Am J Cancer Res 2018;8(5):852-865
www.ajcr.us /ISSN:2156-6976/ajcr0077629

Original Article

Downregulation of histone demethylase JMJD1C inhibits colorectal cancer metastasis through targeting ATF2

Cheng Chen, Maimaiti Aihemaiti, Xin Zhang, Hui Qu, Qi-Long Sun, Qing-Si He, Wen-Bin Yu

Department of General Surgery, Qilu Hospital of Shandong University, 107 West Wenhua Road, Jinan 250012, China

Received April 9, 2018; Accepted April 29, 2018; Epub May 1, 2018; Published May 15, 2018

Abstract: Colorectal cancer (CRC) is one of the most common malignant gastrointestinal cancers. Metastasis is a major leading of death in patients with CRC and many patients have metastatic disease at diagnosis. However, the underlying molecular mechanisms are still elusive. Here, we showed that JMJD1C was overexpressed in colon cancer tissues compared to normal samples and was positively associated with metastasis and poor prognosis. Silencing JMJD1C strongly inhibits CRC migration and invasion both in vitro and in vivo. Further, we found that knockdown of JMJD1C decreased the protein and mRNA levels of ATF2, mechanistically, and JMJD1C regulated the expression of ATF2 by modulating the H3K9me2 but not H3K9me1 activity. In addition, we further performed some "rescues experiments". We found that overexpression of ATF2 could reverse the abrogated migration and invasion ability by knockdown of JMJD1C in CRC. Our results demonstrated that an increase of JMJD1C was observed in colon cancer and knockdown of JMJD1C regulated CRC metastasis by inactivation of the ATF2 pathway. This novel JMJD1C/ATF2 signaling pathway may be a promising therapeutic target for CRC metastasis.

Keywords: Colorectal cancer, JMJD1C, ATF2, metastasis

Introduction

Colorectal cancer (CRC) is one of the most common malignant gastrointestinal cancers [1, 2]. Despite advances in diagnosis and therapy, few therapies have met with limited success and the prognosis of patients with CRC is still poor [3, 4]. Metastasis is a major leading of death in patients with CRC and many patients have metastatic disease at diagnosis [5]. However, the underlying molecular mechanisms are still elusive. Novel targeted therapies for blocking metastasis progress may be of benefit in patients with CRC metastasis [6].

The dysregulation of epigenetic modifications has been suggested to be involved in the development of cancers [7, 8]. Histone-modifying enzymes have been demonstrated to be involved in regulation of epigenetic modification and plays vital roles in the progression of cancer [9-11]. Amounting studies have identified that histone-modifying enzymes contribute to the metastasis progression of several human cancers [12, 13]. JmjC domain-containing proteins are a class of enzymes mainly responsible for histone demethylation. Recent reports have showed that the dyregulation of JmjC domain-containing proteins play diverse roles in tumor development [14]. In squamous cell carcinoma, Ding X *et al.* and his colleagues have showed that KDM4A contributes to the invasion and metastasis by the binding of the AP1 complex to the targeted promoters [15]. In addition, KDM2B overexpression is associated with cancer stem cells in breast cancer and positively correlated with poor prognosis [16]. An increase JMJD3 expression was demonstrated in hepatocellular carcinoma and overexpression JMJD3 promoted metastasis and EMT by regulation of SLUG transcription [17], suggesting that many JmjC domain-containing proteins play an important role in cancer progression. Recently, Mo Chen *et al.* and his colleagues have demonstrated that JMJD1C functions as a coactivator of transcription factors for maintaining the survival of acute myeloid leukemia [18]. JMJD1C has been reported to activate gene transcription by regulating the activity of H3K9me2/

H3K9me1. In gastrointestinal cancers, Cai *et al*. has reported that knockdown of JMJD1C impaired the proliferation of EC cells through inhibiting the YAP signal pathway [19]. However, the role of JMJD1C in the CRC metastasis has not been explored.

ATF2 is a member of the leucine zipper family of DNA binding proteins that binds to the cAMP-responsive element (CRE) involving in several cellular processes such as proliferation, apoptosis and cancer development [20, 21]. Amounting evidence have indicated that P38 or ERK could directly phosphorylate ATF2 and then regulate proliferation and apoptosis, indicating that ATF2 is involved into multi-signal pathways to play its role in cancer development [22]. Recently document has demonstrated that ATF2 expression is associated with clinical outcomes [23]. In addition to the function of ATF2 on proliferation and apoptosis in many types of cancer, in gastric cancer cells, ATF2 could bind to the promoter of miR-132 and then regulate migration and invasion [24]. In addition, Lau *et al.* have suggested that active ATF2 were regulated in advanced melanomas and promoted metastasis by inhibiting protein fucosylation [25]. Above documents have indicated that ATF2 plays a vital role in metastasis in several cancers. However, there is little evidence of the function of ATF2 on CRC metastasis.

In the present study, we established the relationship between JMJD1C and ATF2 in CRC metastasis. We revealed that JMJD1C is upregulated in CRC samples compared with the paired normal tissues and the expression of JMJD1C is strongly associated with lymph node metastasis and poor prognosis. Further, we showed that knockdown of JMJD1C inhibited CRC metastasis through transcriptional regulation of ATF2 expression in vitro, suggesting that JMJD1C might be a novel therapeutic target for CRC metastasis treatment.

Materials and methods

Cell culture, chemicals and antibodies

SW480, SW48, LoVo, HCT116, HT29 and SW620 human colon cancer cell lones were purchased from the American Type Culture Collection. SW480, SW48, SW620 and LoVo cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. HCT116 and HT29 cell lines were maintained in McCoy's 5A medium with 10% FBS and antibiotics at 37°C in a humidified incubator with 5% CO_2. All chemicals and antibodies as follows: JMJD1C, ATF2, GAPDH, E-Cadherin and Vimentin were obtained from Santa Cruz Biotechchnology (Santa Cruz, CA, USA). Lipo2000 reagent was purchased from Invitrogen (Shanghai, China). ShRNA-JMJD1C and the ShRNA negative control (ShNC) were obtained from RiboBio company (Guangzhou, China).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from colon cancer cells with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Then, the RNA was readily reverse-transcribed to the cDNA using the PrimeScript RT reagent kit (TAKARA, Dalian, China). Next, Quantitative real-time PCR (qRT-PCR) was performed with TransStart Green qPCR SuperMix (TransGen Biotech, China) using an ABI7500 real time PCR system (Life Technologies, Grand Island, NY, USA).The qRT-PCR cycles were designated as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s, 55°C for 60 s, 72°C for 30 s; 95°C for 15 s, 55°C for 5 s; and 95°C for 30 s. Expression levels of the indicated genes were normalized to that GAPDH. The relative expression of the indicated genes was determined using $2^{-\Delta\Delta CT}$ method. The qRT-PCR assays were performed with the following primers: 5'-GGAGCGAGATCCCTCCAAAAT-3' (forward), 5'-GGCTGTTGTCATACTTCTCATGG-3' (reverse) for the human GAPDH gene; 5'-CAGGTCTCGTGCCAATCAAAA-3' (forward), 5'-GCTGTTGCTGGTGTGTATTCT-3' (reverse) for the human JMJD1C gene; 5'-AATTGAGGAGCCTTCTGTTGTAG-3' (forward), 5'-CATCACTGGTAGTAGACTCTGGG-3' (reverse) for the human GAPDH gene.

Western blotting

Colon cancer cells were harvested and lysed in NP40 (150 mM NaCl, 0.1% SDS, 1% $NaMoO_4$, 1% NP40, 50 mM Tris-HCl (PH 7.5), and 0.02% NaN_3) buffer containing protease and phosphatase inhibitors. Equal amount of proteins were separated on SDS-PAGE gels and transferred onto polyvinylidene difluoride membrane (PVDF; Millipore, USA). Then, the membrane was separately incubated with the correspond-

ing primary antibodies at 4°C overnight. After incubated with the second antibodies for 1 h at room temperature, the membranes were visualized with ECL.

Clinical samples and immunohistochemical staining

Human CRC samples and the paried adjacent normal tissues were obtained from Qilu Hospital, Shandong University, Shandong, China. The informed consent was obtained from each recruiter involved in this study. This investigation was approved by the Shandong University Institute Research Ethics Committee. Surgically resected samples were collected between January 2001 and December 2015. All specimens were quickly stored in liquid nitrogen. For immunohistochemical staining experiment, the expression levels of JMJD1C in CRC samples and adjacent normal tissues were scored as the proportion of staining area (0%, 0; 1-50%, 1; 50%-100%, 2) multiplied by the intensity of staining (0, negative; 1, weak; 2, moderate; 3, high). Each section was independently assessed by two pathologists without prior knowledge of patient data. The median score was determined as the cut-off value for defining high and low expression.

