



Histone demethylase JMJD1A promotes colorectal cancer growth and metastasis by enhancing Wnt/ β -catenin signaling

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Kesong Peng^{‡1}, Guoqiang Su^{§1}, Jinmeng Ji[‡], Xiaojia Yang[‡], Mengmeng Miao[‡], Pingli Mo[‡], Ming Li[¶], Jianming Xu^{||}, Wengang Li^{¶2}, and Chundong Yu^{‡3}

From the [‡]State Key Laboratory of Cellular Stress Biology, Innovation Center for Cell Biology, School of Life Sciences, Xiamen University, Xiamen, Fujian 361102, China, [§]The First Affiliated Hospital of Xiamen University, Xiamen, Fujian 361003, China, the [¶]Xiamen City Key Laboratory of Biliary Tract Diseases, Xiang'an Hospital of Xiamen University, Xiamen, Fujian 361101, China, and the ^{||}Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030

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The histone demethylase Jumonji domain containing 1A (JMJD1A) is overexpressed in multiple tumors and promotes cancer progression. JMJD1A has been shown to promote colorectal cancer (CRC) progression, but its molecular role in CRC is unclear. Here, we report that JMJD1A is overexpressed in CRC specimens and that its expression is positively correlated with that of proliferating cell nuclear antigen (PCNA). JMJD1A knockdown decreased the expression of proliferative genes such as *c-Myc*, cyclin D1, and *PCNA*, suppressed CRC cell proliferation, arrested cell cycle progression, and reduced xenograft tumorigenesis. Furthermore, JMJD1A knockdown inhibited CRC cell migration, invasion, and lung metastasis by decreasing matrix metalloproteinase 9 (MMP9) expression and enzymatic activity. Moreover, bioinformatics analysis of GEO profile datasets revealed that JMJD1A expression in human CRC specimens is positively correlated with the expression of Wnt/ β -catenin target genes, including *c-Myc*, cyclin D1, and *MMP9*. Mechanistically, JMJD1A enhanced Wnt/ β -catenin signaling by promoting β -catenin expression and interacting with β -catenin to enhance its transactivation. JMJD1A removed the methyl groups of H3K9me2 at the promoters of *c-Myc* and *MMP9* genes. In contrast, the JMJD1A^{H1120Y} variant, which lacked demethylase activity, did not demethylate H3K9me2 at these promoters, failed to assist β -catenin to induce the expression of Wnt/ β -catenin target genes, and failed to promote CRC pro-

gression. These findings suggest that JMJD1A's demethylase activity is required for Wnt/ β -catenin activation. Of note, high JMJD1A levels in CRC specimens predicted poor cancer outcomes. In summary, JMJD1A promotes CRC progression by enhancing Wnt/ β -catenin signaling, implicating JMJD1A as a potential molecular target for CRC management.

Colorectal cancer (CRC)⁴ is the third most commonly diagnosed cancer in males and the second in females, with an established 1.4 million new cases and 693,900 deaths occurring worldwide in 2012 (1). In China, colorectal cancer is the fifth most commonly diagnosed cancer in males and the fourth in females, and it is the fifth leading cause of cancer death in 2015 (2). The high incidence and mortality of CRC urge us to figure out the molecular mechanism of CRC progression and develop effective therapeutics. However, the potential mechanism which triggers CRC development is not clearly identified.

Aberrant activation of the Wnt/ β -catenin signaling pathway, mostly caused by the mutations of the tumor suppressor adenomatous polyposis coli or oncogene β -catenin, is found in 90% of human CRC specimens and plays an essential role in CRC progression (3, 4). The canonical Wnt signaling initiates from the Wnt proteins interacting with frizzled, promoting the stabilization, nuclear translocation, and transcriptional activity of β -catenin through its interaction with transcription factors such as lymphoid enhancer-binding factor 1/T cell-specific transcription factor to transactivate their target genes, such as *c-Myc*, cyclin D1, and *MMP9* (5–7). Targeting Wnt/ β -catenin signaling pathway has been considered as a potential therapeutic strategy for CRC.

Epigenetic regulation is implicated in tumor development as it profoundly controls the transcription profile and phenotype of cancer cells (8). Histone methylation is a critical epigenetic modification that determines whether a gene is transcription-

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This article contains Figs. S1–S6.

¹ Both authors contributed equally to this work.

² To whom correspondence may be addressed. E-mail: lwg11861@163.com.

³ To whom correspondence may be addressed. Tel.: 86-592-2182013; Fax: 86-592-2182083; E-mail: cdyu@xmu.edu.cn.

⁴ The abbreviations used are: CRC, colorectal cancer; JMJD1A, Jumonji domain containing 1A; PCNA, proliferating cell nuclear antigen; MMP9, matrix metalloproteinase 9; IP, immunoprecipitation; ChIP, chromatin immunoprecipitation; H3K9, histone-3 lysine-9; GEO, Gene Expression Omnibus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AOM/DSS, azoxymethane/dextran sodium sulfate.

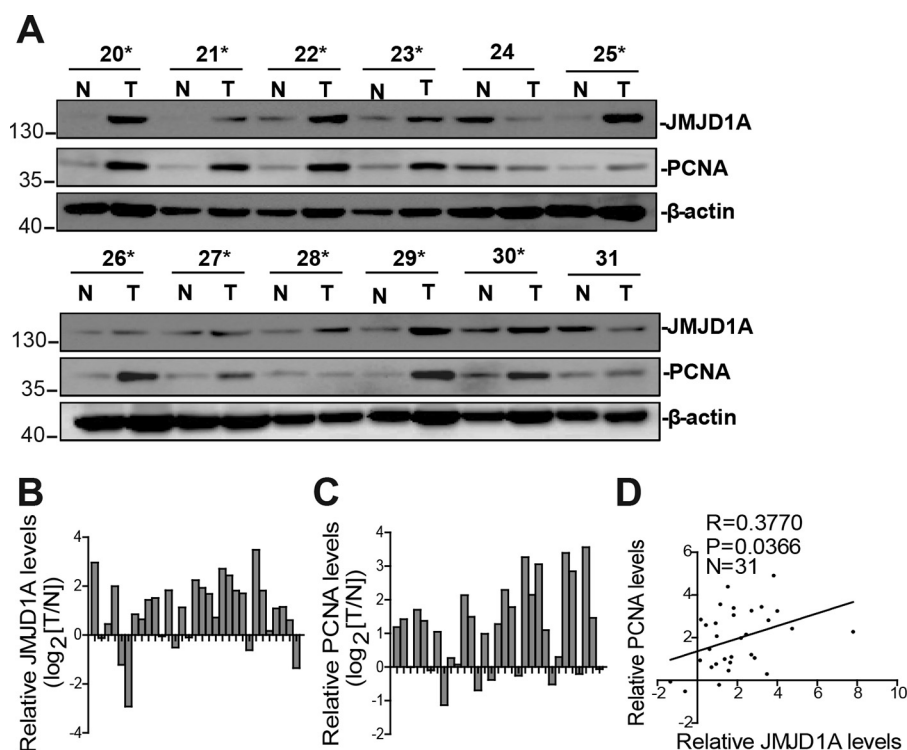


Figure 1. JMJD1A is frequently overexpressed in human CRC specimens and positively correlated with PCNA. A, JMJD1A was frequently overexpressed in human CRC specimens. The protein levels of JMJD1A and PCNA in human CRC specimens and the surrounding nontumorous tissues were measured by Western blot analysis ($n = 31$), and representative blots are shown. N, nontumorous tissue; T, tumor tissue; *, JMJD1A-positive CRC specimen. β -Actin serves as a loading control. B and C, expression of JMJD1A and PCNA was up-regulated in the majority of CRC specimens. Quantitation of relative protein levels of JMJD1A and PCNA in CRC specimens was performed by ImageJ software. Band density of JMJD1A and PCNA was quantified and normalized to β -actin level. D, protein levels of JMJD1A were positively correlated to the protein levels of PCNA.

ally active or inactive (9). Both histone methylation and demethylation are dynamically regulated by respective methyl transferases and demethylases. Methylation of histone-3 lysine-9 (H3K9) is a repressive histone marker associated with transcriptional inactivation. Histone demethylase JMJD1A (Jumonji domain containing 1A, also named KDM3A or JHDM2A) belongs to the Jumonji C-domain containing histone demethylase family. It serves as a positive regulator of transcription of several growth-promoting genes by removing mono- and di-methyl groups from H3K9 (specifically, from H3K9me1 or H3K9me2) *in vitro* and *in vivo* (10–12). JMJD1A functions as a key regulator in spermatogenesis, germ cell development, metabolism, sex determination, stem cell self-renewal, and differentiation, as well as a coactivator for androgen receptor or hypoxia-inducing factor-1 α (HIF-1 α) (12–17). Although JMJD1A recently was shown to be able to maintain tumorigenic potentials of human colorectal cancer stem cells via enhancing Wnt/ β -catenin signaling (18), the role of JMJD1A in CRC growth and metastasis as well as how JMJD1A enhances Wnt/ β -catenin signaling remain to be further elucidated.

