

METTL3 antagonizes 5-FU chemotherapy and confers drug resistance in colorectal carcinoma

MIN LI^{1*}, MINGYUE XIA^{2*}, ZIYU ZHANG^{2*}, YANYIN TAN¹, ENJIE LI²,
ZHIGANG GUO², MINGZHI FANG¹, YONG ZHU³ and ZHIGANG HU²

¹Department of Oncology, Nanjing Hospital of Chinese Medicine Affiliated to Nanjing University of Chinese Medicine;

²Jiangsu Key Laboratory for Molecular and Medical Biotechnology, College of Life Sciences, Nanjing Normal University;

³National Centre of Colorectal Disease, Nanjing Hospital of Chinese Medicine Affiliated to Nanjing University of Chinese Medicine, Nanjing, Jiangsu 210023, P.R. China

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Abstract. Colorectal cancer (CRC) is one of top five leading causes of cancer-associated mortalities worldwide. 5-Fluorouracil (5-FU) is the first-line chemotherapeutic drug in the treatment of CRC; however, its antineoplastic efficiency is limited due to acquired drug resistance. The regulatory mechanism underlying 5-FU chemotherapeutic response and drug resistance in CRC remains largely unknown. The present study identified that silencing of methyltransferase-like 3 (METTL3) suppressed the proliferation and migration of CRC HCT-8 cells. Using cell survival assays, flow cytometric and colony formation analyses, it was revealed that inhibition of METTL3 sensitized HCT-8 cells to 5-FU by enhancing DNA damage and inducing apoptosis in HCT-8 cells under 5-FU treatment. Furthermore, the expression of METTL3 was upregulated in 5-FU-resistant CRC cells (HCT-8R), which contributed to drug resistance through regulation of RAD51 associated Protein 1 (RAD51AP1) expression. Western blotting, immunofluorescence staining and drug

sensitivity assays demonstrated that knockdown of METTL3 augmented 5-FU-induced DNA damage and overcame 5-FU-resistance in HCT-8R cells, which could be mimicked by inhibition of RAD51AP1. The present study revealed that the METTL3/RAD51AP1 axis plays an important role in the acquisition of 5-FU resistance in CRC, and targeting METTL3/RAD51AP1 may be a promising adjuvant therapeutic strategy for patients with CRC, particularly for those with 5-FU-resistant CRC.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer type worldwide, with ~2,000,000 new cases in 2020 (1). It is the third most common cancer type in males, and the second most common cancer type in females after breast cancer (2). CRC is the second most common cause of cancer-associated mortalities worldwide (2). Thanks to the advances in treatments, including endoscopic and surgical excision, radiotherapy, targeted therapy, immunotherapy and chemotherapy, CRC-based therapies have improved, and promising results have been reported (1,3).

5-Fluorouracil (5-FU) is the first-line chemotherapeutic agent for CRC treatment (4). As an anti-metabolite drug that has been widely used in cancer treatments and clinical studies, 5-FU induces cytotoxicity, mainly by interfering with essential biosynthetic processes through the inhibition of thymidylate synthase or by leading to errors in base pairing during RNA and DNA synthesis (5). Furthermore, 5-FU exposure induces DNA damage, thereby promoting apoptosis in cancer cells (6). However, despite its several advantages in cancer management, a critical limitation to the clinical application of 5-FU is the development of chemotherapeutic drug resistance in cancer cells. Resistance to 5-FU has been proposed to develop through drug uptake inhibition, target alterations, elevated DNA repair abilities and resistance to apoptosis (5). Numerous genes have been detected to be involved in the development of resistance to 5-FU in cancer cells (7). Thus, targeting key genes or signaling pathways involved in drug resistance has the potential as a therapeutic strategy to overcome 5-FU resistance during cancer treatment (5,7).

Correspondence to: Professor Yong Zhu, National Centre of Colorectal Disease, Nanjing Hospital of Chinese Medicine Affiliated to Nanjing University of Chinese Medicine, 157 Daming Road, Nanjing, Jiangsu 210023, P.R. China
E-mail: fsyy00233@njucm.edu.cn

Professor Zhigang Hu, Jiangsu Key Laboratory for Molecular and Medical Biotechnology, College of Life Sciences, Nanjing Normal University, 1 Wenyuan Road, Nanjing, Jiangsu 210023, P.R. China
E-mail: huzg_2000@126.com

*Contributed equally

Abbreviations: METTL3, methyltransferase-like 3; FBS, fetal bovine serum; HR, homologous recombination; PBS, phosphate-buffered saline; PI, propidium iodide

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Methyltransferase-like 3 (METTL3) is a key m^6 -methyladenosine (m^6A) methyltransferase enzyme that catalyzes the m^6A modification of its target transcripts. Due to its role in m^6A modification, METTL3 regulates various biological processes, including the cell cycle, cell proliferation, apoptosis, migration invasion and differentiation, and inflammatory response (8). Previous studies have demonstrated the functions of METTL3 and m^6A modification in tumorigenesis and cancer progression in different cancer types, including CRC (9,10). METTL3 was consistently overexpressed in CRC and facilitated CRC progression (11). Additionally, higher METTL3 expression in CRC was associated with poor prognosis. However, the function and mechanism of METTL3 chemotherapeutic efficacy and drug resistance in CRC remain unexplored.

In the present study, a series molecular, biochemical and cellular experiments were performed to explore the function of METTL3 with regard to chemotherapeutic response and drug resistance in CRC. The present study demonstrated the function of METTL3 in regulating cell proliferation, chemotherapeutic response and 5-FU resistance in CRC cells. It was observed that silencing METTL3 suppressed cell proliferation, enhanced chemotherapeutic response and overcame 5-FU resistance in CRC cells through the regulation of RAD51 associated Protein 1 (RAD51AP1), which is a key homologous recombination (HR) repair protein. Therefore, the present results suggest the potential use of METTL3 as a therapeutic target for CRC treatment, either alone or as a combined treatment strategy.

Materials and methods

Plasmid construction. For the knockdown of METTL3 and RAD51AP1, silencing plasmids containing small hairpin RNA (shRNA) METTL3, 5'-CTCAGTGGATCTGTTGTG ATA-3' (12); and RAD51AP1, 5'-GCACTAGCTTTATCA GTGA-3' sequences (13) were constructed based on the psilencer3.0-H1. The sequence of the shRNA negative control (sh-CN) was 5'-GTCAGGCTATCGCGTATCG-3'. The METTL3-overexpressing plasmid was obtained from WZ Biosciences, Inc. ORF of human METTL3 was constructed in pENTER vector, with C terminal Flag and His tag. All plasmids were verified through sequencing. For plasmid transfection, cells were seeded at a density of 3×10^5 cells/well in 6-well plates. Subsequently, 24 h later, the cells were transfected with sh-CN, sh-METTL3, or shRAD51AP1 plasmid (2.0 μ g per well) using HighGene transfection reagent (cat. no. RM09014; Abclonal Biotech Co., Ltd.) at 37°C, following the manufacturer's instructions. Finally, 48 h after transfection, the cells were collected for reverse transcription-quantitative PCR (RT-qPCR) analysis, western blotting assay, or subsequent experiments assay.

METTL3-knockdown (KD) and METTL3-overexpressing lentiviruses were constructed by Corues Biotechnology Company, using the oligonucleotide METTL3-shRNA and METTL3-overexpressing vector (pENTER), respectively. For lentiviral particle production, 293T cells (cat. no. GNHu17; Shanghai Institute of Cell Biology, Chinese Academy of Sciences) were seeded 24 h before lentiviral infection. Lentiviruses were packaged by transfecting the aforementioned

vectors (sh-METTL3, sh-CN transfer vector (psilencer3.0-H1), control pENTER or METTL3-overexpressing vector), psPAX2, and pMD2.G under a 4.5:2:1 ratio into 293T cells. The collected viral mix was purified by ultracentrifugation at $30,000 \times g$ for 2 h at 4°C. Purified viral particles were used to infect target cells with polybrene (Sigma-Aldrich-Merck KGaA). After a 48-h incubation at 37°C, infected cells were screened with 1 mg/ml of puromycin (InvivoGen, Inc.) for two weeks.

Cell lines and cell culture. The human CRC HCT-8 cell line was obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (cat. no. TCHU 18). In addition, human normal epithelial cell line, NCM460, was obtained from INCELL Corporation LLC, and cultured in M3 media supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a 95% humidified atmosphere with 5% CO₂. HCT-8 cells were cultured in the recommended RMPI-1640 medium (cat. no. KGM31800N-500; Nanjing KeyGEN BioTECH Co, Ltd.) and F12 medium (cat. no. KGM21700-500; Nanjing KeyGEN BioTECH Co., Ltd.), supplemented with 10% fetal bovine serum (FBS; Invigentech, Inc.), 100 U/ml penicillin, and 100 U/ml streptomycin, following incubation at 5% CO₂ and a temperature of 37°C. Subsequently, 5-FU-resistant HCT-8 cells (HCT-8R) were selected and established from HCT-8 cells as previously described (7). For METTL3-overexpressing or METTL3-KD HCT-8 and HCT-8R stable cells, the cells were infected with specific lentivirus vectors (MOI, 10) for 48 h and then selected with puromycin (1 mg/ml) for two weeks. These HCT-8 cells were then treated using stepwise dose-dependent 5-FU (product no. F6627; Sigma-Aldrich-Merck KGaA) concentrations (0, 0.01, 0.1, 0.5, 2, and 10 μ M) over 5 months. Subsequently, the acquired drug-resistant HCT-8R cells were cultured and stabilized in a 10- μ M 5-FU-containing medium.