Wound healing and transwell assays

Cells were plated into six-well plates overnight and were carefully scratched with a sterile 200 μl pipette tips. Cells were observed every 24 h using a microscope. For transwell migration assay, about 40000 cells suspended in 200 μl serum-free medium were seeded into the upper chamber and complete medium supplemented with 10% FBS was added into the lower chamber. After incubation for 16 hours, the cells on the lower surface were fixed with 4% methanol and stained with 0.1% crystal violet and counted using a microscope. At least three visual areas were photographed. For transwell invasion assay, about 60000 cells suspended in 200 μl serum-free medium were seeded into the upper chamber pretreated with matrix gel (BD Biosciences, Sparks, MD, USA). The remaining steps were similar to the transwell migration assay.

Luciferase reporter assay

The promoter of ATF2 gene was cloned into the PGL6 vector (PGL6-ATF2). Cells were seeded into 24-well plates. Overnight, PGL6-ATF2 and pRL-TK plasmids were co-transfected into indicated cells with JMJD1C siRNA or ATF2 plasmids. After 48 hours, luciferase activity was analyzed with the Dual-Luciferase Reporter Assay System (Promega, CA, USA) according to the instruction. The PCR was performed with the following primers: 5'-AGTTGGGTAGATGCATCATGG-3' (forward), 5'-GGCATCCCCACCTTCCTTCTG-3' (reverse) for the human ATF2 gene promoter.

Chromatin immunoprecipitation (ChIP) assay

CRC cells were seeded into 10 cm culture dishes and were treated according to the requirement of experiment. Protein-DNA complexes were fixed with 1% formaldehyde and DNA was collected according to the ChIP Assay Kit (Thermo Fisher Scientific, USA). The ChIP-DNA was used for PCR. The primers were designed to examine the promoter region of JMJD1C from 1745 to 2020. The ChIP PCR was performed with the following primers: 5'-TTGTATTTTTCGTTTGCTCCTAG-3' (forward), 5'-TCCACTTTTTCAGTGTACAC-3' (reverse) for the human ATF2 gene promoter; 5'-TACTAGCGGTTTTACGGGCG-3' (forward) and 5'-TCGAACAGGAGGAGCAGAGAGCGA-3' (reverse) for the human *GAPDH* promoter.

Animal experiments

All 4 weeks male nude mice were obtained from the Vital River Experimental Animal Center (Beijing, China). 2×10^6 SW480 stable cells were subcutaneously injected into nude mice. After 8 weeks, the mice were killed and the metastatic lung tissues were collected for weight. H&E staining was used to examine tumor metastatic nodules in lung and the number of metastatic nodules in lung was counted. All animal experiments were approved by the Institutional Animal Care and Use Committee of Shandong University.

Statistical analysis

SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) were used for statistical analysis. The results are shown as means ± standard deviation (SD). The chi-square test was used to examine the association with JMJD1C expression and clini-

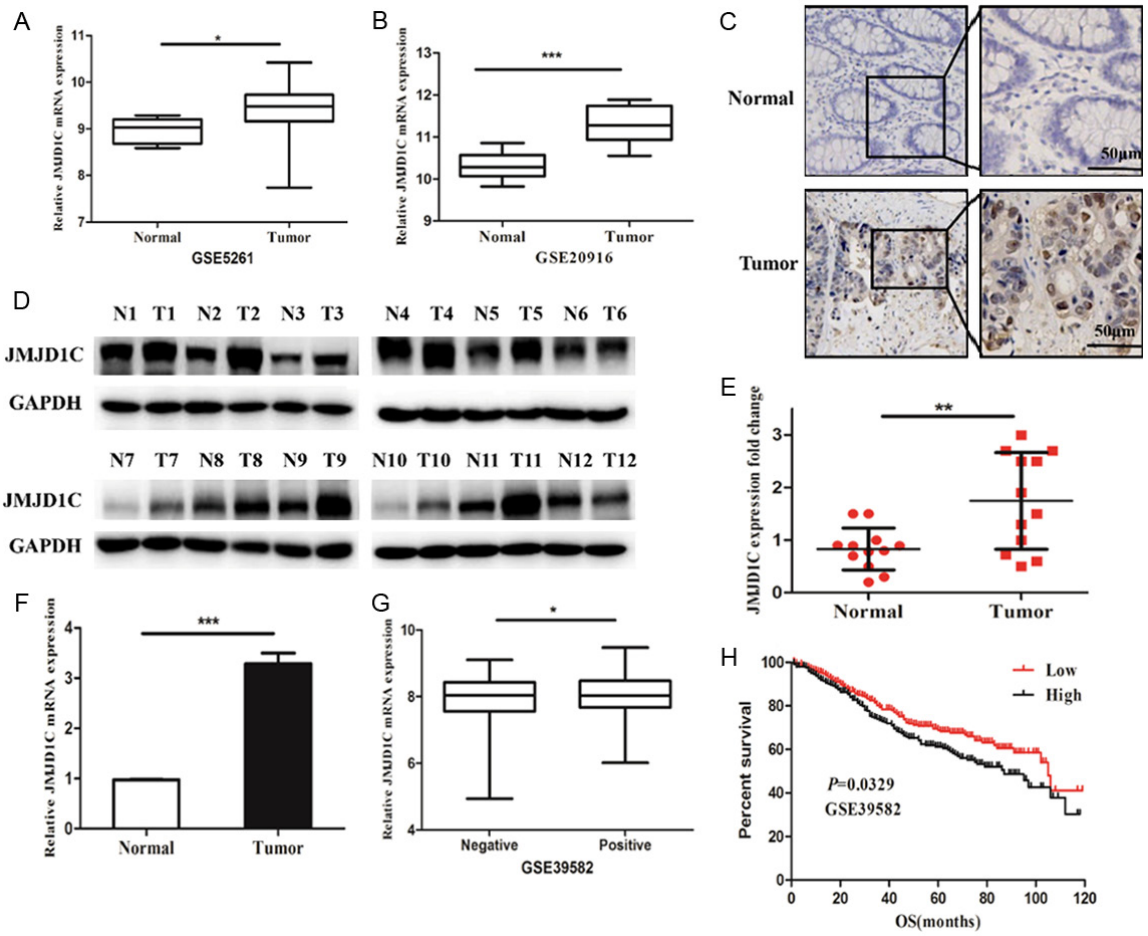


Figure 1. Overexpression of JMJD1C associates with poor prognosis in colon cancer. A, B. Data from GEO databases showed an increase of JMJD1C expression in CRC tissues compared to normal samples, **P*<0.05, ****P*<0.001. C. Expression of JMJD1C was examined in clinical tissues by IHC staining. Scale Bar. 50 μm. D, E. Western blotting assay showed the protein levels of JMJD1C in 12 paried CRC tissues and normal samples. N: normal samples, T: CRC tumor tissue, ***P*<0.01. F. Qpcr assay showed the mRNA expression of JMJD1C in 12 paried CRC tissues and normal samples, ****P*<0.001. G. Data from GSE39582 showed JMJD1C was upregulated in patients with lymph node metastasis (Positive) compared to patients without lymph node metastasis (Negative), **P*<0.05. H. Kaplan-Meier survival analyses of overall survival in GSE39582 database.

copathological characteristics and the Spearman rank correlation coefficient was used to detect the relationship between JMJD1C and ATF2. *P*<0.05 was considered significant. Each experiment was performed at least three times.