In this study, we showed that JMJD1A was overexpressed in human CRC specimens and promoted CRC cell proliferation, colony formation, migration, invasion, tumorigenesis, and metastasis by interacting with β -catenin to enhance Wnt/ β -catenin signaling, and the demethylase activity of JMJD1A was required for its functions.

Results

JMJD1A is frequently overexpressed in human CRC specimens and positively correlated with PCNA

To determine the role of JMJD1A in CRC, Western blot analysis was used to determine the protein levels of JMJD1A in a set of 31 pairs of human CRC specimens and the surrounding nontumorous tissues. The results showed that the protein levels of JMJD1A were overexpressed in 23 (74.2%) of 31 human CRC specimens compared with the surrounding nontumorous tissues (Fig. 1, A and B). In addition, the proliferation marker PCNA was also up-regulated in tumor specimens (Fig. 1, A and C). Moreover, a positive correlation between the expression of JMJD1A and PCNA was identified (Fig. 1D). These results implicate that JMJD1A might play a promoting role in CRC progression.

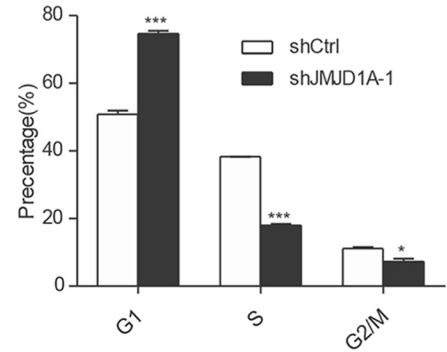
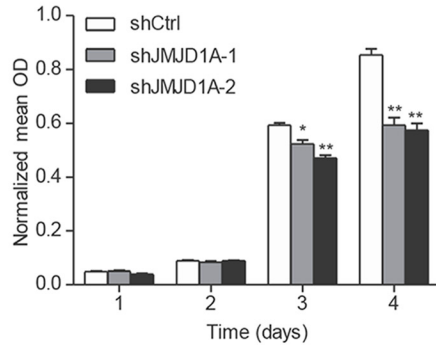
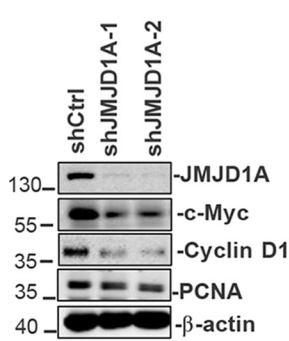
Down-regulation of JMJD1A inhibits CRC cell proliferation, colony formation, and xenograft tumor formation

Because JMJD1A was overexpressed in CRC tumor specimens and positively correlated with PCNA, we postulated that JMJD1A might promote CRC cell proliferation. To test this, human CRC cell lines HCT116 and SW480 were stably transfected with two different human JMJD1A-knockdown shRNA constructs (pll3.7-shJMJD1A-1 and pll3.7-shJMJD1A-2), respectively, and then MTT assay and cell cycle progression assay were performed to measure the effects of JMJD1A knockdown on CRC cell proliferation. As shown in Fig. 2, A and B,

JMJD1A promotes colorectal cancer growth and metastasis

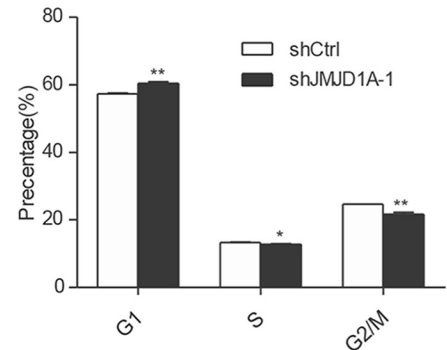
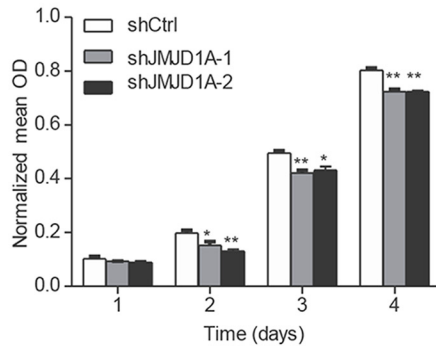
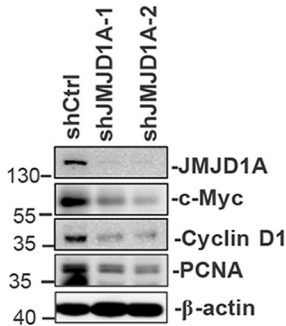
A

HCT116 cells

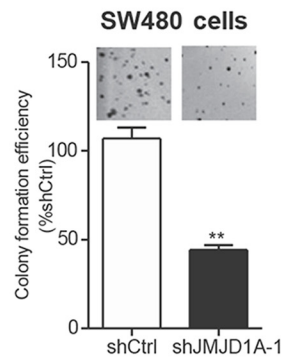
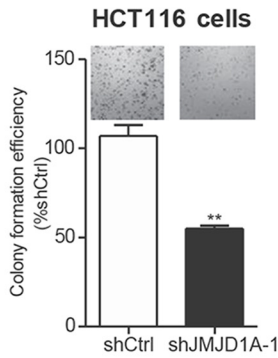


B

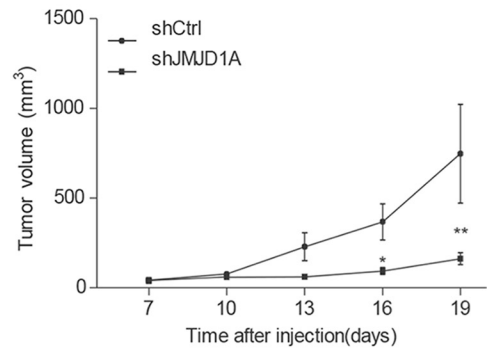
SW480 cells



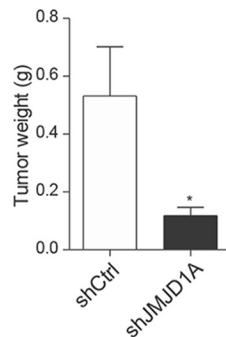
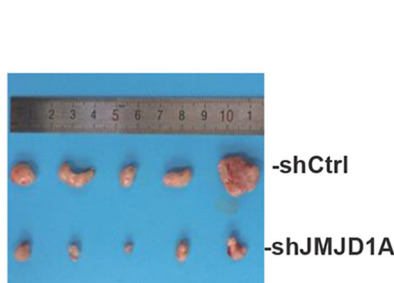
C



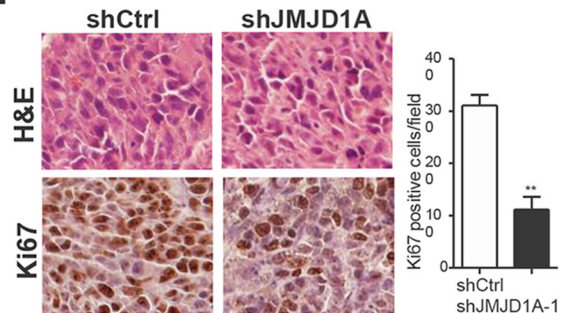
D



E



F



JMJD1A knockdown led to a down-regulation of several proliferation-related genes such as *c-Myc*, cyclin D1, and *PCNA*, a reduction of cell proliferation rate, and an arrest of cell cycle progression at the G₁ phase. Furthermore, rescue of *c-Myc* or cyclin D1 expression in JMJD1A-knockdown HCT116 cells restored cell proliferation (Fig. S1, A and B). These results suggest that knockdown of JMJD1A could inhibit CRC cell proliferation at least in part through reducing the expression of *c-Myc* and cyclin D1.

To determine the effect of JMJD1A knockdown on the tumorigenesis of CRC cells, the colony formation assay and xenograft tumor formation assay were performed. Knockdown of JMJD1A decreased the colony formation abilities of both JMJD1A-knockdown HCT116 and SW480 cells (Fig. 2C). Knockdown of JMJD1A in HCT116 cells markedly reduced xenograft tumor growth and tumor weight (Fig. 2, D and E). Furthermore, knockdown of JMJD1A significantly reduced the number of Ki67-positive CRC cells in tissue sections from JMJD1A-knockdown tumors (Fig. 2F). These results indicate that JMJD1A knockdown inhibits the tumorigenesis of CRC cells.

Knockdown of JMJD1A inhibits CRC cell migration, invasion, and metastasis

Invasion and metastasis are two important characteristics of malignant tumor cells. Therefore, we determined whether JMJD1A plays a role in CRC cell invasion and metastasis. We first measured the effects of JMJD1A knockdown on the migratory and invasive abilities of CRC cells by using transwell cell migration and invasion assays. The results showed that knockdown of JMJD1A reduced the migratory and invasive abilities of CRC cells (Fig. 3, A and B). Furthermore, knockdown of JMJD1A reduced the protein levels and enzyme activity of MMP9 (Fig. 3, A and B), a matrix metalloproteinase that plays an important role in the migration and invasion of a variety of tumor cells. Furthermore, rescue of MMP9 expression in JMJD1A-knockdown HCT116 cells restored cell migration and invasion (Fig. S2). These results indicate that JMJD1A knockdown inhibits the migratory and invasive potential of CRC cells at least in part through down-regulation of MMP9.