Alkaline comet assay. Alkaline comet assay analysis was performed with a commercial Comet Assay Kit (cat. no. KGA240-100; Nanjing KeyGEN BioTECH Co., Ltd.) following the protocol as previously described (14). Briefly, 1×10^5 cells/ml were mixed with molten LM agarose at 37°C at a ratio of 1:10 (vol/vol) and pipetted onto a COMET slide. The slides were placed for 10 min in the dark at 4°C and were immersed in pre-chilled lysis solution. The slides were then removed from the lysis buffer, washed in Tris-HCl buffer and transferred to a horizontal electrophoresis chamber. Voltage (20 V) was applied for 20 min. After being washed in distilled water, the slides were immersed in 70% ethanol for 5 min and allowed to air dry. Slides were stained with propidium iodide (PI), in the dark, at room temperature for 10 min, and then analyzed by fluorescence microscopy (Nikon 80I; Nikon Corporation). A total of 70-90 cells were evaluated in each sample using the COMET Assay Software Project (CASP software version caspl.2.3b1; CaspLab).

Wound healing assay. The wound healing assay was performed as previously described with some modifications (15). Various cells (control, METTL3-KD or METTL3-overexpressing HCT-8) were seeded in a 6-well plate at a concentration of

5×10^5 cells/well until they reached 80% of confluence. The cultured cells were scratched with a 200- μ l sterile pipette tip in a line. The cells were then washed three times with PBS and cultured with RPMI-1640 medium without FBS for another 12 h or 24 h. The scratches were imaged using a fluorescence microscope at a magnification of x200 (Nikon 80I; Nikon Corporation) and analyzed by ImageJ software (1.52v; National Institutes of Health).

Apoptosis assay through flow cytometry. For the apoptosis assay, cells were first treated with different concentrations of 5-FU close to the IC_{50} value. For the HCT-8 cells, the concentrations were 2 and 4 μ g/ml, and for HCT-8R cells, 60 μ g/ml was used. The cells were then trypsinized, washed, and resuspended in 1 ml of phosphate-buffered saline (PBS) containing 5% FBS. Subsequently, the cells were washed again twice with ice-cold PBS, then fixed in 3 ml ice-cold ethanol. Following centrifugation at $3,000 \times g$ for 3 min at room temperature, the cells were collected and stained with both Annexin V-FITC and PI (Annexin V-FITC/PI Double Staining Apoptosis Detection Kit; cat. no. KGA106; Nanjing KeyGEN BioTECH Co., Ltd. at room temperature for 15 min according to the manufacturer's instructions. Subsequently, apoptosis was analyzed using BD FACSverse flow cytometer and FACSSuite Clinical software (BD Biosciences).

Colony-forming assay. The colony-forming assay was conducted using HCT-8 stable cell lines in which METTL3 was knocked down or overexpressed as previously described (14). Briefly, ~500 cells were plated in a 6-well plate and incubated for approximately 15 days at 37°C. For HCT-8R cell colony formation assay, the cultured cells were treated with 0, 10 and 20 μ g/ml 5-FU, which were lower than the IC_{50} value. Colonies in each well were fixed with 4% paraformaldehyde, and then washed using PBS and stained with 0.05% crystal violet at room temperature for 30 min. Stained plates were washed again and dried before scoring the colonies. Images of the colony-forming units (>50 cells) were captured and recorded using a fluorescence microscope (Nikon 80I) and Adobe Photoshop CC 2017 software.

Immunofluorescence staining. For immunofluorescence assays, HCT-8 stable cell lines were cultured in 6-well plates containing an acid-treated cover slide per well overnight to make these cells adhere to the slide. Cells (~ 5×10^4 cells per slide) on cover slides were then washed with PBS thrice. Subsequently, these cells were fixed in PBS-containing 4% formaldehyde at room temperature for 30 min, then washed with PBS again. Following permeabilization with 0.1% Triton X-100 for 10 min, the cells were blocked with 3% BSA at room temperature for 1 h, and then incubated with a primary antibody against phosphorylated (p)- γ -H2AX (1:300; product no. 9718S; Cell Signaling Technology, Inc.), KI67 (1:300; cat. no. 27309-1-AP; ProteinTech Group, Inc.) and 53BP1 (1:300; product code ab175933; Abcam) overnight at 4°C. Finally, washing with PBST was performed twice, after which these cells were incubated with fluorescent secondary antibodies at room temperature for 90 min. The secondary antibodies were as follows: Alexa Fluor™ 594 Tyramide SuperBoost™ Kit, goat anti-mouse IgG (1:200 dilution; cat. no. B40942),

and Alexa Fluor™ 488 Tyramide SuperBoost™ Kit, goat anti-rabbit IgG (1:200 dilution; cat. no. B40943; both from Invitrogen; Thermo Fisher Scientific, Inc.). The cells were then stained with DAPI at 37°C for 15 min. The mounted slides were visualized under a fluorescence microscope (Nikon 80I; Nikon Corporation).

m⁶A dot blot assays. Briefly, total RNA was isolated using the TRIzol (Sigma-Aldrich; Merck KGaA) kit, following the manufacturer's instructions. RNA concentration was measured using a NanoDrop ultrafine Ultraviolet spectrophotometer (ND-1000; Thermo Fisher Scientific, Inc.). A total of 2 μ g RNA samples were then spotted onto a positively-charged nylon membrane (GE Healthcare; Cytiva), and air-dried at room temperature for 5 min. The membranes were subsequently UV cross-linked, washed with PBST containing 0.1% Tween-20 (cat. no. T104863; Aladdin) for 5 min, blocked with 5% nonfat milk in TBST containing 0.1 % Tween-20 at room temperature for 90 min, and incubated with an anti-m⁶A antibody (1:1,000 dilution; cat. no. A17924; ABclonal Biotech Co., Ltd.) overnight at 4°C. Subsequently, the HRP-conjugated anti-rabbit IgG secondary antibody (1:10,000 dilution; cat. no. AS014; ABclonal Biotech Co., Ltd.) was added to the membrane with gentle shaking for 1.5 h at room temperature, followed by development with enhanced chemiluminescence (cat. no. FD8020; Hangzhou Fude Biological Technology Co., Ltd.). A total of 1% methylene blue staining agent (cat. no. M196501; Aladdin) was used at room temperature for 1 h for mRNA loading.

Drug sensitivity assay. Approximately 3,000 METTL3 stabilized knockdown or overexpression of HCT-8/HCT-8R cells were seeded per well for at least four parallel experiments. Cells were treated with multiple dilutions of 5-FU for 24 h. The cell viability of 5-FU-treated cells was then assayed using the Cell Counting Kit-8 (CCK-8) assay (cat. no. IV08-1000T; Invigentech, Inc.) according to the manufacturer's instructions. Using CCK-8 treated for 90 min, the cell survival curves of METTL3-KD HCT-8 or HCT-8R cells were determined with various concentrations of 5-FU treatment (METTL3-KD HCT-8 cells: 0, 0.5, 1, 1.5, 3 μ g/ml; METTL3-KD HCT-8R cells: 0, 10, 20, 40, 60 μ g/ml) due to their different sensitivity. In addition, HCT-8 and HCT-8R cells were treated with 0, 3, 6, 9, 12 μ g/ml 5-Fu. In subsequent experiments, RAD51AP1-KD HCT-8 cells were treated with different concentrations of 5-Fu (0, 0.5, 1, 3, 12 μ g/ml). Data were expressed as the percentage of growth relative to the untreated controls.

Reverse transcription-quantitative PCR (RT-qPCR) assay. RNA extraction and real-time PCR Total RNA from cells were isolated by TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA with HiScript II Q RT SuperMix kit (cat. no. R222-01; Vazyme Biotech Co., Ltd.). RT-qPCR was performed using SYBR Green (cat. no. Q341-02; Vazyme Biotech Co., Ltd.) and operated on an ABI StepOne PCR system. Each reaction was repeated three times. Gene relative expression was determined relative to β -actin in each reaction. The qPCR cycling conditions were as follows: Denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 sec, primer annealing and extension at 60°C for 1 min. The Cq value was determined