Results

Overexpression of JMJD1C associates with poor prognosis in colon cancer

To evaluate the role of JMJD1C in colon cancer, we first analyzed the expression of JMJD1C in different online GEO databases. As shown in **Figure 1A**, **1B**, we interestingly found that an increase JMJD1C was observed in colon cancer tissues compared to the normal colon samples both in GSE5261 [26] and GSE20916 [27] databases. To confirm this phenomenon, we further examined our clinic 100 paired colon cancer and normal colon tissues by using immunohistochemical staining assay. As shown in **Table 1**, we found that the overexpression of JMJD1C, as revealed by immunohistochemical staining, correlated significantly with TNM stage and Lymph node metastasis. Meanwhile, similar to the above results observed in published databases, we showed that JMJD1C was overexpression in colon cancer tissues compared to the normal colon samples by using immunohistochemical staining assay (**Fi-**

Table 1. Association between clinicopathological characteristics and expression of JMJD1C in the CRC patients (n=100)

Characteristics	n	JMJD1		*P*-value
		High expression	Low expression	
Sex				
Male	54	30 (55.6)	24 (54.4)	0.735
Female	46	24 (52.2)	22 (47.8)	
Age				
<45	40	25 (62.5)	15 (37.5)	0.435
≥45	60	42 (70)	18 (30)	
Tumor size				
<5	52	24 (46.2)	28 (53.8)	0.553
≥5	48	25 (52.1)	23 (47.9)	
Differential grade				
Poor	22	12 (54.5)	10 (45.5)	0.556
Middle	35	14 (40)	21 (60)	
Well	43	19 (44.2)	24 (55.8)	
LN metastasis				
Positive	38	26 (68.4)	12 (31.6)	0.035
Negative	62	29 (46.8)	33 (53.2)	
TNM stage				
I-II	58	27 (46.6)	31 (53.4)	0.025
III-IV	42	29 (69)	13 (31)	

LN, lymph node; TNM, tumor, node, metastasis.

gure 1C**) and Western blotting assay (**Figure 1D**, **1E**). Furthermore, QPCR illustrated that the JMJD1C mRNA was strongly upregulated in colon cancer tissues compared to the normal colon samples (**Figure 1F**). The above results suggested that JMJD1C might play an important role in CRC development and especially in metastasis.

To investigate the correlation of JMJD1C expression with CRC metastasis, we further analyzed if the expression of JMJD1C associated with CRC metastasis and prognosis in published database. Similar to our clinic results, we expectedly found that an increase JMJD1C positively associated with lymph node metastasis in GEO database [28] (**Figure 1G**). More important, we found that the increase of JMJD1C expression, as shown in **Figure 1H**, correlated significantly with poor overall survival, indicating that upregulation of JMJD1C may be an independent risk factor for CRC development and result in CRC metastasis.

Loss of JMJD1C inhibits CRC metastasis in vitro

To confirm the above observed phenomenon and to further validate the function of JMJD1C in CRC, we first examined the expression levels of JMJD1C in different CRC cell lines, an increase of JMJD1C was observed in LoVo and SW620 cell lines, which have high ability of metastasis in CRC cell lines, compared to others CRC cell lines (Supplementary Figure 1). This data suggested that JMJD1C may be involved in CRC metastasis.

To validate this hypothesis, we transfected with two shRNA-JMJD1C sequences in SW48 and SW480 cells. As shown in **Figure 2A**, **2B**, both qPCR and Western blotting assays showed that the expression of JMJD1C was actually reduced in cells expressing different ShRNA-JMJD1C. Next, we used them in subsequent functional studies. Cell migration and invasion assays were performed between in control cells and in cells expressing different ShRNA-JMJD1C. As shown in **Figure 2C**, **2D**, we found that the ability of migration was significantly attenuated in SW48 and SW480 cells expressing ShRNA-JMJD1C. Similar to this result, we further found that the ability of invasion was also strongly decreased in SW48 and SW480 cells expressing different ShRNA-JMJD1C (**Figure 2E**, **2F**). Taken together, those data strongly suggested that JMJD1C was involved in CRC development by promoting metastasis.

ATF2 is a directly target of JMJD1C

Above data have showed that JMJD1C is overexpression in CRC and silencing JMJD1C inhibits its CRC metastasis. To further explore the precise molecular mechanism of regulation metastasis by JMJD1C, we used gene set enrichment analysis (GSEA) to analyze microarray data (GSE40367) [29]. As previously reported, the published database included 7 paired primary colon cancer tissues and liver metastasis samples. We found that ATF2, KRAS, RPS14, Notch, SUZ12, STK33 and Pten were significantly enriched in metastatic site. To find the molecular mechanism of regulation colon cancer metastasis by JMJD1C, we found that the mRNA level of ATF2 was the most decreased in ShJMJD1C SW48 cells compared to control cells (Supplementary Figure 2). As shown in **Figure 3A**, we found that ATF2 was strongly

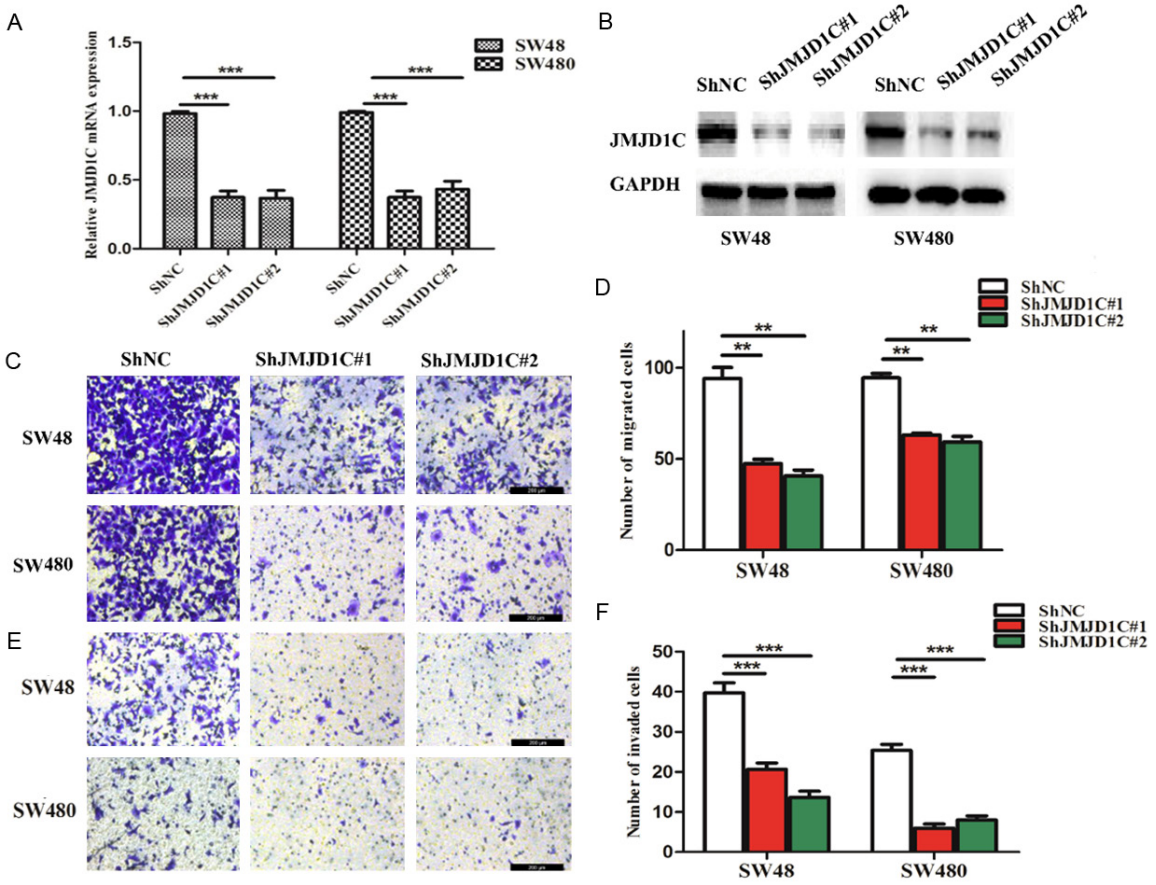


Figure 2. Loss of JMJD1C inhibits CRC metastasis in vitro. A. Qpcr assay showed the mRNA levels of JMJD1C in control cells compared to ShJMJD1C cells, ***P<0.001. B. Western blotting assay showed the protein levels of JMJD1C in control cells compared to ShJMJD1C cells. GAPDH acts as a loading control. C, D. SW48 and SW480 cells transduced with control ShRNA or ShRNA-JMJD1C. Cells were examined for their migration ability using migration chambers. Representative micrographs and quantification of the migration ability of ShJMJD1C cells compared to the control cells. Scale Bar. 200 μm, **P<0.01. E, F. SW48 and SW480 cells transduced with control ShRNA or ShRNA-JMJD1C. Cells were examined for their invasion ability using Matrigel coated invasion chambers. Representative micrographs and quantification of the invasion ability of ShJMJD1C cells compared to the control cells. Scale Bar. 200 μm, ***P<0.001.

enriched in metastasis. More important, we further analyzed some reference parameters including the normalized enrichment score (NES), *P*-value, and fold change and found ATF2 was associated with metastasis. So, it prompts us to hypothesize whether JMJD1C promotes CRC metastasis through regulation of ATF2. To confirm this hypothesis, we examined the protein and mRNA levels of ATF2 in between cells expressing control shRNA and cells expressing shRNA-JMJD1C. As shown in **Figure 3B**, **3C**, we interestingly found that the lower protein and mRNA levels of ATF2 in cells silencing JMJD1C, compared to control cells. This result indicated that JMJD1C probably modulate ATF2 expression on the transcription regulation.