We next measured the effects of JMJD1A knockdown on CRC metastasis *in vivo* by injecting JMJD1A knockdown and control HCT116 cells into nude mice through the tail vein, respectively. Twenty days after cell injection, the lung tissues were harvested for analysis. Knockdown of JMJD1A significantly decreased lung metastasis (Fig. 3C). Ki67 staining for lung tissue sections from JMJD1A knockdown and control HCT116 tumors displayed that knockdown of JMJD1A significantly reduced the number of Ki67-positive HCT116 cells (Fig. 3D). These results indicate that JMJD1A knockdown inhibits CRC metastasis.

JMJD1A enhances Wnt/ β -catenin signaling by promoting β -catenin expression and interacting with β -catenin to enhance its transactivation

Having shown that *c-Myc*, cyclin D1, and *MMP9*, the downstream target genes of the Wnt/ β -catenin signaling pathway (Fig. S3), were down-regulated in JMJD1A knockdown cells, we postulated that JMJD1A might enhance Wnt/ β -catenin signaling to up-regulate the transcription of *c-Myc*, cyclin D1, and *MMP9* genes. To test this, we first detected whether JMJD1A knockdown could reduce the mRNA levels of *c-Myc*, cyclin D1, and *MMP9*. As shown in Fig. 4A, knockdown of JMJD1A decreased the mRNA levels of *c-Myc*, cyclin D1, and *MMP9*, implicating that JMJD1A may regulate the expression of *c-Myc*, cyclin D1, and *MMP9* at the transcriptional level. Next, we transfected JMJD1A and/or β -catenin expression plasmids, together with *c-Myc* or the *MMP9* promoter reporter into 293T cells, respectively, and then performed dual-luciferase activity assays. The results showed that overexpression of JMJD1A and β -catenin synergistically enhanced the promoter activities of *c-Myc* and *MMP9* (Fig. 4B). Furthermore, we analyzed the effects of overexpression of JMJD1A and β -catenin on the activity of the Topflash reporter, a well-established Wnt/ β -catenin reporter. As shown in Fig. 4C, JMJD1A and β -catenin synergistically enhanced Topflash reporter activity in the absence or presence of LiCl, a Wnt/ β -catenin activator. Taken together, these results suggest that JMJD1A can up-regulate the expression of *c-Myc*, cyclin D1, and *MMP9* by cooperating with β -catenin to enhance Wnt/ β -catenin signaling.

Because JMJD1A can enhance Wnt/ β -catenin signaling, we first examined whether JMJD1A can regulate β -catenin expression. The results showed that knockdown of JMJD1A reduced the protein and mRNA levels of β -catenin (Fig. 4, D and E), whereas overexpression of JMJD1A increased the protein and mRNA levels of β -catenin (Fig. 4, F and G), suggesting that JMJD1A can positively regulate β -catenin expression. To determine whether JMJD1A regulates β -catenin expression at transcriptional level, we co-transfected the β -catenin promoter reporter and JMJD1A expression plasmid into 293T cells and then performed dual-luciferase activity assays. As shown in Fig. 4H, JMJD1A dose-dependently enhanced the β -catenin promoter reporter activity. Furthermore, we used ChIP assay to detect whether JMJD1A can be recruited to the β -catenin promoter to demethylate H3K9me2. Our results showed that overexpressed JMJD1A increased the recruitment of JMJD1A to the β -catenin promoter (Fig. 4I) but reduced the H3K9me2 levels at the β -catenin promoter (Fig. 4J). In contrast, knockdown of JMJD1A reduced the recruitment of JMJD1A (Fig. 4K) but increased the H3K9me2 levels at the β -catenin promoter (Fig. 4L). These results suggest that JMJD1A enhances Wnt/ β -

Figure 2. JMJD1A promotes CRC cell proliferation and xenograft tumor formation. A and B, knockdown of JMJD1A suppressed the cell proliferation of HCT116 (A) and SW480 (B). The protein levels of JMJD1A, *c-Myc*, cyclin D1, and *PCNA* were measured by Western blot analysis. Cell growth and cell cycle progression were measured by MTT and flow cytometry assays. The experiments were repeated three times independently. C, knockdown of JMJD1A decreased the colony formation of HCT116 and SW480 cells. The experiments were repeated three times independently. D and E, knockdown of JMJD1A inhibited HCT116 xenograft tumor growth and reduced tumor weight in nude mice. *n* = 5. The experiments were repeated twice independently. F, number of Ki67-positive cells in tumor tissue sections was reduced in JMJD1A-knockdown tumors compared with control tumors. The experiments were repeated twice independently. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001.

JMJD1A promotes colorectal cancer growth and metastasis

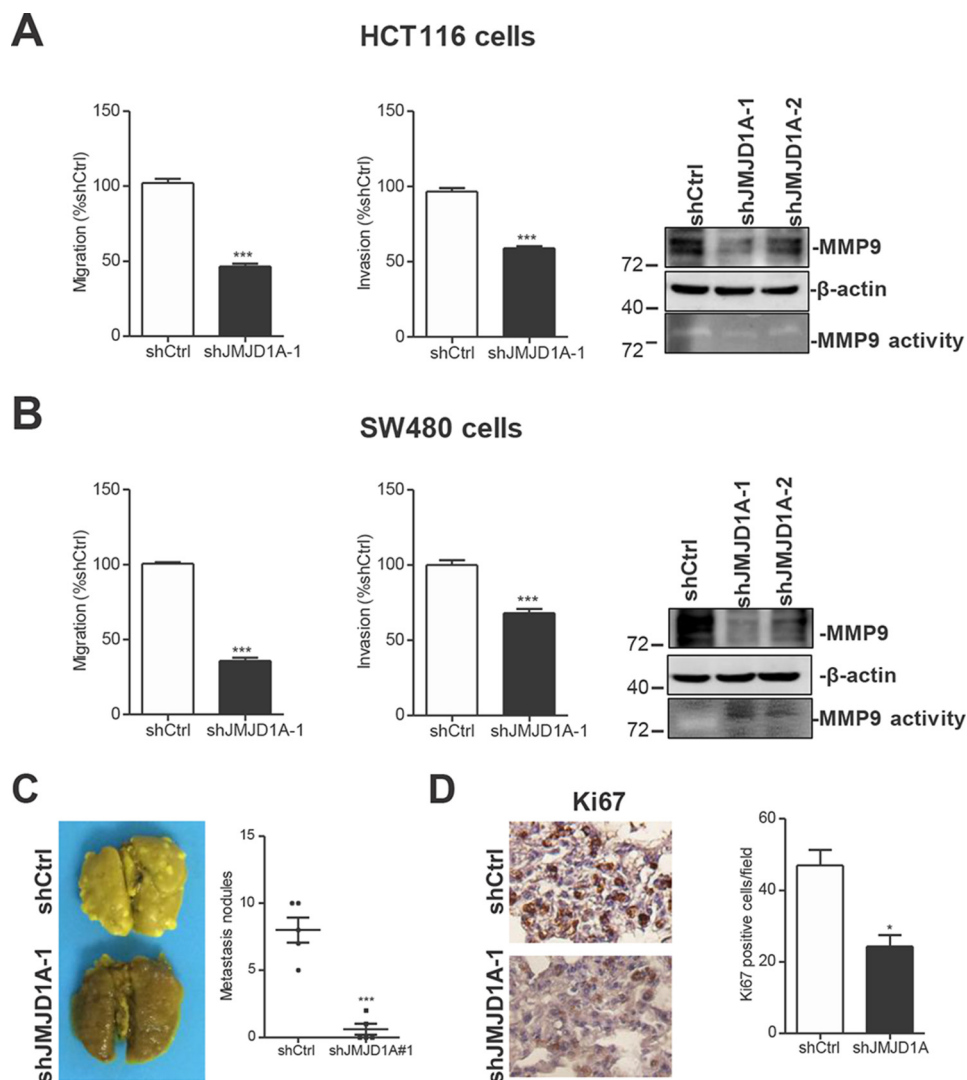


Figure 3. JMJD1A promotes CRC cell migration, invasion, and metastasis. A and B, knockdown of JMJD1A suppressed migration and invasion of HCT116 (A) and SW480 (B) cells. The protein expression and enzymatic activity of MMP9 were assessed by Western blotting and gel zymography assays, respectively. Cell migration and invasion were performed by transwell assays. The experiments were repeated three times independently. C, knockdown of JMJD1A decreased the lung metastasis of HCT116 cells. The experiments were repeated twice independently. D, number of Ki67-positive cells in tumor tissue sections was reduced in JMJD1A-knockdown tumors compared with control tumors. The experiments were repeated twice independently. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

catenin signaling at least in part by promoting β -catenin expression.