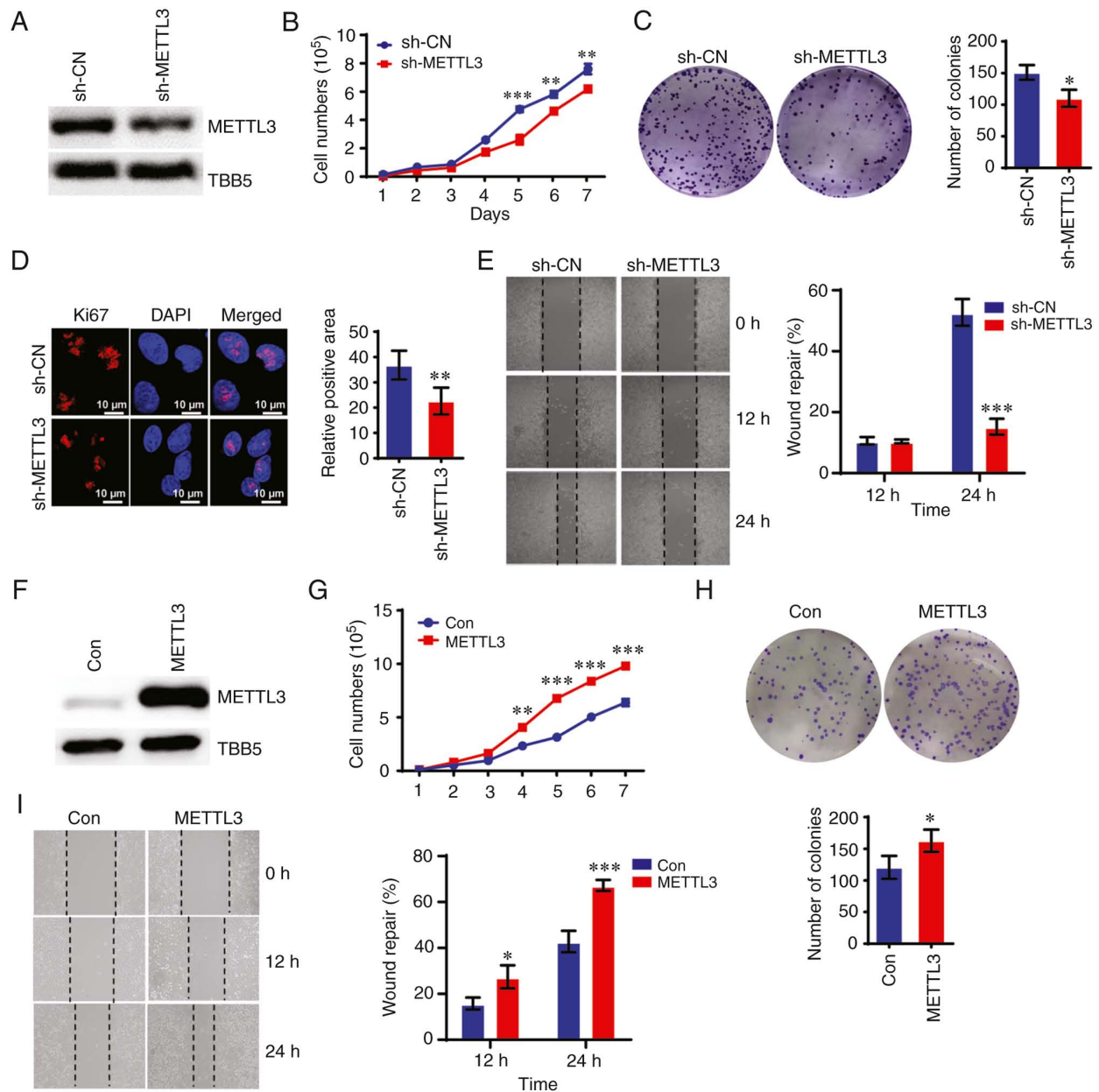


Figure 1. METTL3 regulates colorectal cancer cells proliferation and migration. (A) Western blotting confirmed the knockdown of METTL3 by shRNA. (B) Downregulation of METTL3 suppresses cell proliferation in HCT-8 cells. (C) Knockdown of METTL3 inhibits colony formation of HCT-8 cells. (D) The positive Ki67 staining rates were downregulated in METTL3-KD HCT-8 cells. (E) Scratch wound healing assay using METTL3-KD and control HCT-8 cells (magnification, x200). (F) Overexpression of METTL3 in HCT-8 cells. (G) Overexpression of METTL3 promotes cell proliferation in HCT-8 cells. (H) Overexpression of METTL3 promotes colony formation of HCT-8 cells. (I) Scratch wound healing assay using METTL3-overexpressing and control HCT-8 cells (magnification, x200). Data are presented as the means \pm SD. * P <0.05, ** P <0.01 and *** P <0.001. METTL3, methyltransferase-like 3; KD, knock-down; sh-, small hairpin; CN, control.

from the same threshold fluorescence value for the analyzed genes. The relative quantities of mRNA were calculated using the comparative quantification cycle method ($2^{-\Delta\Delta C_q}$) (16). The primers for RT-qPCR are listed in Table SI.

Western blotting. Total cellular protein was extracted using RIPA lysis buffer (P0013B; Beyotime Institute of Biotechnology) which contained 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS. Total protein concentration was determined by bicinchoninic acid analysis (Hangzhou Fude Biological Technology Co., Ltd), and then proteins in lysed samples

(30 μ g per well) were separated on a 10% acrylamide gel. Protein was then transferred to polyvinylidene difluoride membranes. Following 5% skimmed milk blocking for 90 min at room temperature, the membranes were incubated overnight at 4°C with a primary antibody. Subsequently, the membranes were incubated with a secondary antibody for 90 min at room temperature following extensive TBST (containing 0.1% Tween-20) washing. The blots were filmed by enhanced chemiluminescence (cat. no. FD8020; Hangzhou Fude Biological Technology Co., Ltd.) and developed via Tanon 4500 luminescent imaging workstation (Tanon Science and Technology Co., Ltd.). The following antibodies were used for western

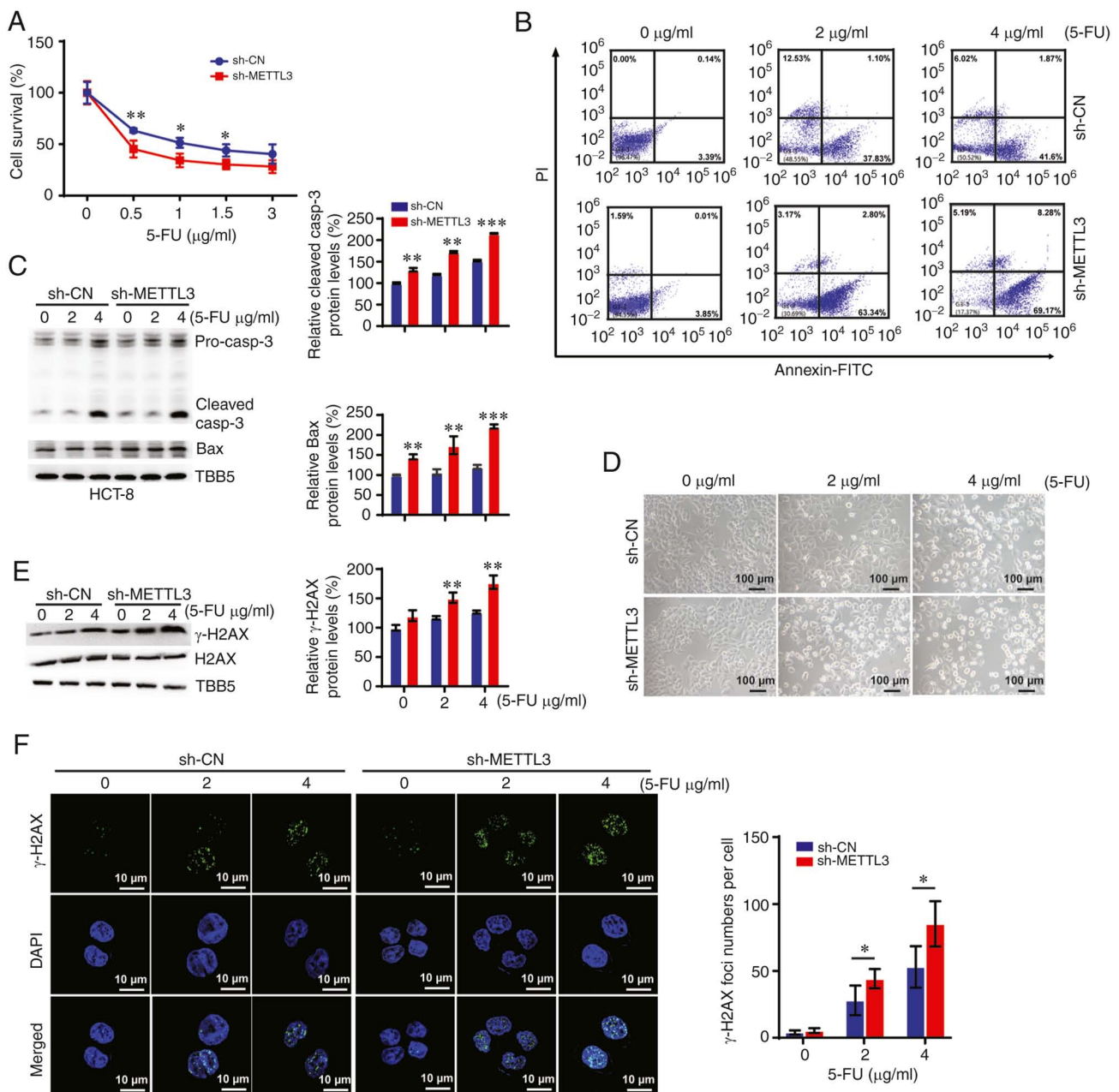


Figure 2. Knockdown of METTL3 induces DNA damage accumulation and sensitizes colorectal cancer cells to 5-FU. (A) Cell survival analysis in METTL3-KD and control HCT-8 cells with 5-FU treatment. (B) Cell apoptosis was analyzed by flow cytometry. (C) Knockdown of METTL3 enhanced the expression of pro-apoptotic proteins caspase-3 and Bax in HCT-8 cells treated with 5-FU. (D) Morphological analysis of control and METTL3-KD HCT-8 cells with various doses (0, 2 and 4 μg/ml) of 5-FU treatment for 24 h. (E) Western blotting to determine γ-H2AX levels in control and METTL3-KD HCT-8 cells following various doses (0, 2 and 4 μg/ml) of 5-FU treatments for 4 h. (F) Immunofluorescence staining of γ-H2AX foci in different cells treated with 5-FU for 4 h. The quantification of the foci numbers per cells are presented on the right panel. Data are presented as the means ± SD. *P<0.05, **P<0.01 and ***P<0.001. METTL3, methyltransferase-like 3; 5-FU, 5-fluorouracil; KD, knockdown; sh-, small hairpin; CN or Con, control.

blotting: Anti-METTL3 (1:1,000 dilution; cat. no. 15073-1-AP; ProteinTech Group, Inc.), anti-RAD51AP1 (1:1,000 dilution; cat. no. 11255-1-AP; ProteinTech Group, Inc.), anti-Flag (1:1,000 dilution; cat. no. AP0007MH; Bioworld Technology, Inc.), anti-p-γH2AX (1:1,000 dilution; product no. 9718S; Cell Signaling Technology, Inc.), anti-H2AX (1:1,000 dilution; cat. no. A11540; ABclonal Biotech Co., Ltd.), anti-caspase-3 (1:1,000 dilution; cat. no. 19677-1-AP; ProteinTech Group, Inc.), anti-Bax (1:1,000 dilution; cat. no. A7626; ABclonal Biotech Co., Ltd.) and anti-TBB5 (1:1,000 dilution; cat. no. AM1031A; Abgent, Inc.). The HRP-conjugated secondary antibody was

obtained from ABclonal Biotech Co., Ltd. (1:10,000 dilution; cat. no. AS014; anti-rabbit IgG; and cat. no. AS003; anti-mouse IgG). The densitometry of western blot protein bands was analyzed by ImageJ software (1.52v; National Institutes of Health).