Previously documents have illustrated that JMJD1C mainly activated gene expression through regulation the H3K9me1 and/or H3K9me2 activity for activating gene promoter activity. To explore the molecular mechanism underling regulation of ATF2 by JMJD1C, we first examined the promoter activity of ATF2 gene. Dual-Luciferase assay clearly showed that a reduced promoter activity of ATF2 gene was observed in cells silencing JMJD1C compared to the control cells (**Figure 3D**). Further, we observed increased expression level of H3K9me2 in cells knockdown JMJD1C cells compared to the control cells, but the expression levels of H3K9me1 and K3K9me3 had no changes among groups, strongly indicating that

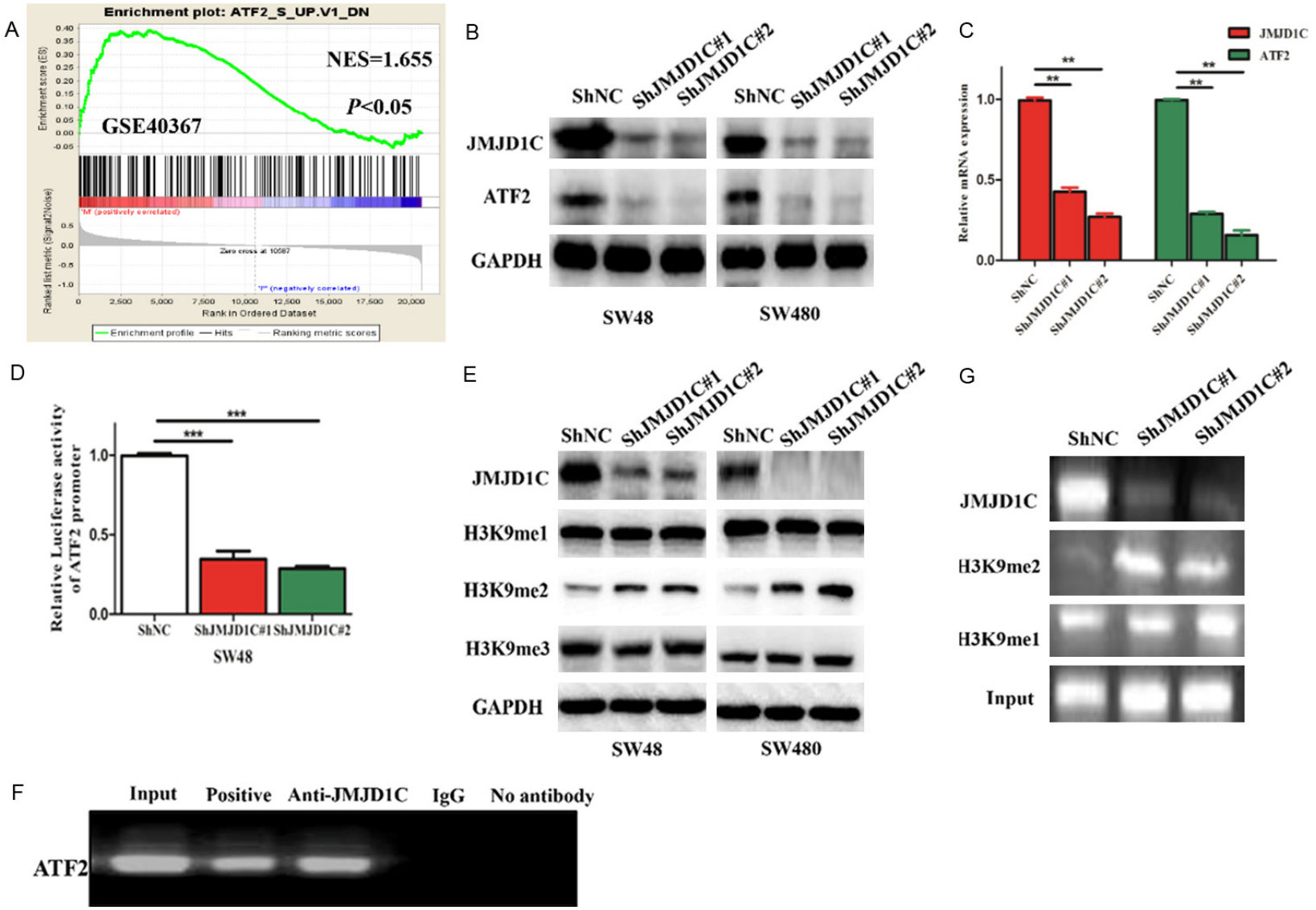


Figure 3. ATF2 is a directly target of JMJD1C. A. GSEA plot showed that ATF2 expression positively with CRC metastasis in published CRC database. B, C. Western blotting assay and Qpcr assay demonstrated that the protein and mRNA levels of JMJD1C and ATF2 expression in the indicated cells, $^{**}P<0.01$. D. Dual-Luciferase assay showed that the promoter activity of ATF2 gene in the indicated cells, $^{***}P<0.001$. E. Western blot showed that the protein expression of H3K9me1, H3K9me2 and H3K9me3 in the indicated cells. F. CHIP assay was performed in SW48 cells. Data showed that the promoter of ATF2 gene was amplified by PCR

from the complexes immunoprecipitated by the JMJD1C antibody, but not the IgG negative control. G. CHIP assay was performed in SW48 control cells and ShJMJD1C cells. Results showed that an increase of H3K9me2 binding to the promoter of ATF2 gene was observed in ShJMJD1C cells compared to control cells, but not and H3K9me1, *P<0.05, **P<0.01; NS, not significant.

the regulation of ATF2 by JMJD1C is dependent on H3K9me2 (**Figure 3E**). Meanwhile, we asked whether JmjC domain involved in this regulation. To test this hypothesis, we generated JMJD1C mutation plasmid without JmjC domain (JMJD1C ΔD) and JMJD1C wild type plasmid (JMJD1C WT). Next, we transfected with Vector control, JMJD1C WT or JMJD1C ΔD in SW48 cells, respectively. Western blot showed that overexpression of JMJD1C WT could increase the expression of ATF2 and decrease the expression of H3K9me2, but JMJD1C ΔD could not (Supplementary Figure 3), suggesting JmjC domain was involved in this regulation. Next, to further confirm the previously published result, we found that JMJD1C could directly bind to the promoter of ATF2 gene in SW48 cells (**Figure 3F**). Meanwhile, previously reports have demonstrated that JMJD1C regulated the H3K9me1 and/or H3K9me2 activity in a cell-type dependent manner. Here, we also examined the H3K9me1and H3K9me2 activity between in cells silencing JMJD1C and in control cells. As shown in **Figure 3G**, we showed that the H3K9me2 but not H3K9me1 activity was reduced in cells silencing JMJD1C, suggesting that JMJD1C regulates the ATF2 expression mainly dependent on the H3K9me2 but not H3K9me1 activity.

JMJD1C regulates CRC metastasis dependent on ATF2 expression

Above data have showed that JMJD1C could directly regulate ATF2 expression and biological line of evidence has demonstrated that ATF2 was involved in CRC metastasis. Thus, we examined the dependence of the reduction in CRC metastasis due to silencing JMJD1C on ATF2. Firstly, we conducted the plasmid including the ATF2 gene (ATF2 plasmid). Then, we cotransfected with the ATF2 plasmids and ShJMJD1C into CRC cells. As shown in **Figure 4A**, **4B**, western blotting showed the JMJD1C and ATF2 protein expression. Subsequently, we used them in subsequent functional studies. Cell migration and invasion assays were performed in those cells. As shown in **Figure 4C**, **4D**, the migration activity abrogated by silencing JMJD1C was reversed by overexpressing ATF2. Consistent with it, as shown in **Figure 4E**, **4F**, the invasion activity decreased by silencing JMJD1C was also reversed by overexpressing ATF2. Taken together, these results demonstrated that knockdown of JMJD1C abrogated CRC metastasis dependent on ATF2.