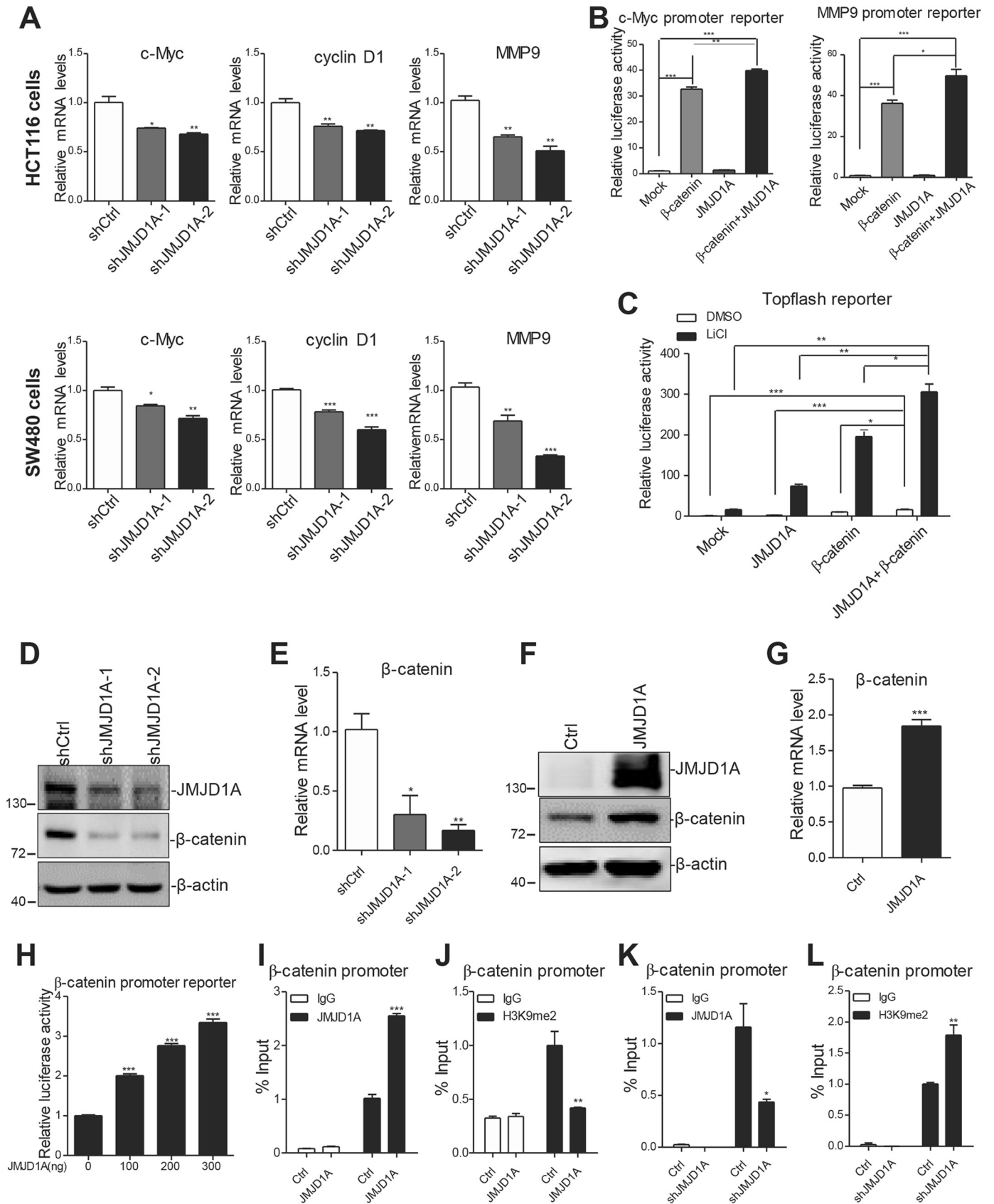
We next asked whether JMJD1A could interact with β -catenin. As shown in Fig. 5, A and B, the interaction between endogenous JMJD1A and β -catenin in HCT116 and SW480 cells was able to be detected by Co-IP assay using anti-JMJD1A and anti- β -catenin antibodies, respectively. This result is consistent with a previous report showing that JMJD1A can interact with β -catenin (18). To determine whether JMJD1A can directly bind to β -catenin, the GST pull-down assay was performed by incubating *Escherichia coli*-produced GST- β -catenin protein with JMJD1A protein produced by an *E. coli* extract-based cell-free protein synthesis system. The results showed that the GST- β -catenin protein, but not GST, was able to pull down JMJD1A (Fig. 5C), indicating that JMJD1A directly binds to β -catenin. Furthermore, GST pull-down assays showed that β -catenin interacted with the zinc finger domain of

JMJD1A (Fig. 5D), whereas JMJD1A interacted with the Arm 3–10 domain of β -catenin (Fig. 5E).

Taken together, our data suggest that JMJD1A enhances Wnt/ β -catenin signaling at least through two mechanisms: one is to increase the expression of β -catenin, and the other is to interact with β -catenin to enhance its transactivation.

JMJD1A decreases the levels of H3K9me2 at the promoters of c-Myc and MMP9 to enhance β -catenin transactivation

Because JMJD1A can cooperate with β -catenin to enhance Wnt/ β -catenin signaling, it was expected that knockdown of JMJD1A reduced the promoter activities of c-Myc, MMP9, and Topflash in HCT116 cells in the absence or presence of LiCl (Fig. 6A). To determine whether JMJD1A could be recruited to the promoters of c-Myc and MMP9 to demethylate H3K9me2 when Wnt/ β -catenin signaling is activated, HCT116 cells were treated with LiCl, and then the recruitment of β -catenin and



JMJD1A promotes colorectal cancer growth and metastasis

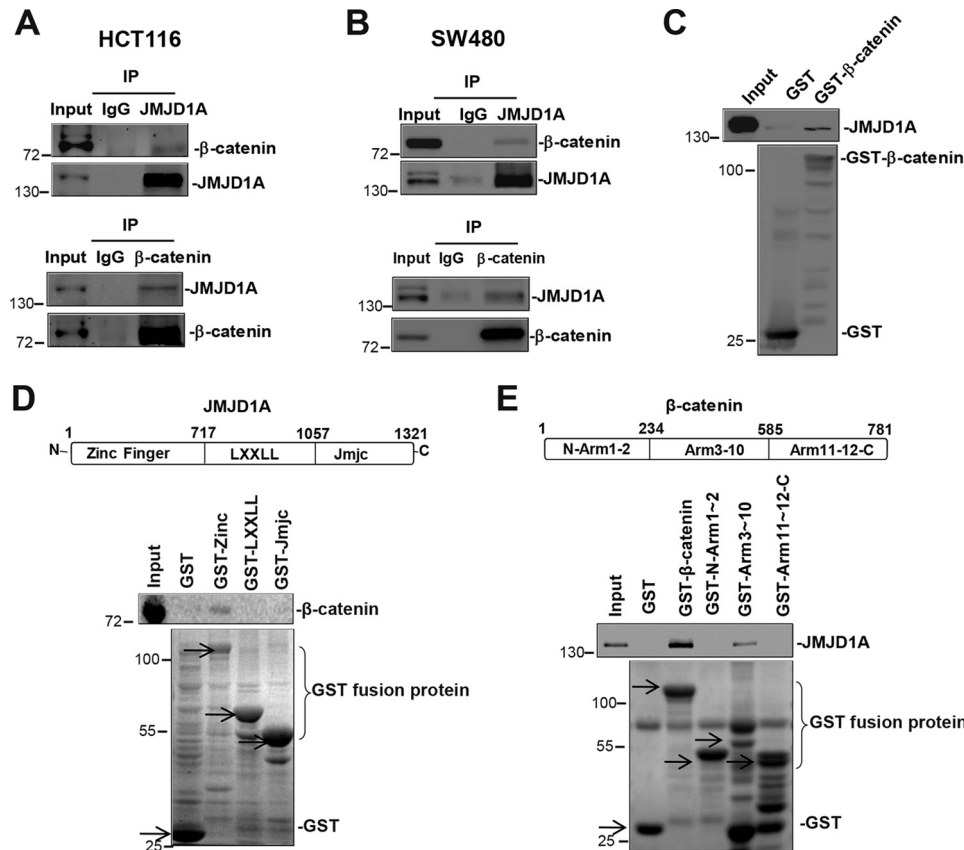


Figure 5. JMJD1A directly interacts with β -catenin. A and B, JMJD1A interacted with β -catenin. The interaction between endogenous JMJD1A and β -catenin in HCT116 and SW480 cells was detected by Co-IP assay, respectively. C, GST pull-down analysis of the direct interaction between JMJD1A and β -catenin *in vitro*. D, GST pull-down analysis of the interaction between β -catenin and different domains of JMJD1A. E, GST pull-down analysis of the interaction between JMJD1A and different domains of β -catenin. All experiments were repeated three times independently.