Bioinformatics analysis. The m⁶A modification in gene exonic 5' untranslated region (5'UTR), or 3'UTR mRNA regions was screened by the m⁶A-Atlas, which is a comprehensive knowledgebase for unraveling the m⁶A epitranscriptome (<http://180.208.58.19/m6A-Atlas/>). The translational studies on

the expression of METTL3 and RAD51AP1 were conducted by LinkedOmics, which is a publicly available portal that includes multi-omics data from all 32 TCGA Cancer types and 10 Clinical Proteomics Tumor Analysis Consortium (CPTAC) cancer cohorts (<http://www.linkedomics.org/>) (17).

Statistical analyses. Each experiment was performed at least three independent times. Statistical analysis was conducted using GraphPad Prism 8.0 (GraphPad Software, Inc.). Data were analyzed for the assumption of normal distribution using the Kolmogorov-Smirnov test and homogeneity test of variance using Bartlett's test. The statistical evaluation was performed by unpaired two-tailed Student's t-test between two groups (sh-METTL3 vs. sh-CN, METTL3 vs. Con, HCT-8 vs. HCT-8R and sh-RAD51AP1 vs. sh-CN), and one-way analysis of variance (ANOVA) for relative m⁶A densities and relative protein levels among multiple groups. Tukey's test was employed to assess the post hoc comparisons for variables that were different among multiple groups. For correlation analysis between the expression of METTL3 and RAD51AP1 observed in CRC from the LinkedOmics database (<http://www.linkedomics.org/>), Pearson's correlation test was used. $P < 0.05$ was considered to indicate statistically significant differences.

Results

METTL3 promotes the proliferation of CRC cells. Elevated expression of METTL3, which has been detected in human CRC tissues, was associated with poor prognosis in patients with CRC (11,18). Therefore, to investigate the regulatory effect of METTL3 on CRC, a METTL3-KD stable cell line was constructed with METTL3 shRNA lentivirus in CRC HCT-8 cells. Western blotting and RT-qPCR demonstrated that METTL3 expression was significantly downregulated in sh-METTL3 cells compared with that in control HCT-8 cells (Figs. 1A and S1A). Downregulating METTL3 markedly suppressed the proliferation of HCT-8 cells, as measured using CCK-8 and colony formation assays (Fig. 1B and C). Ki67 expression was downregulated in sh-METTL3 cells compared with that in control cells (Fig. 1D). The wound healing assay showed that METTL3 depletion significantly impaired the migration of HCT-8 cells compared with that of control cells (Fig. 1E). By contrast, overexpressing METTL3 promoted the proliferation, colony formation ability and migration ability of CRC cells (Figs. 1F-I and S1B). As expected, knockdown of METTL3 reduced global m⁶A levels in HCT-8 cells, whereas overexpression of METTL3 increased m⁶A levels in these cells compared with control cells (Fig. S1C and D).

Knockdown of METTL3 sensitizes CRC cells to 5-FU. 5-FU is currently the most commonly used chemotherapeutic agent to improve survival in patients with CRC (19). Therefore, the present study investigated whether knocking down METTL3 would affect 5-FU treatment in CRC. The cell survival assay demonstrated that knocking down METTL3 sensitized CRC cells to 5-FU (Fig. 2A). Furthermore, downregulation of METTL3 promoted apoptosis in a concentration-dependent manner [quadrant (Q)2 indicates late apoptosis and Q4 indicates early apoptosis] in HCT-8 cells (Fig. 2B). In addition, the expression of pro-apoptotic proteins (caspase-3 and Bax)

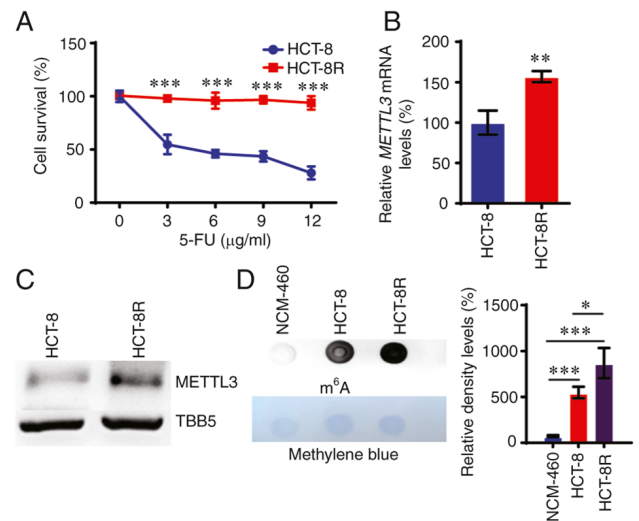


Figure 3. METTL3 and m⁶A levels are upregulated in 5-FU-resistant colorectal cancer cells. (A) Cell survival assay of HCT-8R and HCT-8 cells under 5-FU treatment to confirm the establishment of 5-FU-resistant CRC cells. (B and C) The mRNA and protein levels of METTL3 increased in the HCT-8R cells compared with the control HCT-8 cells. (D) Dot blot analysis for the m⁶A modification levels of total RNA in HCT-8 and HCT-8R cells. The quantification of the m⁶A levels are presented on the right panel. Data are presented as the means \pm SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. METTL3, methyltransferase-like 3; m⁶A, N⁶-methyladenosine; 5-FU, 5-fluorouracil; HCT-8R, 5-FU-resistant HCT-8 cells.

was upregulated in 5-FU-treated cells, which was enhanced by METTL3 depletion (Fig. 2C). These results were verified using morphological analysis, which showed that knockdown of METTL3 promoted 5-FU treatment-induced cell death in CRC cells with higher apoptosis as revealed by several smaller rounded cell fragments and cell loss (Fig. 2D).

A previous study showed that 5-FU could cause DNA damage, specifically, double-strand (and single-strand) breaks during the S phase of the cell cycle due to the misincorporation of 5-fluorodeoxyuridine triphosphate (5-FdUTP) into DNA (20). Therefore, the present study investigated whether METTL3 modified the levels of DNA damage in HCT-8 cells following 5-FU treatment. As shown in Fig. 2E, downregulation of METTL3 enhanced the expression level of phosphorylated histone γ -H2AX (an established marker of DNA damage), which was induced by 5-FU. Furthermore, the present study detected γ -H2AX- and p53-binding Protein 1 (53BP1)-positive foci in control and sh-METTL3 5-FU-treated HCT-8 cells via immunofluorescence assays. The present data revealed that 5-FU treatment dose-dependently increased the foci numbers of γ -H2AX and 53BP1, which were augmented upon knockdown of METTL3 (Figs. 2F and S2). By contrast, overexpression of METTL3 enhanced DNA repair and decreased DNA damage in HCT-8 cells, which further impeded apoptosis and triggered the resistance of HCT-8 cells to 5-FU (Fig. S2B-E).

METTL3 and m⁶A levels are upregulated in 5-FU resistant CRC cells. To investigate whether METTL3 contributes to 5-FU-induced resistance in CRC, 5-FU-resistant cells were obtained using parental HCT-8 CRC cells treated with increasing concentrations of 5-FU, as previously described (7). A cell survival assay was then used to confirm the acquired

