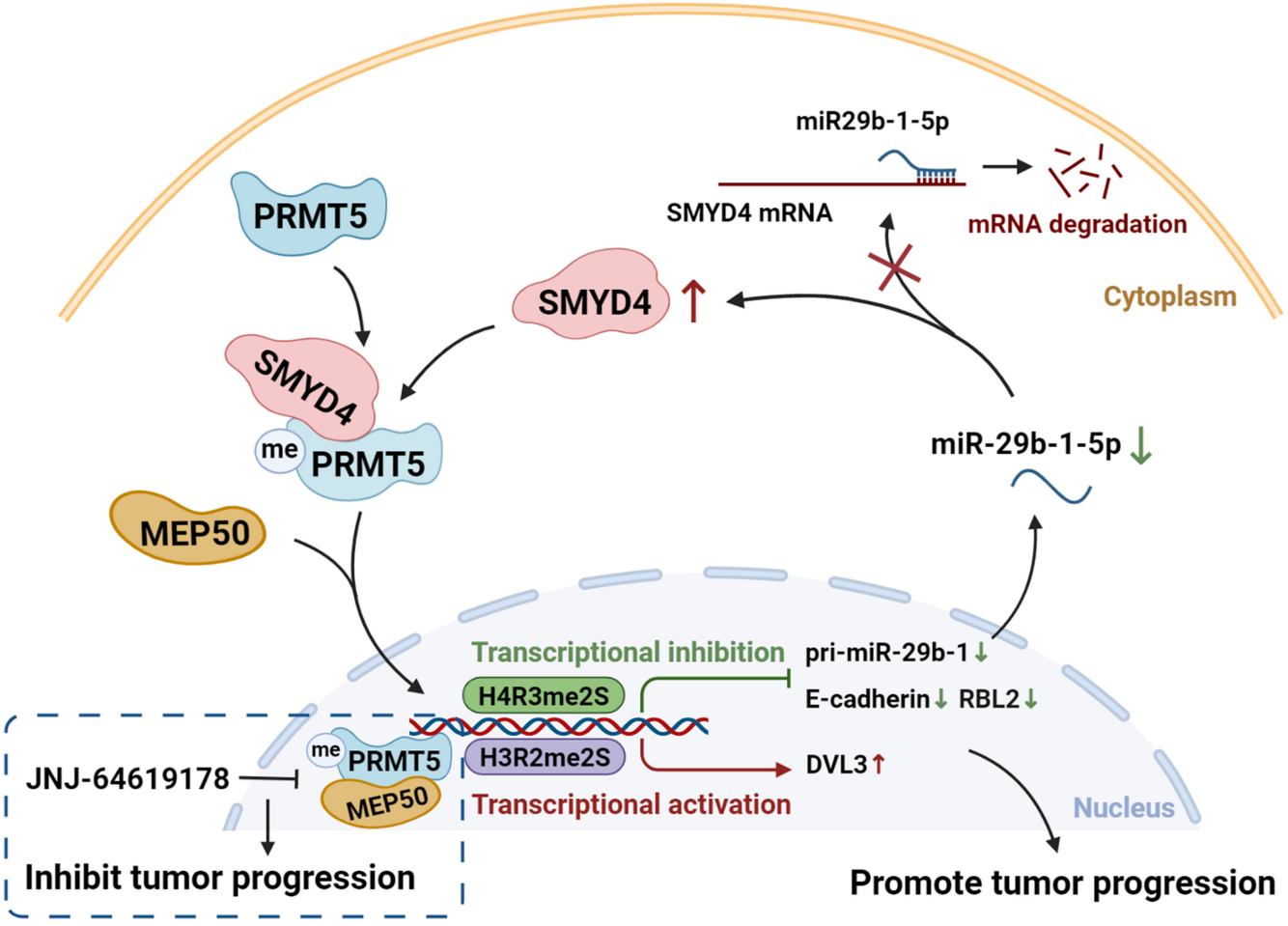

FIGURE 9 The proposed mechanism model of this study. The proposed mechanism model of this study. SMYD4 specially binds to PRMT5 and monomethylates PRMT5 in cytoplasm, which enhances the interaction between PRMT5 and MEP50. The PRMT5-MEP50 complex then promotes the symmetrical dimethylation of H3R2 and H4R3, which respectively activates DVL3 transcription and inhibits transcription of E-cadherin, RBL2, and pri-miR-29b-1. Subsequently, the downregulation of miR-29b-1-5p increases SMYD4 expression, forming a positive feedback loop. Together, this pathway promotes hepatocellular carcinoma (HCC) progression. PRMT5 inhibitor JNJ64619178 can interrupt the feedback loop and thus inhibit the progression of HCC cases with SMYD4 high expression.

homodimerization in erythroleukemia.[32] To date, few studies have reported lysine methylation of PRMT5. Our findings broaden the understanding of PRMT5 regulation mechanism in malignant tumors.

Both arginine methyltransferases and lysine methyltransferases are thought to be promising therapeutic targets for malignant tumors.[3,13] At this time, we could not identify a suitable inhibitor of SMYD4. Considering the positive feedback loop formed by SMYD4 and PRMT5, we employed a PRMT5 inhibitor to treat SMYD4-overexpressing cells. JNJ-64619178 is a novel, highly selective, potent, and clinical-stage PRMT5 inhibitor.[34,35] Unlike other reported PRMT5 inhibitors, JNJ-64619178 can simultaneously bind to S-adenosylmethionine (SAM) and substrate pockets of the PRMT5/MEP50 complex, thus exhibiting potent inhibitory effect.[34,35] Our results showed that JNJ-64619178 could significantly inhibit the SMYD4-mediated oncogenic effect in HCC, suggesting PRMT5 inhibitor may be a treatment choice for HCC cases with high SMYD4 expression. However, perhaps a combined treatment with both SMYD4 and PRMT5 inhibitors may achieve a synergistic effect.

In summary, we report in this study that SMYD4 acts as an oncogene in HCC by methylating and activating PRMT5. Furthermore, we found that SMYD4 forms a positive feedback loop with PRMT5. Inhibition of PRMT5 by JNJ-64619178 attenuates the SMYD4-mediated oncogenic effects in HCC (Figure 9). Therefore, our findings delineate the functional importance of SMYD4 in HCC progression and broaden the understanding of the PRMT5 regulatory mechanism, which provides a model of the crosstalk between lysine methyltransferases and arginine methyltransferases in HCC. Targeting SMYD4-PRMT5 signaling may provide potential therapeutic opportunities for HCC.