JMJD1A to the promoters of *c-Myc* and *MMP9* was detected by ChIP assay. As shown in Fig. 6B, LiCl treatment increased the recruitment of β -catenin, indicating that Wnt/ β -catenin signaling is activated by LiCl. LiCl treatment led to an increase of JMJD1A recruitment (Fig. 6C), but a decrease of H3K9me2 levels at the promoters of *c-Myc* and *MMP9* (Fig. 6D), indicating that activation of Wnt/ β -catenin signaling could enhance the recruitment of JMJD1A to the promoters of *c-Myc* and *MMP9* to demethylate H3K9me2. In contrast, down-regulation of β -catenin reduced the recruitment of β -catenin (Fig. 6E) and JMJD1A (Fig. 6F) to the promoters of *c-Myc* and *MMP9* but increased the H3K9me2 levels at the promoters of *c-Myc* and *MMP9* (Fig. 6G). When down-regulation of JMJD1A reduced the recruitment of JMJD1A (Fig. 6H), but increased the levels of H3K9me2 at the promoters of *c-Myc* and *MMP9* (Fig. 6I), the recruitment of β -catenin was markedly decreased (Fig. 6J), indicating that JMJD1A can enhance the recruitment of β -catenin to the promoters of *c-Myc* and *MMP9*.

Demethylase activity of JMJD1A is required for Wnt/ β -catenin activation and enhancement of CRC cell proliferation, migration, invasion, xenograft tumor formation, and metastasis

It has been reported that the histidine at amino acid 1120 of JMJD1A (His-1120) is essential for its histone demethylase function (12). We found that JMJD1A^{H1120Y} variant failed to reduce the levels of H3K9me2 at the promoters of *c-Myc* and *MMP9* as compared with WT JMJD1A (Fig. 7A), suggesting that the demethylase activity of JMJD1A is required for reducing the levels of H3K9me2 at the promoters of *c-Myc* and *MMP9*. Furthermore, we found that although the JMJD1A^{H1120Y} variant could bind to β -catenin (Fig. S4), it was unable to assist β -catenin to enhance the promoter activities of *c-Myc*, *MMP9*, and *Topflash* (Fig. 7, B and C), and it was unable to induce the expression of β -catenin target genes such as *c-Myc*, *cyclin D1*, and *MMP9* (Fig. 7D).

Figure 4. JMJD1A enhances Wnt/ β -catenin signaling by promoting β -catenin expression. A, knockdown of JMJD1A reduced the mRNA levels of *c-Myc*, *cyclin D1*, and *MMP9*. The mRNA levels were analyzed by real-time PCR. B, JMJD1A cooperated with β -catenin to enhance the promoter activities of *c-Myc* and *MMP9*. C, JMJD1A cooperated with β -catenin to enhance the *Topflash* reporter activity in 293T cells. D and E, knockdown of JMJD1A reduced the protein and mRNA levels of β -catenin in HCT116 cells. F and G, overexpression of JMJD1A increased the protein and mRNA levels of β -catenin in HCT116 cells. H, JMJD1A dose-dependently increased the activities of β -catenin promoter in 293T cells. I and J, overexpression of JMJD1A increased the levels of JMJD1A (I) but reduced the levels of H3K9me2 (J) at the promoter of β -catenin. K and L, knockdown of JMJD1A decreased the levels of JMJD1A (K) but increased the levels of H3K9me2 (L) at the promoter of β -catenin. All experiments were repeated three times independently. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

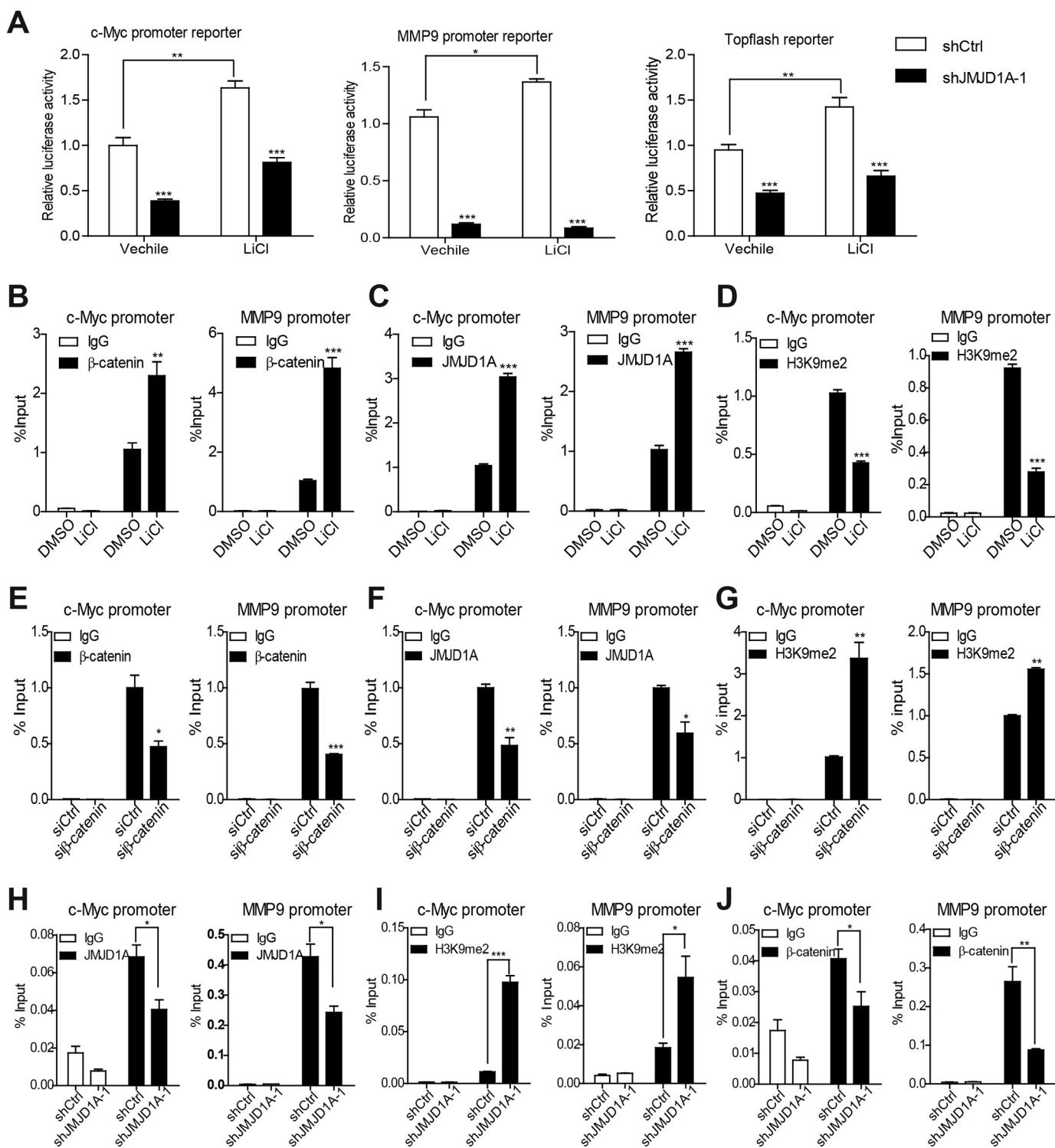


Figure 6. JMJD1A decreases H3K9me2 levels at the promoters of c-Myc and MMP9 to enhance β -catenin transactivation in HCT116 cells. *A*, knockdown of JMJD1A reduced the activities of c-Myc and MMP9 promoters and Topflash reporter with or without LiCl treatment. *B*, ChIP analysis of the levels of β -catenin at the promoters of c-Myc and MMP9 with or without LiCl treatment. *C*, ChIP analysis of the levels of JMJD1A at the promoters of c-Myc and MMP9 with or without LiCl treatment. *D*, ChIP analysis of the levels of H3K9me2 at the promoters of c-Myc and MMP9 with or without LiCl treatment. *E*, ChIP analysis of the levels of β -catenin at the promoters of c-Myc and MMP9 in β -catenin knockdown HCT116 cells. *F*, ChIP analysis of the levels of JMJD1A at the promoters of c-Myc and MMP9 in β -catenin knockdown HCT116 cells. *G*, ChIP analysis of the levels of H3K9me2 at the promoters of c-Myc and MMP9 in β -catenin knockdown HCT116 cells. *H* and *I*, knockdown of JMJD1A decreased the levels of JMJD1A (*H*) but increased the levels of H3K9me2 (*I*) at the promoters of c-Myc and MMP9. *J*, knockdown of JMJD1A decreased the levels of β -catenin at the promoters of c-Myc and MMP9. All experiments were repeated three times independently. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

These results indicate that the demethylase activity of JMJD1A is required for JMJD1A to enhance Wnt/ β -catenin signaling.