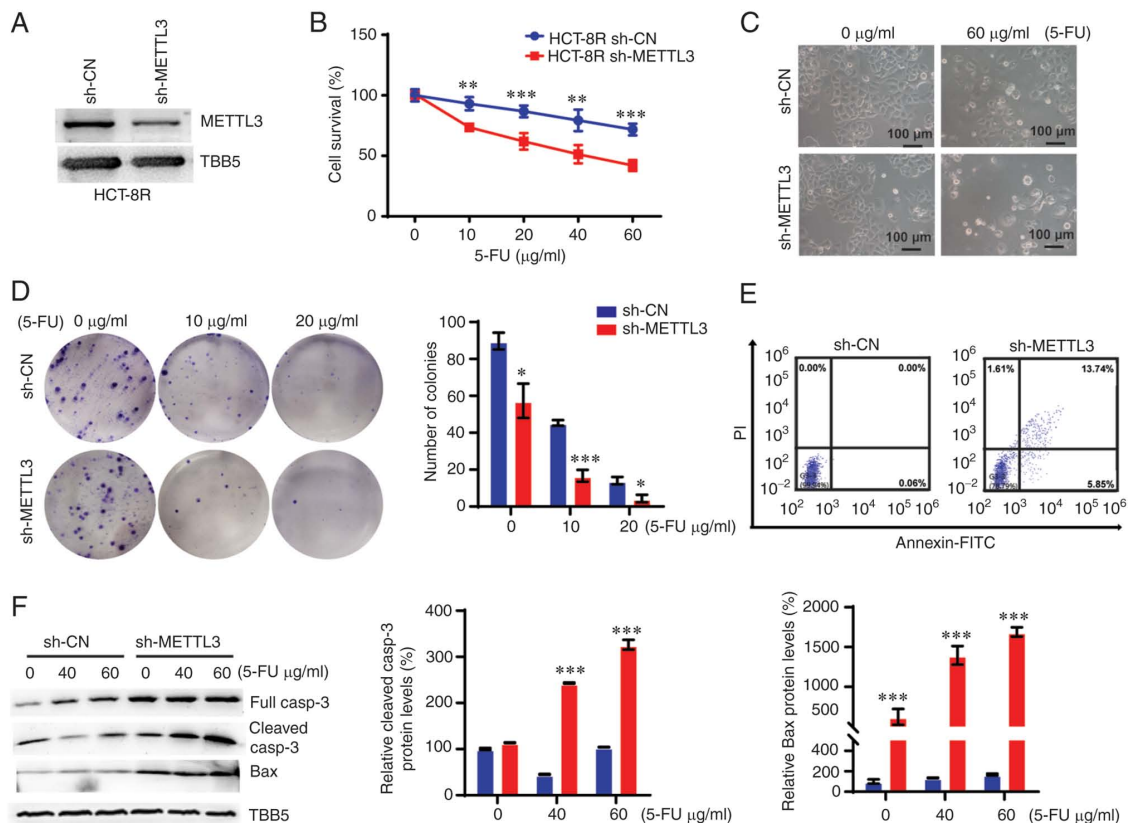


Figure 4. Knockdown of METTL3 overcomes 5-FU-resistance in colorectal cancer cells. (A) Western blotting confirmed the knockdown of METTL3 in HCT-8R cells. (B) Cell survival assay in control and METTL3-KD HCT-8R cells. (C) Morphological analysis of control and METTL3-KD HCT-8R cells with 60 $\mu\text{g/ml}$ 5-FU treatment for 48 h. (D) Colony formation assay in control and METTL3-KD HCT-8R cells treated with low doses (0, 10 and 20 $\mu\text{g/ml}$) of 5-FU. (E) Knockdown of METTL3 enhances apoptosis in HCT-8R cells with 5-FU treatment. (F) Silencing of METTL3 induces caspase-3 and Bax expression in HCT-8R cells following 5-FU treatment. Data are presented as the means \pm SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. METTL3, methyltransferase-like 3; 5-FU, 5-fluorouracil; HCT-8R, 5-FU-resistant HCT-8 cells; KD, knockdown; sh-, small hairpin; CN, control.

resistance to 5-FU by HCT-8R cells (Fig. 3A). Next, the METTL3 and RNA m⁶A expression levels were assessed in these HCT-8R cells. As shown in Fig. 3B and C, both the mRNA and protein levels of METTL3 were upregulated in HCT-8R cells compared with those in control HCT-8 cells. Dot blot assay showed that the total m⁶A RNA levels were elevated in HCT-8R cells compared with those in HCT-8 control cells (Fig. 3D).

Depletion of METTL3 overcomes 5-FU-induced resistance in CRC cells. Since METTL3 was abundantly overexpressed in HCT-8R cells, it was hypothesized that manipulating METTL3 could alter the 5-FU-induced resistance in CRC. To confirm this hypothesis, shRNA was used to knock down the expression of METTL3 in HCT-8R cells (Figs. 4A and S3). A cell survival assay and morphological analysis demonstrated that down-regulation of METTL3 overcame 5-FU-induced resistance in HCT-8R cells (Fig. 4B and C). A colony formation assay also confirmed this role of METTL3 on 5-FU-induced resistance in CRC cells (Fig. 4D). Furthermore, the effect of METTL3 on 5-FU-induced apoptosis in HCT-8R cells was detected. As expected, treatment with 10 $\mu\text{g/ml}$ 5-FU for 12 h did not promote apoptosis in 5-FU-resistant HCT-8R cells, whereas the same treatment induced ~20% apoptosis in METTL3-KD HCT-8R cells (Fig. 4E). Knocking down METTL3 increased the caspase-3 and Bax expression levels in HCT-8R cells (Fig. 4F).

Knockdown of METTL3 enhances 5-FU-induced DNA damage in HCT-8R cells. Elevated DNA repair abilities lead to drug resistance, which severely limits the efficacy of chemotherapeutic drugs in cancer cells (14,21). The role of DNA repair in 5-FU response and resistance has been observed in numerous studies (5,6). The present study further investigated whether knocking down METTL3 would modulate DNA damage accumulation in HCT-8R cells. The present data showed that the expression levels of γ -H2AX were significantly increased in METTL3-KD HCT-8R cells compared with those in HCT-8R control cells treated with 40 or 60 $\mu\text{g/ml}$ 5-FU (Fig. 5A). Consistently, an increasing number of positive γ -H2AX and 53BP1 nuclei foci were detected in METTL3-KD HCT-8R cells following 5-FU treatment (Fig. 5B and C). Comet assays also revealed that METTL3-KD HCT-8R cells had higher levels of spontaneous DNA strand break than HCT-8R control cells upon 5-FU treatment (Fig. 5D). Therefore, these results indicated that downregulating METTL3 increased DNA damage accumulation in 5-FU-resistant CRC cells treated with 5-FU.

METTL3 regulates RAD51AP1 expression in CRC cells. A previous study by our research group identified that the protein levels of essential enzymes participating in the base excision repair (BER) pathway did not show significant changes in HCT-8R cells versus HCT-8 cells (7). Several studies have

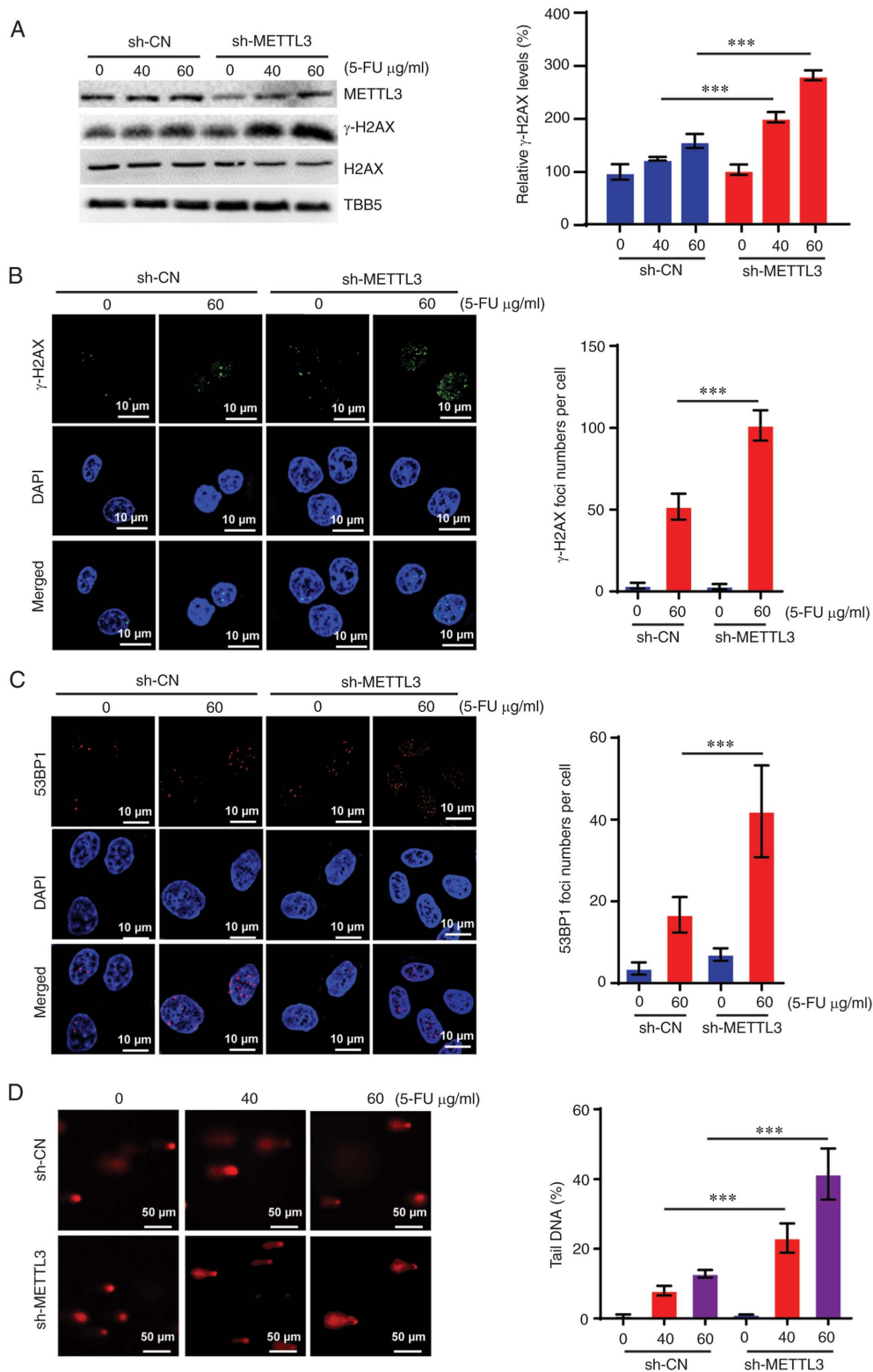


Figure 5. Silencing of METTL3 enhances 5-FU-induced DNA damage in drug-resistant colorectal cancer cells. (A) Western blot assay to determine γ -H2AX levels in METTL3-KD and control HCT-8 cells following treatment with various doses (0, 40 and 60 μ g/ml) of 5-FU. (B and C) Immunofluorescence staining of γ -H2AX foci and 53BP1 foci in cells. The quantification of the foci numbers per cells are presented on the right panel. (D) Comet assay of METTL3-KD and control HCT-8 cells treated with different dose of 5-FU. Representative images for the quantification of the average tail moment reported for each treatment condition. Each experiment was repeated three times. *** P <0.001. METTL3, methyltransferase-like 3; 5-FU, 5-fluorouracil; KD, knockdown; sh-, small hairpin; CN, control.

verified the effect of METTL3 on HR repair (10,22,23). Thus, the present study applied a comprehensive strategy to identify potential genes involved in the METTL3-mediated response

to 5-FU in HCT-8R cells (Fig. 6A). RT-qPCR was used to assess key genes involved in HR in order to detect which genes were modulated in METTL3-KD or 5-FU-resistant