Knockdown of JMJD1C inhibits CRC metastasis in vivo

In an effort to understand the effect of JMJD1C on metastasis, we conducted the SW48 cells stably expressing shRNA-JMJD1C. As shown in **Figure 5A**, western blotting confirmed the effective of shRNA-JMJD1C in SW48 cells. Then, we intravenously injected different CRC cells group into Nude mice. About 8 weeks after inoculation, all mice were sacrificed. As shown in **Figure 5B**, all mice generated lung metastases by SW48 control cells. However, only three quarters of mice generated lung metastases by knockdown of JMJD1C in SW48 cells. Meanwhile, the metastatic nodules were counted, as shown in **Figure 5C**, **5D**, we found that the number of metastatic nodules were lower in SW48-silenced cells compared to the control cells. In addition, we examined the lung weight between two groups. Consistent with above result, we found that the lung weight in control cells group was heavier than in knockdown of JMJD1C groups. Collectively, these findings markedly demonstrated that the promotion role of JMJD1C in CRC metastasis.

Correlation between JMJD1C and ATF2 expression in CRC

To further explore the friendship between JMJD1C and ATF2 in CRC, we examined the correlation between JMJD1C and ATF2 expression in 12CRC samples by western blotting assay. As shown in **Figure 6A**, **6B**, we clearly found that JMJD1C and ATF2 exists strongly positive correlation. Meanwhile, we examined the JMJD1C and ATF2 expression in CRC samples by IHC, correlation studies demonstrated that JMJD1C expression positively correlated with the ATF2 expression (**Figure 6C**, **6D**). Importantly, we also found that JMJD1C expression positive-

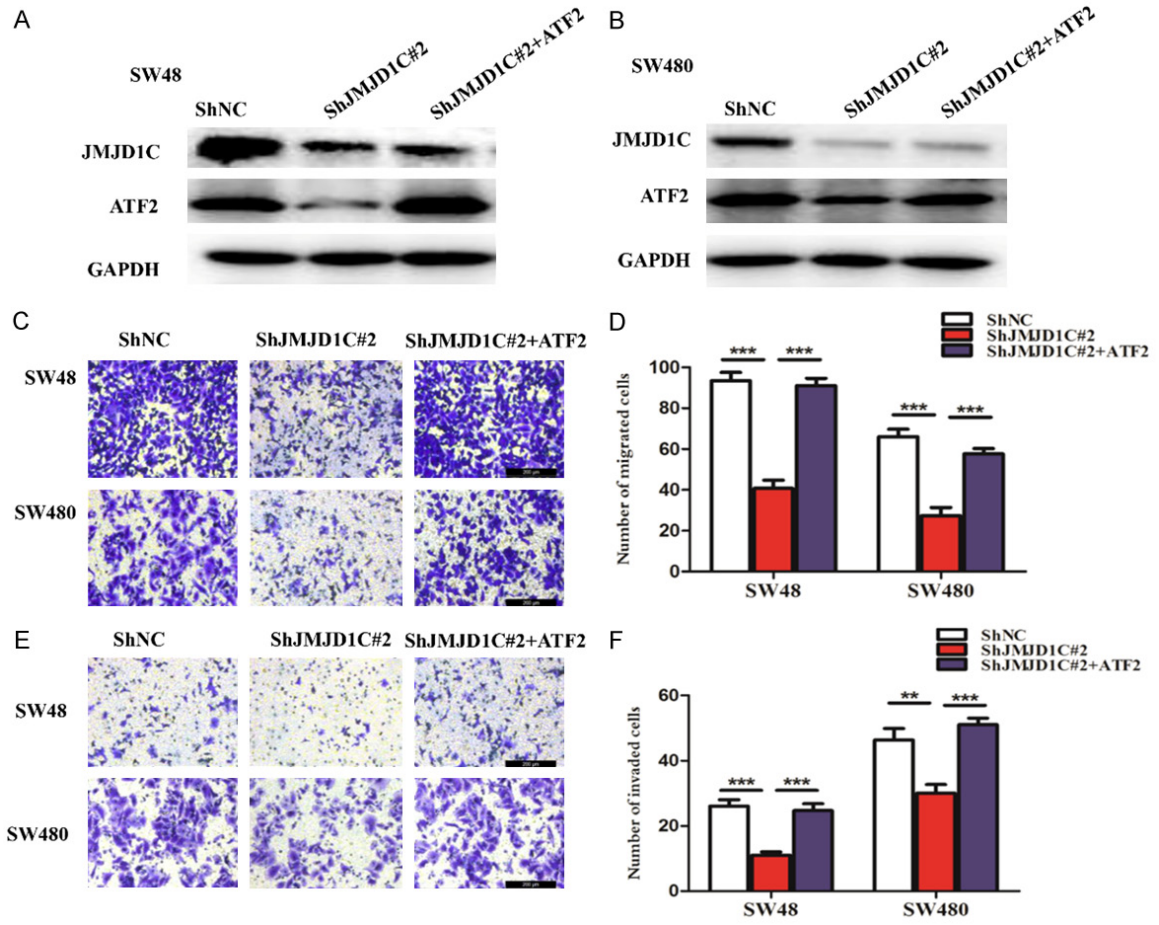


Figure 4. JMJD1C regulates CRC metastasis dependent on ATF2 expression. A, B. Western blotting assay demonstrated that the protein levels of JMJD1C and ATF2 expression in the indicated cells, GAPDH served as the internal control. ***P*<0.01. C, D. Representative micrographs and quantification of the migration ability of ShJMJD1C cells, ShJMJD1C cells with transiently overexpression of ATF2 compared to the control cells. Scale Bar. 200 μm, ****P*<0.001. E, F. Representative micrographs and quantification of the invasion ability of ShJMJD1C cells, ShJMJD1C cells with transiently overexpression of ATF2 compared to the control cells. Scale Bar. 200 μm, **P<0.01, ****P*<0.001.

ly correlated with ATF2 expression in previously published CRC databases (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi) (Supplementary Figure 4).

Discussion

Amounting evidence have demonstrated that epigenetic modification has involved in several cellular processes [30, 31]. Histone-modifying enzymes have been implicated in cancer development and progression. In this study, we demonstrated that JMJD1C is upregulated in CRC tissues. Moreover, the expression of JMJD1C is strongly associated with tumor TNM and lymphatic metastasis. In addition, we found that overexpression JMJD1C is also related with patient overall survival by analyzing several online databases. Further, we showed that knockdown JMJD1C markedly inhibited CRC metastasis through transcriptional regulation of ATF2 pathway in vitro and in vivo.

The deregulation of JmjC domain-containing proteins has been indicated to be the cause of cancer development [32-34]. For example, recent investigation has showed that the histone demethylase KDM3A promoted Ewing Sarcoma cell metastasis through directly regulation of MCAM expression [35]. In addition, in melanoma JMJD3 could regulate the tumor microenvironment and accelerate cancer metastasis [36], suggesting that JmjC domain-containing proteins play a crucial role in several