Furthermore, we determined whether the demethylase activity of JMJD1A is required for cell proliferation, migration, invasion, xenograft tumor formation, and metastasis. We trans-

JMJD1A promotes colorectal cancer growth and metastasis

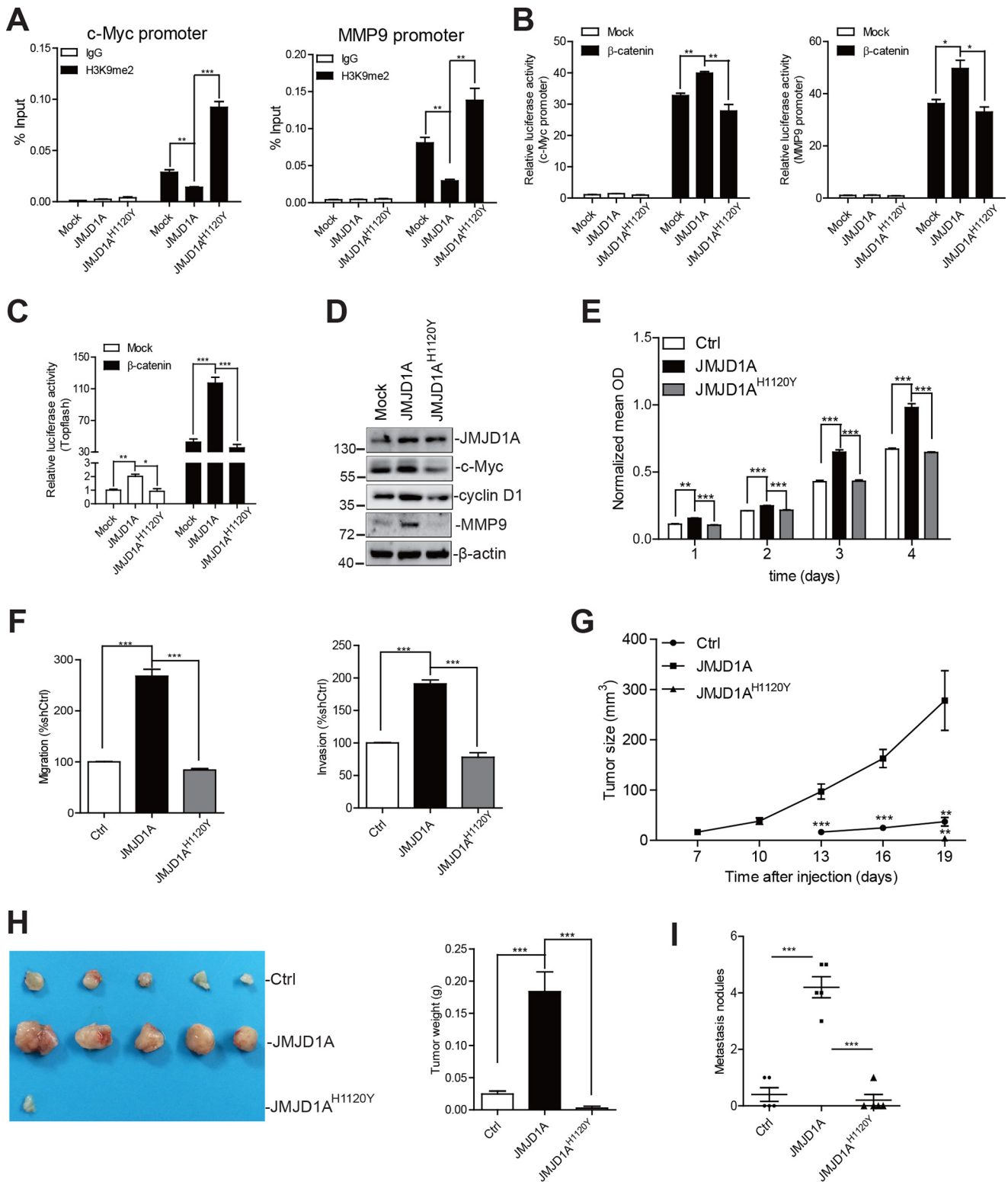


Figure 7. Demethylase activity of JMJD1A is required for the activation of Wnt/ β -catenin signaling and the enhancement of CRC cell growth, migration, invasion, xenograft tumor growth, and lung metastasis. *A*, overexpression of JMJD1A^{H1120Y} variant failed to decrease the levels of H3K9me2 at the promoters of c-Myc and MMP9 as compared with WT JMJD1A. *B* and *C*, JMJD1A^{H1120Y} variant could not assist β -catenin to enhance the promoter activities of c-Myc and MMP9 (*B*) and Topflash reporter (*C*) in 293T cells. *D*, JMJD1A^{H1120Y} variant could not increase the expression of c-Myc, cyclin D1, and MMP9 in HCT116 cells as compared with WT JMJD1A. *E*, WT JMJD1A could but JMJD1A^{H1120Y} variant could not increase the proliferation of shJMJD1A-expressing HCT116 cells. *F*, WT JMJD1A could but JMJD1A^{H1120Y} variant could not increase migration and invasion of shJMJD1A-expressing HCT116 cells. *G* and *H*, WT JMJD1A could but JMJD1A^{H1120Y} variant could not increase shJMJD1A-expressing HCT116 xenograft tumor size (*G*) and weight (*H*). *I*, WT JMJD1A could but JMJD1A^{H1120Y} variant could not enhance shJMJD1A-expressing HCT116 lung metastasis. All cell experiments were repeated three times, and animal experiments were repeated twice independently. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

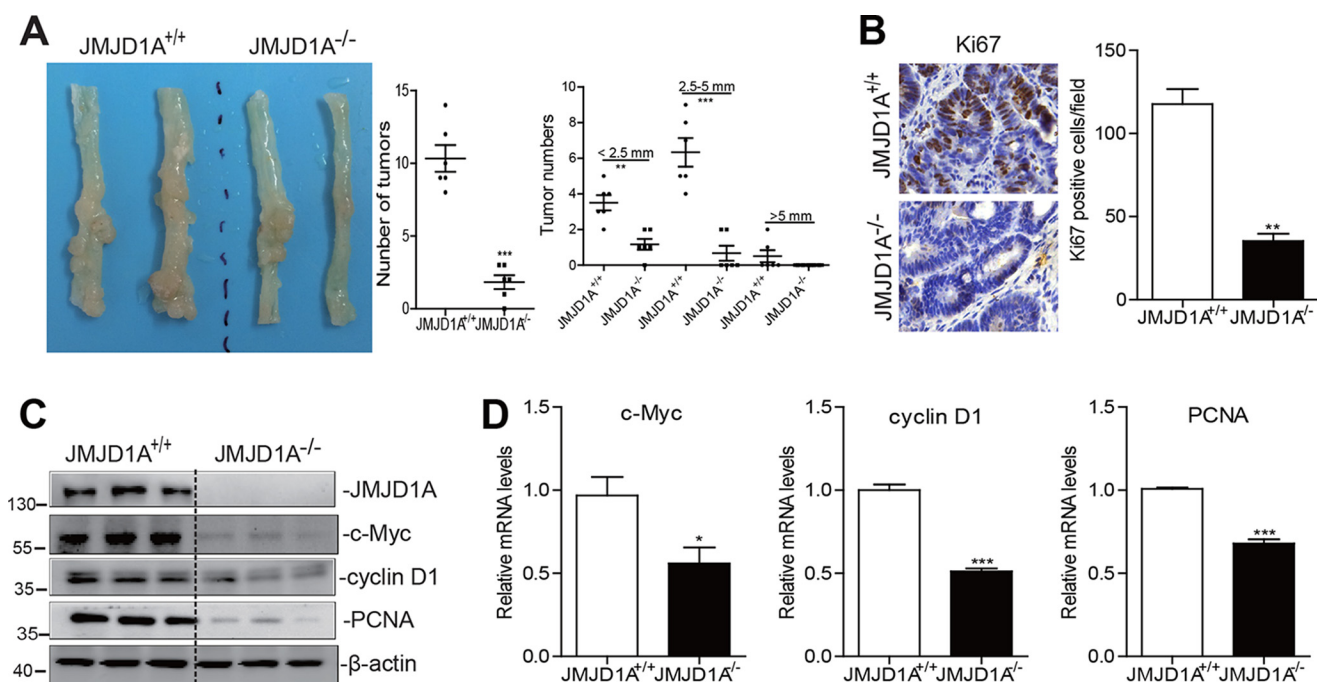


Figure 8. JMJD1A deficiency protects mice against AOM/DSS-induced CRC formation. A, JMJD1A-deficient mice had significantly less and smaller colon tumors as compared with WT mice. Images and quantitation of colorectal tumors from JMJD1A-deficient and WT mice are shown. B, JMJD1A-deficient colon tumors had significantly less Ki67-positive cells as compared with WT tumors. Ki67 staining of tumor sections of colorectal tumors from JMJD1A-deficient and WT mice. C and D, expression of c-Myc, cyclin D1, and PCNA was reduced in JMJD1A-deficient colorectal tumors. The protein and mRNA levels of JMJD1A, c-Myc, cyclin D1, and PCNA were measured in colorectal tumors from JMJD1A-deficient and WT mice, respectively. The experiment of AOM/DSS-induced CRC formation was performed twice independently. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

fecting WT JMJD1A and JMJD1A^{H1120Y} expression plasmids into shJMJD1A-expressing (JMJD1A-knockdown) HCT116 cells, respectively, and then measured cell proliferation, migration, invasion, xenograft tumor formation, and lung metastasis. The results showed that WT JMJD1A could but the JMJD1A^{H1120Y} variant could not increase the shJMJD1A-expressing HCT116 cell proliferation rate (Fig. 7E), migration and invasion rate (Fig. 7F), tumor size (Fig. 7G), tumor weight (Fig. 7H), and lung metastasis (Fig. 7I), implicating that the demethylase activity of JMJD1A is required for enhancement of cell proliferation, migration, invasion, xenograft tumor formation, and metastasis by JMJD1A.