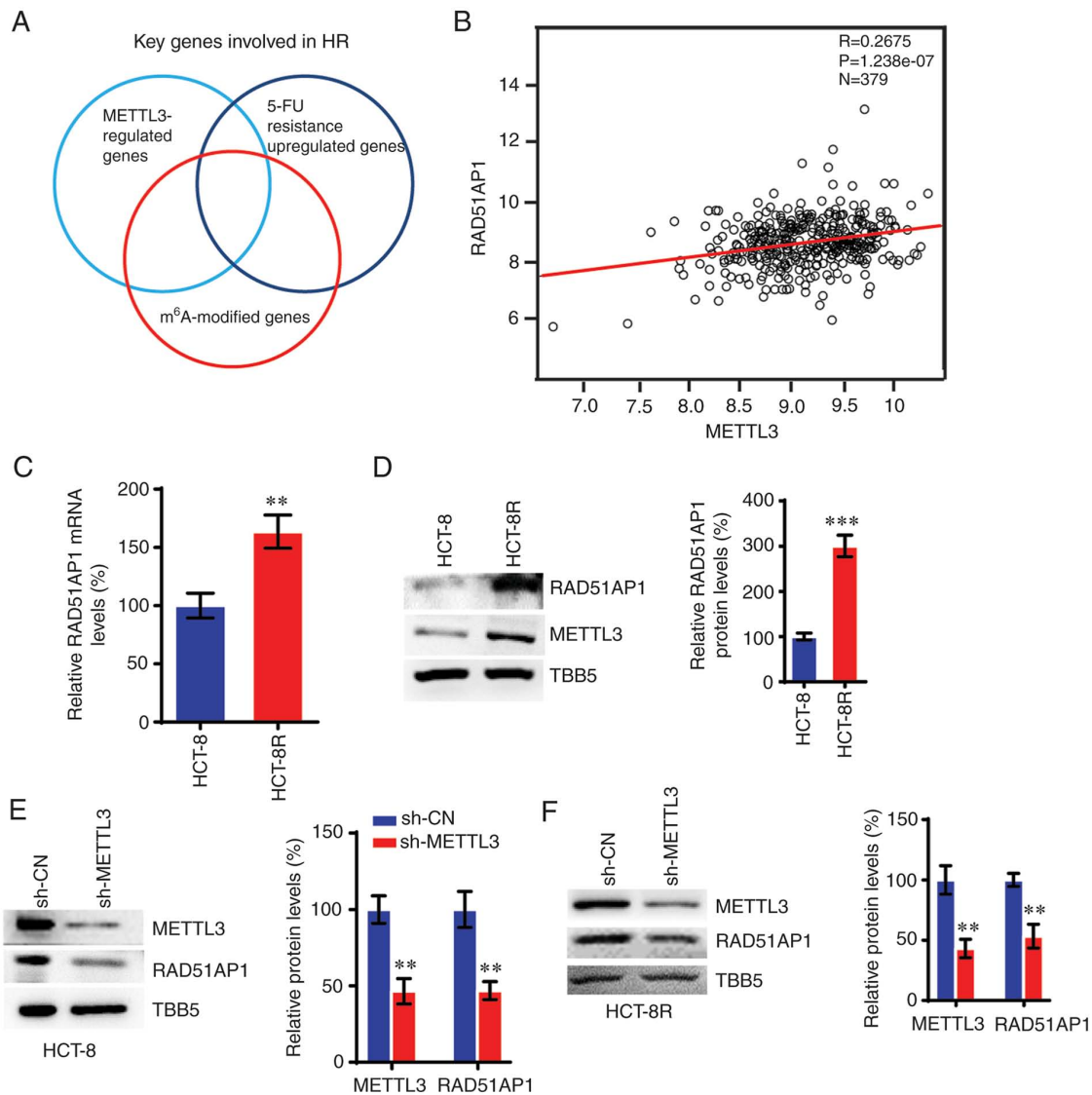


Figure 6. METTL3 regulates RAD51AP1 expression in colorectal cancer cells. (A) Schematic of the screening process of METTL3 targets involved in HR in colorectal cancer cells. (B) The LinkedOmics database (<http://www.linkedomics.org>) analysis of the association of METTL3 with RAD51AP1 using Pearson's correlation test. (C and D) The mRNA and protein levels of RAD51AP1 were higher in HCT-8R cells than those in HCT-8 cells. (E and F) Knockdown of METTL3 suppresses RAD51AP1 expression in HCT-8 cells and HCT-8R cells, respectively. The quantification of the relative band grey intensity is shown on the right panel. Data are presented as the means \pm SD. ** $P < 0.01$ and *** $P < 0.001$. METTL3, methyltransferase-like 3; RAD51AP1, RAD51 associated Protein 1; HR, homologous recombination; HCT-8R, 5-FU-resistant HCT-8 cells; sh-, small hairpin; CN, control.

cells versus control cells (Fig. S4). Subsequently, HR genes that were up- or down-regulated in METTL3-KD and 5-FU-resistant cells were further screened for modification of m⁶A in their exonic, 5'UTR or 3'UTR mRNA regions using the m⁶A-Atlas, which is a comprehensive knowledgebase for unraveling the m⁶A epitranscriptome (24). This screening strategy led us to focus on RAD51AP1, which plays a key role in HR repair by interacting with and enhancing the recombinase activity of RAD51 (25,26). A previous study showed that RAD51AP1 promoted the invasion of RNA transcripts into donor DNA at the double standard break sites in transcribed regions, and stimulated HR repair through the formation of DNA-RNA (DR)-loops (27). A positive correlation between the expression of METTL3 and RAD51AP1 was observed in CRC cells (Fig. 6B), which was consistent with the results of METTL3. The expression levels of RAD51AP1 increased in 5-FU-resistant HCT-8R cells compared with those in control

HCT-8 cells (Fig. 6C and D). Knockdown of METTL3 also resulted in decreased RAD51AP1 expression in both HCT-8 and HCT-8R cells (Fig. 6E and F).

Downregulation of RAD51AP1 promotes DNA damage and sensitizes CRC cells to 5-FU. Considering that the expression pattern of RAD51AP1 is regulated by METTL3, and that METTL3 is involved in 5-FU-dependent responses, the present study investigated whether RAD51AP1 regulated 5-FU-dependent chemotherapy responses. A cell survival assay demonstrated that knockdown of RAD51AP1 significantly sensitized HCT-8 cells to 5-FU (Fig. 7A and B). Furthermore, knocking down RAD51AP1 promoted apoptosis in HCT-8 cells with 5-FU treatment (Fig. 7C). Consistently, the protein levels of caspase-3 and Bax were increased by RAD51AP1 downregulation (Fig. 7D). The present study further detected DNA damage in RAD51AP1-KD cells. The data revealed that