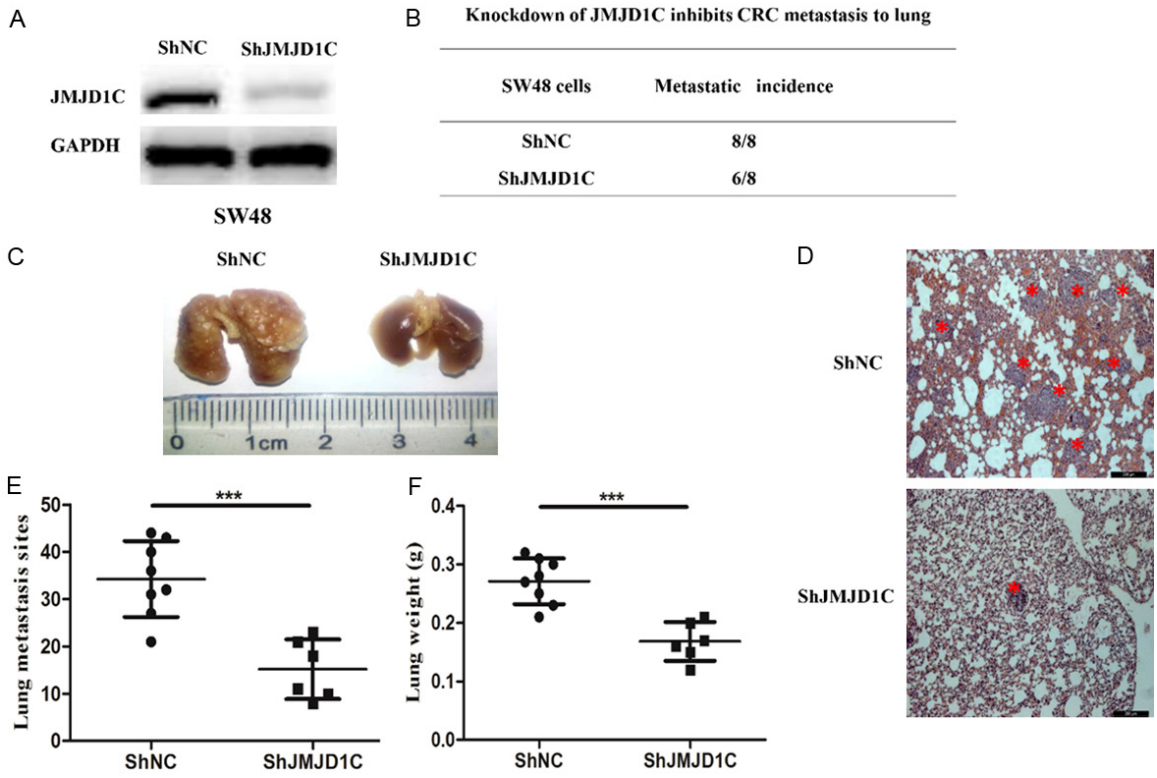


Figure 5. Knockdown of JMJD1C inhibits CRC metastasis in vivo. A. Western blotting assay showed that the protein level of JMJD1C in cells stably expressing ShRNA-JMJD1C compared to control cells. B. Table showed the lung metastatic incidence in ShJMJD1C mice group compared to control mice group. C, D. Representative images of lung and H&E-stained lung sections showed spontaneous metastases generated from SW48 cells after intravenous injection. E. Scatter plot showed lung metastatic nodules in ShJMJD1C-SW48 cells group compared to the control group, ***P<0.001. F. Scatter plot showed the wet weight of lungs in ShJMJD1C-SW48 cells group compared to the control group, ***P<0.001.

cancers. JMJD1C is a member of JmjC domain-containing proteins and functions as a transcriptional coactivator in several gene expression programs. It has been known that JMJD1C mainly demethylased the H3K9me1 and H3K9me2 to modulate gene expression. Interestingly, JMJD1C regulated the activity of H3K9me1 and H3K9me2 in a context-dependent manner. Up to now, it has been reported that an increase JMJD1C were found in EC tissues and knockdown JMJD1C inhibited the proliferation of EC cells [16]. Several lines of evidence implied that JMJD1C is an oncogene as it could promote cancer proliferation and inhibit apoptosis [37]. However, the role and function of JMJD1C in CRC has not been explored and the clinical value of JMJD1C in CRC is yet to be investigated. Here, we showed that JMJD1C is upregulated in CRC tissues and is related with poor clinical outcomes. More important, both in our laboratory clinical samples and online database also showed that the expression of JMJD1C is associated with lymphatic metastasis, strongly suggesting that JMJD1C might be involved in CRC metastasis to regulate the progress of CRC. Next, we provided powerful evidence that knockdown JMJD1C significantly decrease CRC metastasis in vitro and in vivo.

ATF2 is a transcriptional factor and binds to the cAMP-responsive element (CRE) involving in gene expression programs. Undoubtedly, ATF2 has many normal physiological roles including regulate apoptosis and mediate cell death. ATF2 has been revealed to interact with JNK [38] and b-catenin [39], the major component of WNT pathway, suggesting that ATF2 plays an important role in cancer development. Actually, several reports have indicated that ATF2 is involved in cancer development. Lau *et al*. and his colleagues have showed that ATF2 conferred melanoma resistance to therapy [40]. In pancreatic cancer, knockdown of ATF2

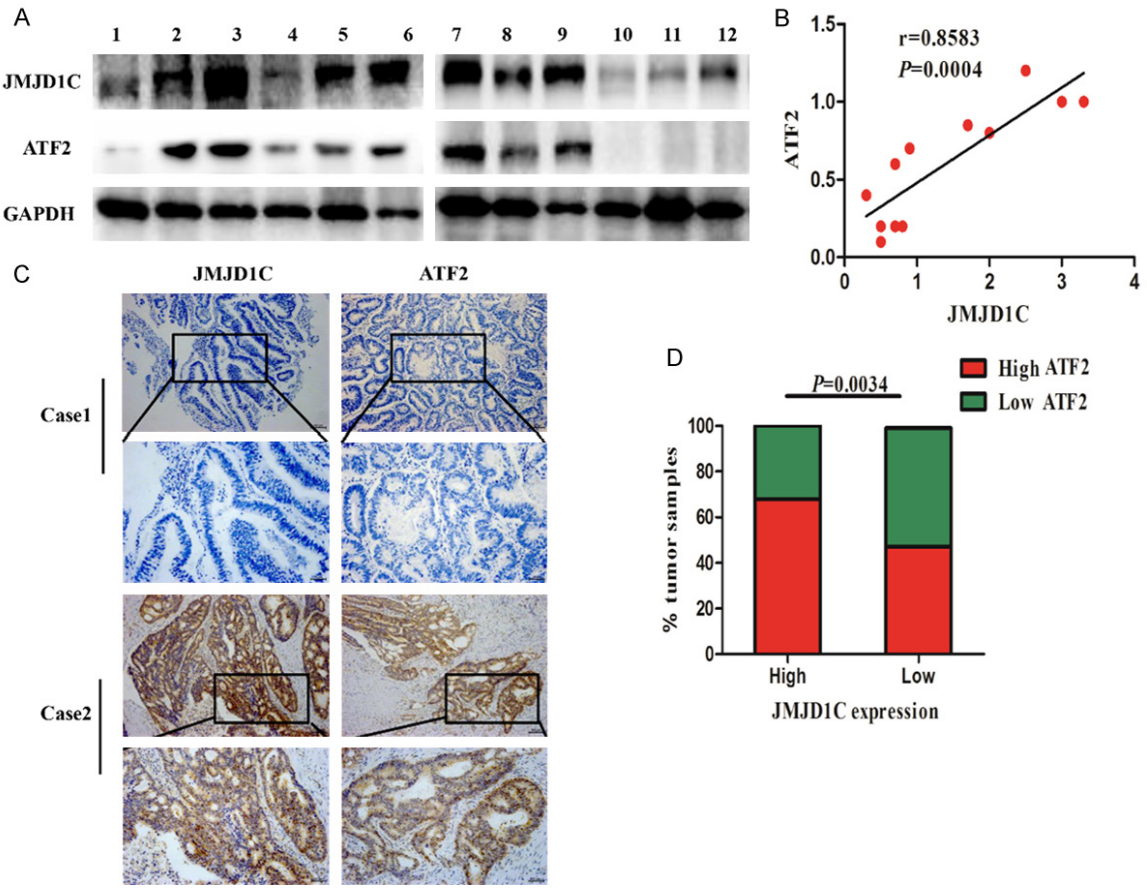


Figure 6. Correlation between JMJD1C and ATF2 expression in CRC. (A, B) Western blotting and correlation analyses showed that the JMJD1C expression was positively associated with ATF2 expression in 12 freshly human CRC tissues. (C, D) The expression of JMJD1C was positively associated with the expression of ATF2 examined by IHC in 100 primary human CRC samples. Representative cases were shown (C) and quantification of IHC was also shown (D).

attenuated cancer cells growth and arrested cell cycle at G1 phase [41], indicating that ATF2 may act as a novel biomarker of pancreatic cancer. The oncogenic characters of ATF2 have been associated with its phosphorylation. However, the transcriptional regulation of ATF2 is still unclear in CRC. Here, we found that the protein and mRNA levels of ATF2 expression was downregulated by silencing JMJD1C in several CRC cells. In addition, we unclosed that ATF2 was involved into CRC metastasis mediated by JMJD1C. In vitro experiments, we confirmed that the decreased metastasis mediated by knockdown JMJD1C partially was reversed by overexpression ATF2, which is consistent with other findings that ATF2 might function as an oncogene in tumor progression. Given the different role of JMJD1C in regulation of H3K9me1 and H3K9me2 in a context-dependent manner, we further explored the precise molecular mechanism underlying the regulation of ATF2 mediated by JMJD1C. By ChIP assay, JMJD1C was been showed to directly bind to the promoter of ATF2, demonstrating that JMJD1C might exhibit its histone demethylase activity to regulate ATF2 expression. Further, we showed that the regulation of ATF2 by JMJD1C was dependent on the H3K9me2 but not H3K9me1 activity. Similar to this data, the regulation of YAP mediated by JMJD1C also dues to H3K9me2 in EC. By previous online database analysis, we showed that ATF2 pathway is associated with CRC metastasis, suggesting that ATF2 may be a novel biomarker for CRC metastasis. In our data, we explored that the decreased metastasis mediated by knockdown JMJD1C was reversed by overexpression of ATF2 in vitro.