AUTHOR CONTRIBUTIONS

Zhenyu Zhou: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; validation; visualization; writing – original draft; writing – review and editing. **Zheng Chen:** Data curation; investigation; methodology; resources; software; validation; visualization. **Qianlei Zhou:** Data curation; investigation; validation. **Shiyu Meng:** Software; writing – review and editing. **Juanyi Shi:** Resources; software; validation; visualization. **Sintim Mui:** Investigation; validation; writing – review and editing. **Hai Jiang:** Conceptualization; resources; supervision. **Jianhong Lin:** Conceptualization; data curation; software. **Gui He:** Investigation; software; visualization. **Wenbin Li:** Data curation; funding acquisition; project administration. **Jianlong Zhang:** Conceptualization; data curation; funding acquisition. **Jie Wang:** Conceptualization; funding acquisition; project administration; supervision. **Chuanchao He:** Conceptualization; funding acquisition; project administration; supervision; writing – review and editing. **Yongcong Yan:** Conceptualization; data curation; formal analysis; funding acquisition; methodology; supervision; validation; visualization; writing – review and editing. **Zhiyu Xiao:** Conceptualization; funding acquisition; project administration; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data regarding the current study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Approval of the research protocol by an institutional review board: The study was approved by the Ethics Committee of Sun Yat-Sen Memorial Hospital (SYSMH).

Informed consent: Informed consent was obtained from each patient.

Registry and the registration no. of the study/trial: N/A.

Animal studies: The mouse studies were approved by the institutional animal care and use committee of Sun Yat-Sen University (SYSU-IACUC-2021-000615).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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