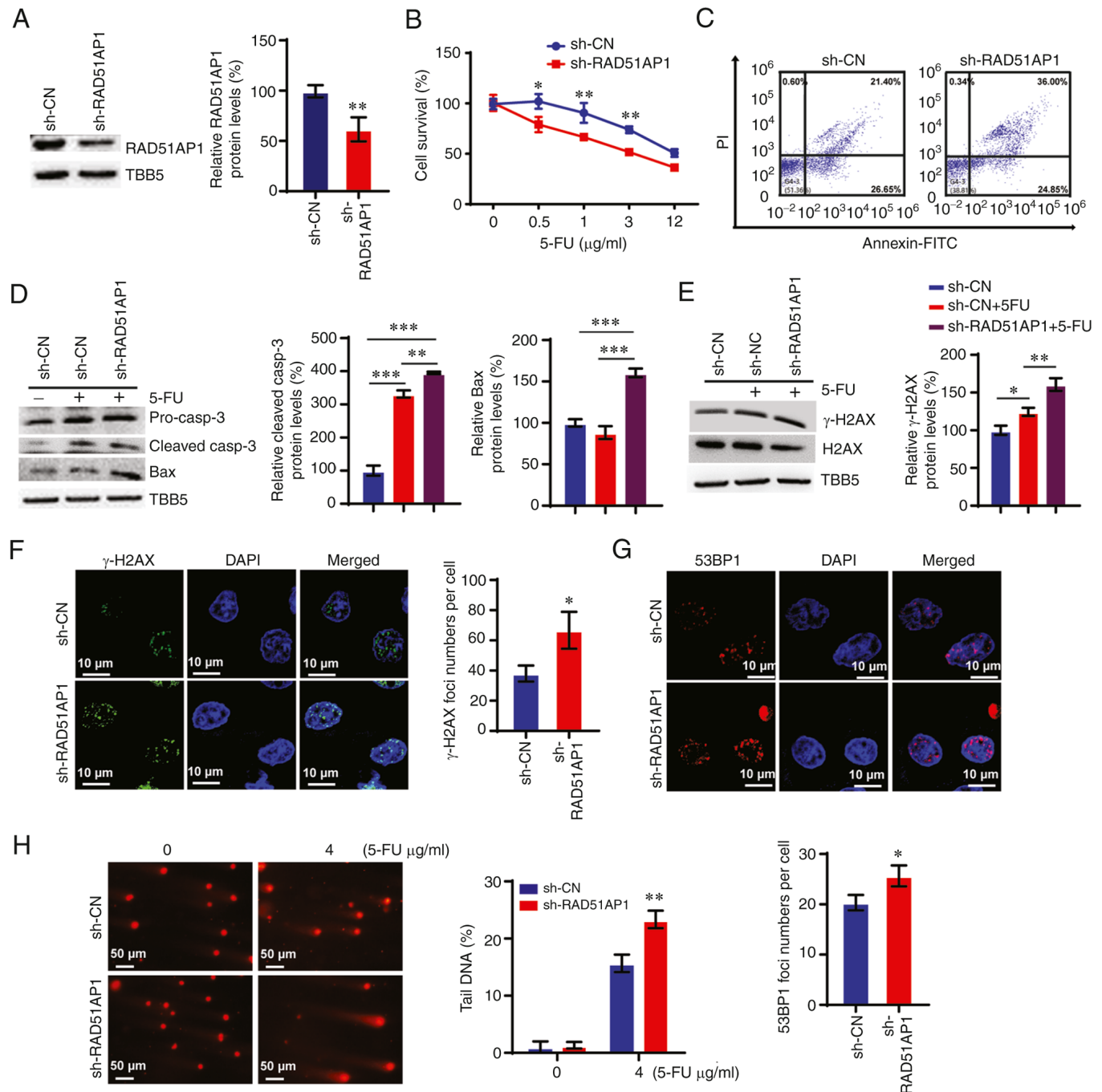


Figure 7. Knockdown of RAD51AP1 promotes DNA damage and sensitizes HCT-8 cells to 5-FU. (A) Western blotting identified the knockdown of RAD51AP1 in HCT-8 cells. (B) A cell survival assay demonstrated that downregulation of RAD51AP1 sensitizes HCT-8 cells to 5-FU. (C) Cell apoptosis analyzed by flow cytometry in control and RAD51AP1-KD HCT-8 cells with 4 $\mu\text{g/ml}$ 5-FU treatment for 24 h. (D) Knockdown of RAD51AP1 promoted the expression of caspase-3 and Bax in HCT-8 cells treated with 4 $\mu\text{g/ml}$ 5-FU for 12 h. (E) Western blotting to determine γ -H2AX levels in control and RAD51AP1-KD HCT-8 cells following 4 $\mu\text{g/ml}$ 5-FU treatments for 4 h. (F and G) Immunofluorescence staining of γ -H2AX foci and 53BP1 foci in different cells treated 4 $\mu\text{g/ml}$ 5-FU for 4 h. (H) Comet assay of RAD51AP1-KD and control HCT-8 cells treated with or without 5-FU. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. RAD51AP1, RAD51 associated Protein 1; 5-FU, 5-fluorouracil; KD, knockdown; sh-, small hairpin; CN, control.

knocking down RAD51AP1 enhanced 5-FU-induced γ -H2AX expression levels in HCT-8 cells (Fig. 7E). The number of positive nuclei foci of both γ -H2AX and 53BP1 increased in RAD51AP1-KD HCT-8 cells subjected to 5-FU treatment (Fig. 7F and G). Furthermore, comet assays revealed higher levels of spontaneous DNA strand breaks in RAD51AP1-KD cells compared with those in control cells following 5-FU treatment (Fig. 7H).

Knockdown of RAD51AP1 overcomes 5-FU-resistance in CRC cells. As aforementioned, RAD51AP1 was elevated in HCT-8R cells. Therefore, the present study further analyzed whether RAD51AP1 contributed to 5-FU resistance in CRC cells. The data showed that knocking down RAD51AP1 suppressed 5-FU resistance in HCT-8R cells (Fig. 8A and B). Cell morphology analysis of RAD51AP1-KD HCT-8R cells verified the above data, which showed more apoptotic bodies

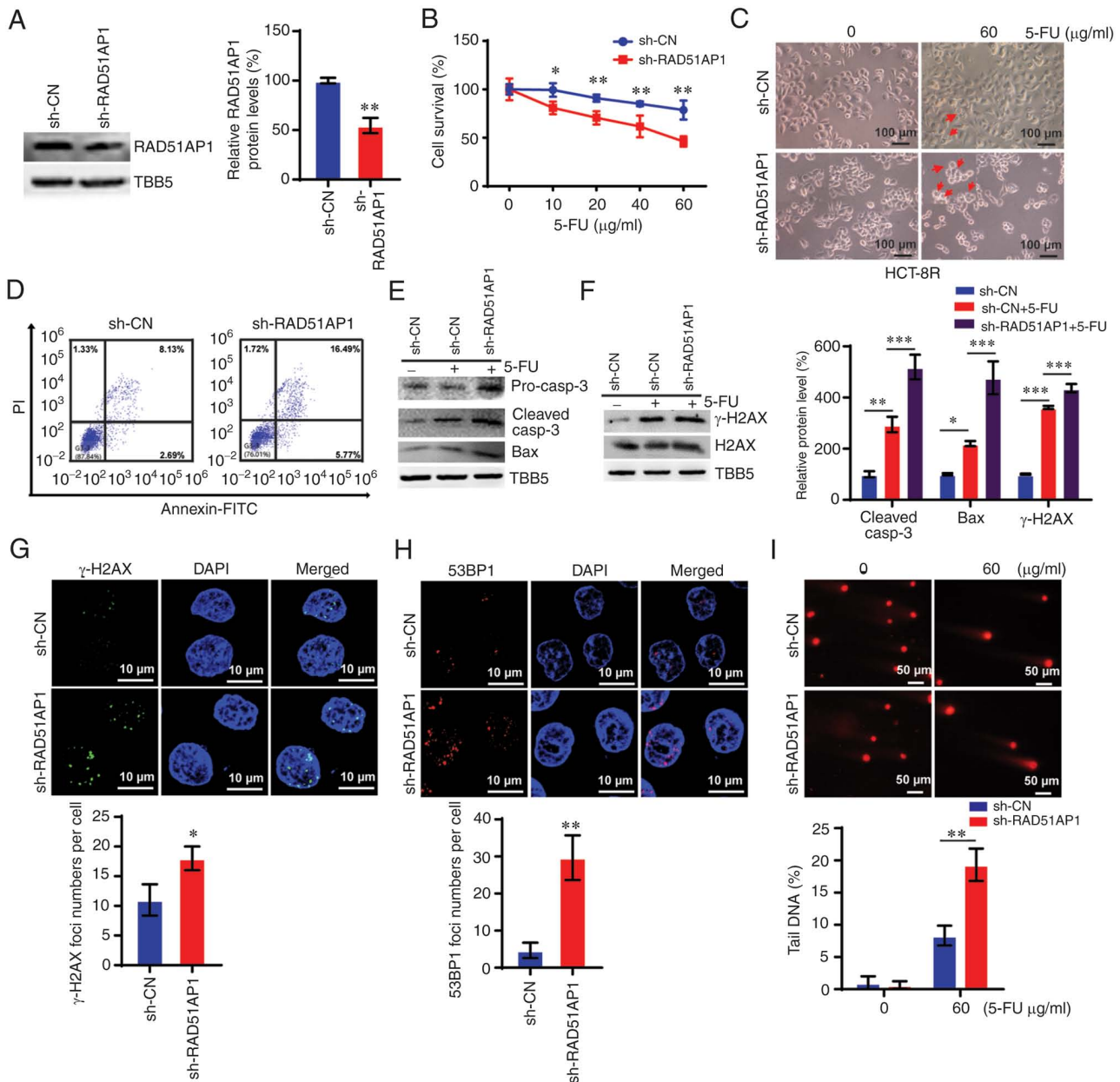


Figure 8. Inhibition of RAD51AP1 overcomes 5-FU-resistance in colorectal cancer cells. (A) Western blot analysis of RAD51AP1 in control and RAD51AP1-KD HCT-8R cells. (B) Cell survival assay in control and RAD51AP1-KD HCT-8R cells. (C) Morphological analysis of control and RAD51AP1-KD HCT-8R cells with 60 $\mu\text{g/ml}$ 5-FU treatment for 48 h. (D) Knockdown of RAD51AP1 promotes apoptosis in HCT-8R cells with 5-FU treatment. (E) Silencing of RAD51AP1 induces caspase-3 and Bax expression in HCT-8R cells following 5-FU treatment. (F) Western blotting to determine γ -H2AX levels in control and RAD51AP1-KD HCT-8R cells following 5-FU treatments for 4 h. (G and H) Immunofluorescence staining of γ -H2AX foci and 53BP1 foci in different cells treated 5-FU for 4 h. (I) Comet assay of RAD51AP1-KD and control HCT-8R cells treated with or without 5-FU (60 $\mu\text{g/ml}$) for 12 h. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. RAD51AP1, RAD51 associated Protein 1; 5-FU, 5-fluorouracil; KD, knockdown; HCT-8R, 5-FU-resistant HCT-8 cells; sh-, small hairpin; CN, control.

and cell loss in the RAD51AP1-KD cells with 60 $\mu\text{g/ml}$ 5-FU treatment (Fig. 8C). Flow cytometric analysis also demonstrated increased apoptosis in RAD51AP1-KD HCT-8R cells (Fig. 8D). By downregulating RAD51AP1 in HCT-8R cells, the expression of caspase-3 and Bax was upregulated (Fig. 8E). Knocking down RAD51AP1 markedly enhanced the DNA damage levels in HCT-8R cells subjected to 5-FU treatments (Fig. 8F-I). These data indicated that knocking down RAD51AP1 overcame 5-FU resistance in CRC cells by enhancing DNA damage accumulation and promoting cell apoptosis.

Discussion

5-FU is one of the most commonly used CRC chemotherapy drugs in the clinics, which can effectively kill gastrointestinal tumor cells or inhibit their proliferation. However, its long-term application can induce drug resistance of tumor cells, leading to failures in clinical treatments (23). Thus patients with CRC urgently need new therapeutic targets to improve the clinical efficacy of 5-FU. Presently, the mechanism of chemotherapy resistance in CRC remains unclear; however, the current study observed that the METTL3 and m⁶A levels were

highly expressed in 5-FU-resistant CRC cells. Knockdown of METTL3 sensitized CRC cells to 5-FU and promoted 5-FU treatment-induced DNA damage in CRC cells, leading to cell death. Downregulation of METTL3 overcame 5-FU-induced resistance in CRC cells. Additionally, it was also found that METTL3 was positively correlated with the expression of the RAD51AP1 protein in CRC cells. Similarly, downregulation of RAD51AP1 significantly sensitized CRC cells to 5-FU, and overcame 5-FU-resistance in CRC cells by enhancing DNA damage accumulation and promoting cell apoptosis. The above experimental results confirmed the effect of METTL3 on 5-FU resistance in CRC cells.