Taken together, our findings demonstrated that JMJD1C was increased in CRC and is associ-

ated with clinical outcomes. Furthermore, in vitro, knockdown JMJD1C strongly inhibited cell migration via transcriptionally regulating the expression of ATF2. More important, we stated that the regulation of ATF2 by JMJD1C was dependent on H3K9me2 but not H3K9me1. In addition, the re-expression ATF2 partially reversed knockdown JMJD1C-meidated cell metastasis. Collectively, we revealed that the novel JMJD1C/ATF2 signaling pathway may be a promising therapeutic target for CRC metastasis.

Acknowledgements

This study was supported by funds to 1) Natural Science Foundation of China (No. 81600441); 2) Shandong Provincial Natural Science Foundation, China (No. ZR2014HQ071).

Disclosure of conflict of interest

None.

Address correspondence to: Wen-Bin Yu, Department of General Surgery, Qilu Hospital of Shandong University, 107 West Wenhua Road, Jinan 250012, China. E-mail: wenbinn_yu1969@126.com

References

[1] Siegel RL, Miller KD, Fedewa SA, Ahnen DJ, Meester RGS, Barzi A and Jemal A. Colorectal cancer statistics, 2017. CA Cancer J Clin 2017; 67: 177-193.

[2] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.

[3] Poston GJ, Figueras J, Giuliante F, Nuzzo G, Sobrero AF, Gigot JF, Nordlinger B, Adam R, Gruenberger T, Choti MA, Bilchik AJ, Van Cutsem EJ, Chiang JM and D'Angelica MI. Urgent need for a new staging system in advanced colorectal cancer. J Clin Oncol 2008; 26: 4828-4833.

[4] Chang GJ, Kaiser AM, Mills S, Rafferty JF and Buie WD. Practice parameters for the management of colon cancer. Dis Colon Rectum 2012; 55: 831-843.

[5] Fidler IJ. Critical factors in the biology of human cancer metastasis. Am Surg 1995; 61: 1065-1066.

[6] Van Cutsem E, Nordlinger B, Adam R, Kohne CH, Pozzo C, Poston G, Ychou M, Rougier P; European Colorectal Metastases Treatment Group. Towards a pan-European consensus on the treatment of patients with colorectal liver metastases. Eur J Cancer 2006; 42: 2212-2221.

[7] Sirchia SM, Ferguson AT, Sironi E, Subramanyan S, Orlandi R, Sukumar S and Sacchi N. Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. Oncogene 2000; 19: 1556-1563.

[8] Jaffe LF. Epigenetic theories of cancer initiation. Adv Cancer Res 2003; 90: 209-230.

[9] Vu LP, Luciani L and Nimer SD. Histone-modifying enzymes: their role in the pathogenesis of acute leukemia and their therapeutic potential. Int J Hematol 2013; 97: 198-209.

[10] Zhang K and Dent SY. Histone modifying enzymes and cancer: going beyond histones. J Cell Biochem 2005; 96: 1137-1148.

[11] Derr RS, van Hoesel AQ, Benard A, Goossens-Beumer IJ, Sajet A, Dekker-Ensink NG, de Kruijf EM, Bastiaannet E, Smit VT, van de Velde CJ and Kuppen PJ. High nuclear expression levels of histone-modifying enzymes LSD1, HDAC2 and SIRT1 in tumor cells correlate with decreased survival and increased relapse in breast cancer patients. BMC Cancer 2014; 14: 604.

[12] Cock-Rada A and Weitzman JB. The methylation landscape of tumour metastasis. Biol Cell 2013; 105: 73-90.

[13] Wang Z, Tang F, Qi G, Yuan S, Zhang G, Tang B and He S. KDM5B is overexpressed in gastric cancer and is required for gastric cancer cell proliferation and metastasis. Am J Cancer Res 2015; 5: 87-100.

[14] Nakamura S, Tan L, Nagata Y, Takemura T, Asahina A, Yokota D, Yagyu T, Shibata K, Fujisawa S and Ohnishi K. JmjC-domain containing histone demethylase 1B-mediated p15(Ink4b) suppression promotes the proliferation of leukemic progenitor cells through modulation of cell cycle progression in acute myeloid leukemia. Mol Carcinog 2013; 52: 57-69.

[15] Ding X, Pan H, Li J, Zhong Q, Chen X, Dry SM and Wang CY. Epigenetic activation of AP1 promotes squamous cell carcinoma metastasis. Sci Signal 2013; 6: ra28.1-13, S0-15.

[16] Kottakis F, Foltopoulou P, Sanidas I, Keller P, Wronski A, Dake BT, Ezell SA, Shen Z, Naber SP, Hinds PW, McNiel E, Kuperwasser C and Tsichlis PN. NDY1/KDM2B functions as a master regulator of polycomb complexes and controls self-renewal of breast cancer stem cells. Cancer Res 2014; 74: 3935-3946.

[17] Tang B, Qi G, Tang F, Yuan S, Wang Z, Liang X, Li B, Yu S, Liu J, Huang Q, Wei Y, Zhai R, Lei B, Yu H, Tomlinson S and He S. Aberrant JMJD3 expression upregulates slug to promote migration, invasion, and stem cell-like behaviors in hepatocellular carcinoma. Cancer Res 2016; 76: 6520-6532.

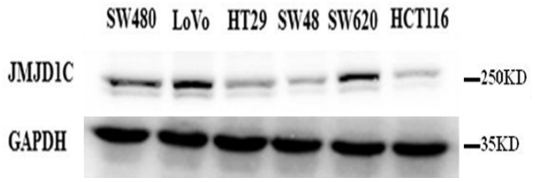
[18] Chen M, Zhu N, Liu X, Laurent B, Tang Z, Eng R, Shi Y, Armstrong SA and Roeder RG. JMJD1C is