JMJD1A deficiency protects mice against AOM/DSS-induced CRC formation

To validate our findings genetically, the AOM/DSS-induced mouse CRC formation model was performed to investigate whether JMJD1A deficiency protects against CRC progression in mice. After three cycles of AOM/DSS treatment, WT mice developed a prominent CRC phenotype featuring the appearance of abundant colon tumors, whereas JMJD1A-deficient mice had significantly less and smaller colon tumors (Fig. 8A). Results from Ki67 staining showed that JMJD1A-deficient colon tumors exhibited markedly less cell proliferation compared with WT tumors (Fig. 8B). Consistently, the protein and mRNA levels of c-Myc, cyclin D1, and PCNA were much lower in colon tumors from JMJD1A-deficient mice than from WT mice (Fig. 8, C and D). These results provide the genetic evidence to show that JMJD1A promotes CRC progression by enhancing Wnt/ β -catenin signaling.

Expression of JMJD1A is positively correlated with the target genes of Wnt/ β -catenin signaling in human CRC specimens and indicates poor prognosis

To corroborate the findings that JMJD1A promotes human CRC progression by enhancing Wnt/ β -catenin signaling clinically, we analyzed the relationship between the expression of JMJD1A and the target genes of Wnt/ β -catenin signaling in human CRC specimens by searching the Gene Expression Omnibus (GEO) dataset (GSE24514). The expression of JMJD1A was much higher in CRC specimens than the surrounding nontumorous specimens (Fig. 9A) and positively correlated with PCNA (Fig. 9B), c-Myc (Fig. 9C), cyclin D1 (Fig. 9D), and MMP9 (Fig. 9E), supporting the findings that JMJD1A promotes human CRC progression by enhancing Wnt/ β -catenin signaling. Furthermore, TCGA data showed that the prognosis of CRC patients who have higher levels of JMJD1A in tumors was poorer than the CRC patients with lower levels of JMJD1A in tumors (Fig. 9F).

Discussion

Increasing evidence indicates that JMJD1A is overexpressed in a multitude of cancers, including breast cancer, prostate cancer, liver cancer, and bladder cancer (8–10, 14, 19–23). Although it has been reported that JMJD1A can promote CRC progression and is a novel prognostic marker for CRC (24), the role of JMJD1A in CRC progression remains to be further elucidated. In this study, we clearly verified a key role of JMJD1A in promoting CRC progression as follows: 1) JMJD1A was frequently overexpressed in human CRC specimens, and knock-

JMJD1A promotes colorectal cancer growth and metastasis

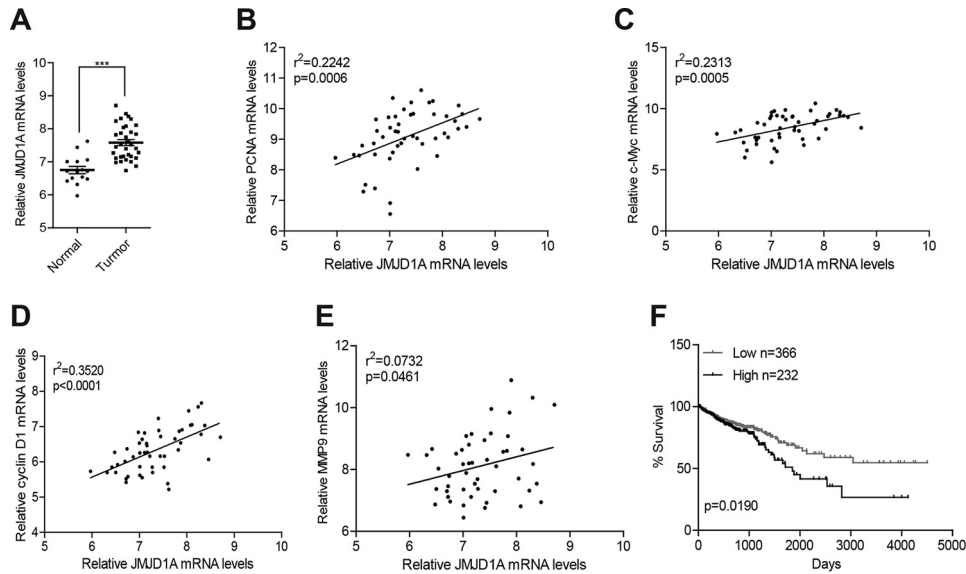


Figure 9. Expression of JMJD1A is positively correlated with the expression of β -catenin target genes in human CRC specimens from a GEO dataset and indicates poor prognosis. A, JMJD1A was overexpressed in many human CRC specimens. Analysis of the mRNA levels of JMJD1A in normal ($n = 15$) and CRC samples (tumor, $n = 34$) from a GEO dataset (GSE24514) is shown. The JMJD1A probe ID used was 212689_s_at. B–E, expression of JMJD1A is positively correlated with the expression of β -catenin target genes. Linear regression of JMJD1A and PCNA, c-Myc, cyclin D1, and MMP9 using normal colonic mucosa ($n = 15$) and CRC samples ($n = 34$) from the GEO database (GSE3167, $n = 46$). The probe IDs used were 212689_s_at (JMJD1A), 201202_s_at (PCNA), 202243_s_at (c-Myc), 208711_s_at (cyclin D1), and 203936_s_at (MMP9). F, prognosis of the CRC patient who expressed higher levels of JMJD1A is poor in TCGA database. ***, $p < 0.001$.

down of JMJD1A significantly suppressed CRC cell proliferation, colony formation, xenograft tumor formation, migration, invasion, and metastasis; 2) knockout of JMJD1A in mice significantly inhibited AOM/DSS-induced CRC formation.

In this study, we observed that knockdown of JMJD1A significantly down-regulated the expression of c-Myc, cyclin D1, and MMP9 at both protein and mRNA levels, indicating that JMJD1A promotes CRC progression at least in part by positively regulating the expression of these genes. Because c-Myc, cyclin D1, and MMP9 are target genes of Wnt/ β -catenin signaling, which is frequently activated in CRC, we were prompted to determine whether JMJD1A promotes CRC progression by enhancement of Wnt/ β -catenin signaling. Our present results demonstrated that JMJD1A directly up-regulated β -catenin expression as well as interacted with β -catenin to enhance β -catenin's transactivation; JMJD1A was recruited to the promoters of c-Myc and MMP9 to facilitate the expression of c-Myc and MMP9 and eventually promoted CRC progression. Therefore, JMJD1A can serve as a coactivator for β -catenin to enhance Wnt/ β -catenin signaling. It is generally considered that JMJD1A activates gene transcription through H3K9 demethylation at the promoter. In agreement to this notion, we found that knockdown of JMJD1A increased the levels of H3K9me2 at the promoters of c-Myc and MMP9, and we found the JMJD1A^{H1120Y} variant, which loses demethylase activity, failed to demethylate H3K9me2 at the promoters of c-Myc and MMP9, failed to assist β -catenin to induce the Topflash reporter activity, failed to induce the expression of c-Myc, cyclin D1, and MMP9, and failed to promote CRC cell proliferation, migration, invasion, and metastasis. These results demonstrate that the demethylase activity of JMJD1A is required for Wnt/ β -catenin activation and enhancement of CRC progression by JMJD1A.

It has been reported that the expression of JMJD1A can be induced by hypoxia in a HIF-1 α -dependent manner (17). Hypoxia and activation of HIF-1 α signaling frequently happen in CRC (25, 26), providing a possible explanation for up-regulation of JMJD1A observed in CRC specimens. Because the expression of JMJD1A can be induced by HIF-1 α and JMJD1A can enhance Wnt/ β -catenin signaling, JMJD1A at least in part mediates the cross-talk between HIF-1 α and Wnt/ β -catenin signaling. Recently, we showed that JMJD1A could serve as a coactivator for HIF-1 α to enhance HIF-1 α signaling and amplify JMJD1A expression in a feedback regulatory manner (27), implicating that JMJD1A may promote CRC progression by simultaneously enhancing HIF-1 α and Wnt/ β -catenin signaling.

Given the essential role of JMJD1A in promoting CRC progression, JMJD1A may serve as a potent pharmacological target for CRC treatment. Indeed, Padi *et al.* (28) recently reported that calcitriol (1 α ,25-dihydroxyvitamin D₃, the active form of vitamin D) could inhibit CRC cell proliferation and xenograft tumor growth in mice by activating the vitamin D receptor to induce the expression of miR-627 for JMJD1A down-regulation. In our system, calcitriol decreased the expression of JMJD1A, c-Myc, cyclin D1, PCNA, and MMP9, and it suppressed the promoter activities of c-Myc and MMP9 and Topflash reporter (Fig. S5). These results indicate that calcitriol inhibits CRC progression at least in part by down-regulating JMJD1A expression to diminish Wnt/ β -catenin signaling. In this study, we demonstrated that the demethylase activity of JMJD1A is required for its coactivation function for β -catenin. Therefore, pharmacological targeting of JMJD1A demethylase activity to block Wnt/ β -catenin signaling could be another attractive therapeutic approach for treating CRC.