required for the survival of acute myeloid leukemia by functioning as a coactivator for key transcription factors. Genes Dev 2015; 29: 2123-2139.
[19] Cai Y, Fu X and Deng Y. Histone demethylase JMJD1C regulates esophageal cancer proliferation Via YAP1 signaling. Am J Cancer Res 2017; 7: 115-124.
[20] Zhang X, Zhang Y, Fan C, Wang L, Liu Y, Li A, Jiang G, Zhou H, Cai L and Miao Y. Noxin promotes proliferation of breast cancer cells via P38-ATF2 signaling pathway. Tumour Biol 2017; 39: 1010428317705515.
[21] Liu Z, Gan L, Wu T, Feng F, Luo D, Gu H, Liu S and Sun C. Adiponectin reduces ER stress-induced apoptosis through PPARalpha transcriptional regulation of ATF2 in mouse adipose. Cell Death Dis 2016; 7: e2487.
[22] Banerjee B, Nandi P, Chakraborty S, Raha S, Sen PC and Jana K. Resveratrol ameliorates benzo(a)pyrene-induced testicular dysfunction and apoptosis: involvement of p38 MAPK/ATF2/iNOS signaling. J Nutr Biochem 2016; 34: 17-29.
[23] You Z, Zhou Y, Guo Y, Chen W, Chen S and Wang X. Activating transcription factor 2 expression mediates cell proliferation and is associated with poor prognosis in human non-small cell lung carcinoma. Oncol Lett 2016; 11: 760-766.
[24] Liu F, Cheng Z, Li X, Li Y, Zhang H, Li J, Liu F, Xu H and Li F. A novel Pak1/ATF2/miR-132 signaling axis is involved in the hematogenous metastasis of gastric cancer cells. Mol Ther Nucleic Acids 2017; 8: 370-382.
[25] Lau E, Feng Y, Claps G, Fukuda MN, Perlina A, Donn D, Jilaveanu L, Kluger H, Freeze HH and Ronai ZA. The transcription factor ATF2 promotes melanoma metastasis by suppressing protein fucosylation. Sci Signal 2015; 8: ra124.
[26] Kaiser S, Park YK, Franklin JL, Halberg RB, Yu M, Jessen WJ, Freudenberg J, Chen X, Haigis K, Jegga AG, Kong S, Sakthivel B, Xu H, Reichling T, Azhar M, Boivin GP, Roberts RB, Bissahoyo AC, Gonzales F, Bloom GC, Eschrich S, Carter SL, Aronow JE, Kleimeyer J, Kleimeyer M, Ramaswamy V, Settle SH, Boone B, Levy S, Graff JM, Doetschman T, Groden J, Dove WF, Threadgill DW, Yeatman TJ, Coffey RJ Jr, Aronow BJ. Transcriptional recapitulation and subversion of embryonic colon development by mouse colon tumor models and human colon cancer. Genome Biol 2007; 8: R131.
[27] Skrzypczak M, Goryca K, Rubel T, Paziewska A, Mikula M, Jarosz D, Pachlewski J, Oledzki J and Ostrowski J. Modeling oncogenic signaling in colon tumors by multidirectional analyses of microarray data directed for maximization of analytical reliability. PLoS One 2010; 5.
[28] Marisa L, de Reynies A, Duval A, Selves J, Gaub MP, Vescovo L, Etienne-Grimaldi MC, Schiappa R, Guenot D, Ayadi M, Kirzin S, Chazal M, Flejou JF, Benchimol D, Berger A, Lagarde A, Pencreach E, Piard F, Elias D, Parc Y, Olschwang S, Milano G, Laurent-Puig P and Boige V. Gene expression classification of colon cancer into molecular subtypes: characterization, validation, and prognostic value. PLoS Med 2013; 10: e1001453.
[29] Roessler S, Lin G, Forgues M, Budhu A, Hoover S, Simpson RM, Wu X, He P, Qin LX, Tang ZY, Ye QH and Wang XW. Integrative genomic and transcriptomic characterization of matched primary and metastatic liver and colorectal carcinoma. Int J Biol Sci 2015; 11: 88-98.
[30] Sutherland JE and Costa M. Epigenetics and the environment. Ann N Y Acad Sci 2003; 983: 151-160.
[31] Vaissiere T, Sawan C and Herceg Z. Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutat Res 2008; 659: 40-48.
[32] Han XR, Zha Z, Yuan HX, Feng X, Xia YK, Lei QY, Guan KL and Xiong Y. KDM2B/FBXL10 targets c-Fos for ubiquitylation and degradation in response to mitogenic stimulation. Oncogene 2016; 35: 4179-4190.
[33] Yi X, Jiang XJ, Li XY and Jiang DS. Histone methyltransferases: novel targets for tumor and developmental defects. Am J Transl Res 2015; 7: 2159-2175.
[34] Van Rechem C, Black JC, Boukhali M, Aryee MJ, Graslund S, Haas W, Benes CH and Whetstine JR. Lysine demethylase KDM4A associates with translation machinery and regulates protein synthesis. Cancer Discov 2015; 5: 255-263.
[35] Sechler M, Parrish JK, Birks DK and Jedlicka P. The histone demethylase KDM3A, and its downstream target MCAM, promote Ewing sarcoma cell migration and metastasis. Oncogene 2017; 36: 4150-4160.
[36] Krieg AJ, Rankin EB, Chan D, Razorenova O, Fernandez S and Giaccia AJ. Regulation of the histone demethylase JMJD1A by hypoxia-inducible factor 1 alpha enhances hypoxic gene expression and tumor growth. Mol Cell Biol 2010; 30: 344-353.
[37] Sroczynska P, Cruickshank VA, Bukowski JP, Miyagi S, Bagger FO, Walfridsson J, Schuster MB, Porse B and Helin K. shRNA screening identifies JMJD1C as being required for leukemia maintenance. Blood 2014; 123: 1870-1882.
[38] Gozdecka M, Lyons S, Kondo S, Taylor J, Li Y, Walczynski J, Thiel G, Breitwieser W and Jones N. JNK suppresses tumor formation via a gene-expression program mediated by ATF2. Cell Rep 2014; 9: 1361-1374.

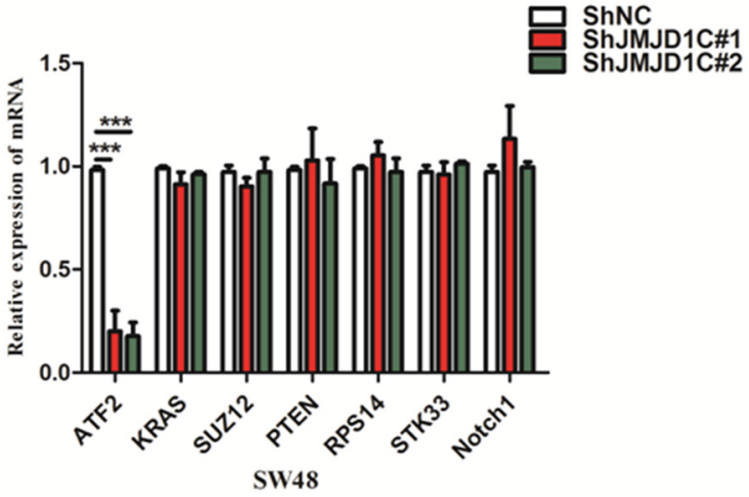
[39] Grumolato L, Liu G, Haremaki T, Mungamuri SK, Mong P, Akiri G, Lopez-Bergami P, Arita A, Anouar Y, Mlodzik M, Ronai ZA, Brody J, Weinstein DC and Aaronson SA. beta-Catenin-independent activation of TCF1/LEF1 in human hematopoietic tumor cells through interaction with ATF2 transcription factors. PLoS Genet 2013; 9: e1003603.

[40] Lau E, Sedy J, Sander C, Shaw MA, Feng Y, Scortegagna M, Claps G, Robinson S, Cheng P, Srivas R, Soonthornvacharin S, Ideker T, Bosenberg M, Gonzalez R, Robinson W, Chanda SK, Ware C, Dummer R, Hoon D, Kirkwood JM and Ronai ZA. Transcriptional repression of IFNbeta1 by ATF2 confers melanoma resistance to therapy. Oncogene 2015; 34: 5739-5748.

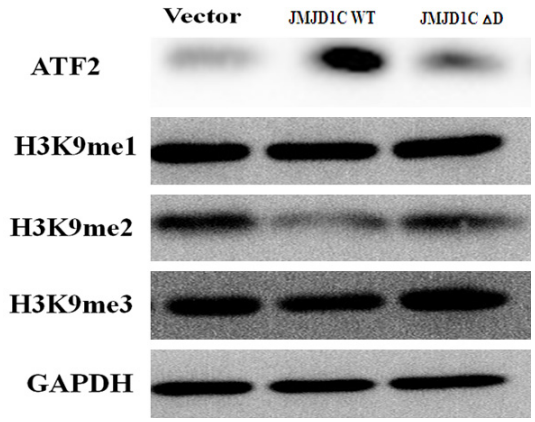
[41] Li M, Wu X, Liu N, Li X, Meng F and Song S. Silencing of ATF2 inhibits growth of pancreatic cancer cells and enhances sensitivity to chemotherapy. Cell Biol Int 2017; 41: 599-610.



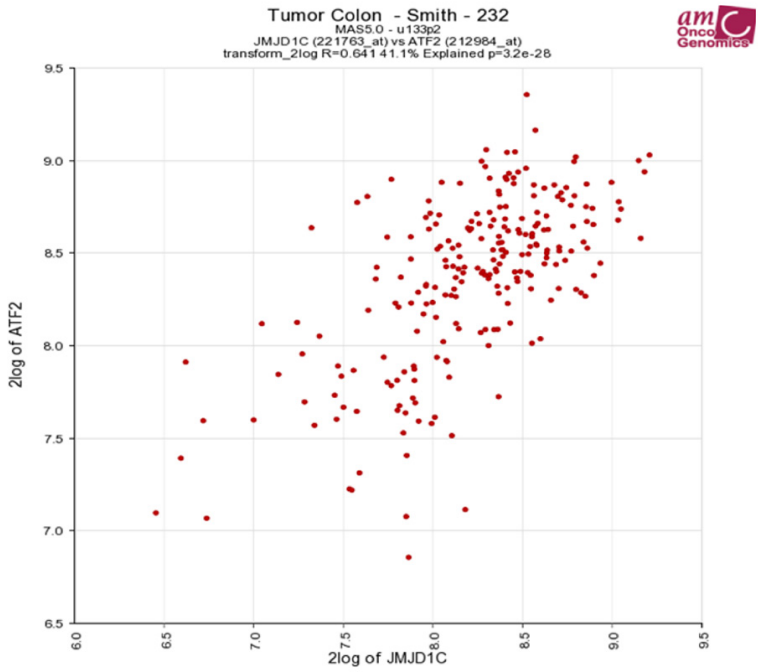
Supplementary Figure 1. Western blotting assay showed the expression of JMJD1C in different CRC cell lines.



Supplementary Figure 2. Qpcr assay showed the mRNA expression of related genes in indicated cell lines.



Supplementary Figure 3. Western blot showed that JMJD1C WT upregulated the expression of ATF2 and increased H3K9me2 expression, but JMJD1C ΔD could not in SW48.



Supplementary Figure 4. Bioinformatics assay showed that the relationship between JMJD1C and ATF2.