Because Wnt/ β -catenin signaling plays an essential role in intestinal homeostasis (29, 30), one of the major concerns for targeting JMJD1A for CRC treatment is that it may cause severe side effects on intestinal homeostasis due to diminished Wnt/ β -catenin signaling. However, JMJD1A-deficient mice showed relatively normal intestinal structures, functions, and growth (Fig. S6), indicating that JMJD1A deficiency does not affect the intestinal homeostasis in general. It implicates that although specific targeting of JMJD1A for CRC treatment reduces the Wnt/ β -catenin signaling in the intestine, it may not cause a severe side effect on intestinal homeostasis.

During the preparation of this manuscript, we noticed that Li *et al.* (18) recently reported that KDM3A/B (JMJD1A/B) were critical epigenetic factors in Wnt/ β -catenin signaling that orchestrates chromatin changes and transcription to maintain tumorigenic potentials of human colorectal cancer stem cells. In their study, the authors showed that JMJD1A interacted with β -catenin to enhance Wnt/ β -catenin-mediated transcription and to maintain tumorigenic potentials and chemoresistance of human colorectal cancer stem cells, and JMJD1A was recruited to the Wnt/ β -catenin target gene promoter to erase H3K9me2 marks and promote H3K4 methylation by MLL1. In this study, we not only demonstrated that JMJD1A promoted CRC cell proliferation and tumorigenesis by interacting and cooperating with β -catenin to enhance Wnt/ β -catenin signaling and that JMJD1A was recruited to Wnt/ β -catenin target gene promoter to demethylate H3K9me2 for enhancing gene expression, but we also showed that JMJD1A could promote CRC cell migration, invasion, and metastasis, and JMJD1A could enhance Wnt/ β -catenin signaling by promoting β -catenin expression. Most importantly, our study provided the genetic evidence to support the notion that JMJD1A plays an essential role in promoting CRC progression by demonstrating that deletion of JMJD1A in mice protects against AOM/DSS-induced CRC development.

Collectively, our study demonstrates that JMJD1A promotes CRC growth and metastasis by directly interacting with β -catenin to enhance Wnt/ β -catenin signaling, and the demethylase activity of JMJD1A is required for its tumor promotion function, indicating that pharmacological targeting JMJD1A by down-regulating its expression or inhibiting its demethylase activity is a promising approach for CRC treatment.

Experimental procedures

Patients and colon tissue samples

Thirty one human CRC specimens and the surrounding non-tumorous colon tissues were obtained from the First Affiliated Hospital of Xiamen University (Xiamen, China). Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Research Ethics Committee at Xiamen University.

Cell lines

The human CRC cell lines HCT116 and SW480 were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Dulbecco's modified Eagle's medium containing 10%

FBS (Gibco) and 100 units/liter penicillin/streptomycin. The cell lines were recently authenticated by short tandem repeat markers by Genetic Testing Biotechnology Corp. (Suzhou, China). For Wnt/ β -catenin activation, the cells were treated with 50 μ M LiCl for 24 h.

Establishment of stable JMJD1A-knockdown cell lines

Two different JMJD1A shRNA plasmids were generated by inserting two different human JMJD1A-specific targeting sequences 5'-AGAAGAAUUC AAGAGAUUCCGGAGG-3' and 5'-AACACAUUCCAGUUGCUCUUAUUGU-3' into pL3.7-puro vector plasmid, respectively. All plasmids were transfected into different cell lines using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Puromycin (1 μ g/ml) was added to select stable JMJD1A-knockdown cell lines.

Cell proliferation

MTT assay was performed to measure cell proliferative rate as described previously (6).

Cell cycle analysis

For this analysis, 4×10^5 cells were seeded into 6-well plates, synchronized by serum starvation for 24 h, and re-entered into the cell cycle by an exchange of medium with 10% FBS for 24 h. Both adherent and nonadherent cells were harvested and fixed in 70% ethanol at 4 °C overnight. Cells were incubated with RNase A at 37 °C for 30 min and then stained with propidium iodide (PI). Cell cycle status was measured by flow cytometry.

Colony formation

For colony formation assays, 500 cells were cultured in 6-well plates. After 2 weeks, cells were stained with 0.005% crystal violet for 10 min to detect foci, and colonies were counted.

Tumor xenograft

Four- to 6-week-old male nude mice were obtained from the Laboratory Animal Center of Xiamen University. A total of 5×10^6 JMJD1A-knockdown or control HCT116 cells were subcutaneously injected into the dorsal flanks of mice. The number of mice in each group was five. From day 7 after injection, the tumor size was measured along two perpendicular axes every 3 days using a vernier caliper. The volume of the tumor was calculated using the following formula: volume = length \times width² \times 0.52. Two investigators were blinded to the group allocation when performing the experiments and assessing the results. All experimental procedures involving animals were performed in accordance with animal protocols approved by the Laboratory Animal Center of Xiamen University.

Cell migration and invasion assays

Cell migration and invasion assays were performed to investigate the cell migration and invasion. The method has been described in our previous study (31).

Lung metastasis

To test tumor metastasis, JMJD1A-knockdown and control HCT116 cells were intravenously injected into 4–6-week-old male nude mice. Three weeks after injection, the mice were

JMJD1A promotes colorectal cancer growth and metastasis

sacrificed, and the lungs were fixed by picric acid/formaldehyde. Tumors in the lungs were quantitated and used for hematoxylin and eosin staining and Ki67 immunohistochemical analysis. Two investigators were blinded to the group allocation when performing the experiments and assessing the results. All experimental procedures involving animals were performed in accordance with animal protocols approved by the Laboratory Animal Center of Xiamen University.

Real-time RT-PCR

Real-time RT-PCR was performed as described previously (6). Primers for JMJD1A, c-Myc, cyclin D1, PCNA, MMP9, and β -actin have been published in previous studies (21, 32).

Antibodies and Western blot analysis

Western blotting has been described in our previous study (31). Filters were probed with the following primary antibodies: anti-JMJD1A (catalog no. 12835-1-AP, Bethyl); anti- β -catenin (catalog no. 2586s, Santa Cruz Biotechnology); anti-PCNA (catalog no. 2586s, Cell Signaling); anti- β -actin (catalog no. A5316, Sigma); anti-cyclin D1 (catalog no. ab134175, Abcam); anti-MMP9 (sc-6840, Santa Cruz Biotechnology); or anti-c-Myc (catalog no. ab32072, Abcam). Blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Pierce) and visualized by chemiluminescence.

Gel zymography assay

The enzymatic activity of MMP9 was performed by gel zymography, as described previously (33).

Luciferase assay

The activities of c-Myc and MMP9 promoters and Topflash reporter were analyzed by the luciferase reporter assay system (Promega), as described previously (27).

Co-IP assay

The interaction between JMJD1A and β -catenin in cells was analyzed by the Co-IP assay as described previously (27).

In vitro expression of JMJD1A protein and GST pulldown assays

E. coli extract-based cell-free *in vitro* expression of JMJD1A protein was performed using the S30 T7 high yield Protein Expression System (Promega) following the manufacturer's protocol. GST pulldown assays were conducted as described previously (27).

ChIP assay

ChIP assay was performed as described previously (27, 34). In brief, the chromatin of HCT116 cells was immunoprecipitated using anti-JMJD1A, anti- β -catenin, and anti-H3K9me2 (catalog no. ab1220, Abcam) antibodies or nonspecific IgG (Santa Cruz Biotechnology). ChIP DNAs were purified and amplified by real-time PCR with specific primers for the c-Myc and MMP9 promoter. Primers used for the ChIP assay were as follows: c-Myc: forward, 5'-ACTCTCCCTGGACTCTTGA-3'; reverse, 5'-ACAAACCGCATCCTTGTCT-3'; MMP9: for-

ward, 5'-TCACAGGAGCGCCTCCTTAA-3'; reverse 5'-AGCAAAGCAGCAGCCCAGCA-3'.

Colon tumor induction

JMJD1A-deficient mice and WT mice were maintained on a C57BL/6 background. To induce colon tumors, 6-week-old female WT and JMJD1A-deficient mice were intraperitoneally injected AOM (10 mg/kg), followed by administration of three cycles of 1 week of 2% DSS water and 2 weeks of water (35). At the experimental end point, mice were sacrificed, and colons were harvested for photographing, quantitation, hematoxylin and eosin staining, and immunohistochemical analysis. Two investigators were blinded to the group allocation when performing the experiments and assessing the results. All experimental procedures involving animals were performed in accordance with animal protocols approved by the Laboratory Animal Center of Xiamen University.

Hematoxylin and eosin and immunohistochemistry

The experiments of hematoxylin and eosin and immunohistochemistry were performed as described previously (27).

Statistical analysis

The data were collected from more than three independent experiments. All data were expressed as mean \pm S.E. Statistically significant differences ($p < 0.05$) were examined using *t* test.

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