Increasing evidence has shown that m⁶A and its key methyltransferase METTL3 influence the occurrence and development of tumors. Li *et al* (28) observed that METTL3 served as an oncogene in CRC. As reported, METTL3 maintained the expression of SRY-box 2 (SOX2) in CRC cells through a m⁶A-insulin like growth factor 2 mRNA binding protein 2-dependent mechanism, which was consistent with the present results. CSC is a tumor cell line with strong carcinogenic and metastatic potential, which is considered a reason for chemotherapy resistance. CSC-labeled SOX2 has been confirmed to have carcinogenic effects. Therefore, inhibiting METTL3 can reduce the expression of its surface antigen, thereby enhancing the chemotherapy response of CRC *in vivo* and *in vitro*. Enhanced chemotherapy-based responses reduce the frequency of stem cells, which effectively stops the recurrence and metastasis of malignant tumors. By contrast, Deng *et al* (29) reported the tumor suppressing effect of METTL3 during CRC cell proliferation, migration, and invasion through the p38/ERK pathway. The authors proposed that the main reason why METTL3 played a dual role in cancer regulation was the difference in targeting pathways and cancer heterogeneity. The results of the present study showed that although METTL3 promoted the proliferation of CRC cells, the proliferative and metastatic abilities of HCT-8 cells were significantly reduced upon downregulation of METTL3. The present study further demonstrated that inhibition of METTL3 sensitized HCT-8 cells to 5-FU and overcome 5-FU resistance in the CRC cells through regulation of RAD51AP1 expression. Although knockdown of METTL3 could not reverse the drug resistance completely, the present data at least demonstrated that suppression of METTL3 could partially overcome the 5-FU-resistance in HCT-8R cells. These data indicated that METTL3 may combine with other genes together to confer drug resistance in CRC. However, understanding the role and mechanism of METTL3 in colon cancer requires further research, including the collection of additional clinical tissue specimens for analysis.

In summary, the present results showed that METTL3 was an oncogene in colon cancer, which regulated the proliferation and metastasis of colon cancer. However, since METTL3 and its regulated HR repair protein, RAD51AP1, are highly expressed in 5-FU-resistant CRC cells, silencing them enhanced 5-FU-induced DNA damage in HCT-8R cells, improved chemotherapy response and overcame 5-FU resistance. Therefore, METTL3 could be used as an important indicator to predict the level of differentiation and metastasis of CRC cells, thereby providing new insights and targets for future treatments and drug developmental processes. However,

the regulatory role of METTL3 in the occurrence and development of CRC as well as its molecular mechanism should be further investigated.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

ZH, MF, YZ and ML designed and supervised the study, and wrote the manuscript. ML, MX, EL and ZZ performed the experiments and analyzed the data. ML, YT, ZG, EL and MF provided technical and material support. ML, MX and ZZ confirm the authenticity of all the raw data. ML, YT, YZ, ZG, MF and ZH conceived, wrote, and edited the manuscript as well as contributed to funding acquisition. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Xi Y and Xu P: Global colorectal cancer burden in 2020 and projections to 2040. *Transl Oncol* 14: 101174, 2021.
2. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 71: 209-249, 2021.
3. Biller LH and Schrag D: Diagnosis and treatment of metastatic colorectal cancer: A review. *Jama* 325: 669-685, 2021.
4. Xie YH, Chen YX and Fang JY: Comprehensive review of targeted therapy for colorectal cancer. *Signal Transduct Target Ther* 5: 22, 2020.
5. Sethy C and Kundu CN: 5-Fluorouracil (5-FU) resistance and the new strategy to enhance the sensitivity against cancer: Implication of DNA repair inhibition. *Biomed Pharmacother* 137: 111285, 2021.

6. Wyatt MD and Wilson DM III: Participation of DNA repair in the response to 5-fluorouracil. *Cell Mol Life Sci* 66: 788-799, 2009.
7. He L, Zhu H, Zhou S, Wu T, Wu H, Yang H, Mao H, SekharKathera C, Janardhan A, Edick AM, *et al*: Wnt pathway is involved in 5-FU drug resistance of colorectal cancer cells. *Exp Mol Med* 50: 1-12, 2018.
8. Liu S, Zhuo L, Wang J, Zhang Q, Li Q, Li G, Yan L, Jin T, Pan T, Sui X, *et al*: METTL3 plays multiple functions in biological processes. *Am J Cancer Res* 10: 1631-1646, 2020.
9. Zeng C, Huang W, Li Y and Weng H: Roles of METTL3 in cancer: Mechanisms and therapeutic targeting. *J Hematol Oncol* 13: 117, 2020.
10. Li E, Xia M, Du Y, Long K, Ji F, Pan F, He L, Hu Z and Guo Z: METTL3 promotes homologous recombination repair and modulates chemotherapeutic response in breast cancer by regulating the EGF/RAD51 axis. *Elife* 11: e75231, 2022.
11. Chen H, Gao S, Liu W, Wong CC, Wu J, Wu J, Liu D, Gou H, Kang W, Zhai J, *et al*: RNA N(6)-Methyladenosine Methyltransferase METTL3 facilitates colorectal cancer by activating the m(6)A-GLUT1-mTORC1 axis and is a therapeutic target. *Gastroenterology* 160: 1284-1300.e16, 2021.
12. Xiang Y, Laurent B, Hsu CH, Nachtergaele S, Lu Z, Sheng W, Xu C, Chen H, Ouyang J, Wang S, *et al*: RNA m(6)A methylation regulates the ultraviolet-induced DNA damage response. *Nature* 543: 573-576, 2017.
13. Wiese C, Dray E, Groesser T, San Filippo J, Shi I, Collins DW, Tsai MS, Williams GJ, Rydberg B, Sung P and Schild D: Promotion of homologous recombination and genomic stability by RAD51AP1 via RAD51 recombinase enhancement. *Mol Cell* 28: 482-490, 2007.
14. Lu X, Liu R, Wang M, Kumar AK, Pan F, He L, Hu Z and Guo Z: MicroRNA-140 impedes DNA repair by targeting FEN1 and enhances chemotherapeutic response in breast cancer. *Oncogene* 39: 234-247, 2020.
15. Grada A, Otero-Vinas M, Prieto-Castrillo F, Obagi Z and Falanga V: Research techniques made simple: Analysis of collective cell migration using the wound healing assay. *J Invest Dermatol* 137: e11-e16, 2017.
16. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* 25: 402-408, 2001.
17. Vasaikar SV, Straub P, Wang J and Zhang B: LinkedOmics: Analyzing multi-omics data within and across 32 cancer types. *Nucleic Acids Res* 46: D956-D963, 2018.
18. Peng W, Li J, Chen R, Gu Q, Yang P, Qian W, Ji D, Wang Q, Zhang Z, Tang J and Sun Y: Upregulated METTL3 promotes metastasis of colorectal cancer via miR-1246/SPRED2/MAPK signaling pathway. *J Exp Clin Cancer Res* 38: 393, 2019.
19. McQuade RM, Stojanovska V, Bornstein JC and Nurgali K: Colorectal cancer chemotherapy: The evolution of treatment and new approaches. *Curr Med Chem* 24: 1537-1557, 2017.
20. De Angelis PM, Svendsrud DH, Kravik KL and Stokke T: Cellular response to 5-fluorouracil (5-FU) in 5-FU-resistant colon cancer cell lines during treatment and recovery. *Mol Cancer* 5: 20, 2006.
21. Li L, Kumar AK, Hu Z and Guo Z: Small molecule inhibitors targeting key proteins in the DNA damage response for cancer therapy. *Curr Med Chem* 28: 963-985, 2021.
22. Zhang C, Chen L, Peng D, Jiang A, He Y, Zeng Y, Xie C, Zhou H, Luo X, Liu H, *et al*: METTL3 and N6-Methyladenosine promote homologous recombination-mediated repair of DSBs by modulating DNA-RNA hybrid accumulation. *Mol Cell* 79: 425-442.e7, 2020.
23. Yu D, Horton JR, Yang J, Hajian T, Vedadi M, Sagum CA, Bedford MT, Blumenthal RM, Zhang X and Cheng X: Human MettL3-MettL14 RNA adenine methyltransferase complex is active on double-stranded DNA containing lesions. *Nucleic Acids Res* 49: 11629-11642, 2021.
24. Tang Y, Chen K, Song B, Ma J, Wu X, Xu Q, Wei Z, Su J, Liu G, Rong R, *et al*: m6A-Atlas: A comprehensive knowledgebase for unraveling the N6-methyladenosine (m6A) epitranscriptome. *Nucleic Acids Res* 49: D134-D143, 2021.
25. Dunlop MH, Dray E, Zhao WX, San Filippo J, Tsai MS, Leung SG, Schild D, Wiese C and Sung P: Mechanistic insights into RAD51-associated Protein 1 (RAD51AP1) action in homologous DNA repair. *J Biol Chem* 287: 12343-12347, 2012.
26. Pires E, Sung P and Wiese C: Role of RAD51AP1 in homologous recombination DNA repair and carcinogenesis. *DNA Repair (Amst)* 59: 76-81, 2017.
27. Ouyang J, Yadav T, Zhang JM, Yang H, Rheinbay E, Guo H, Haber DA, Lan L and Zou L: RNA transcripts stimulate homologous recombination by forming DR-loops. *Nature* 594: 283-288, 2021.
28. Li T, Hu PS, Zuo Z, Lin JF, Li X, Wu QN, Chen ZH, Zeng ZL, Wang F, Zheng J, *et al*: METTL3 facilitates tumor progression via an m(6)A-IGF2BP2-dependent mechanism in colorectal carcinoma. *Mol Cancer* 18: 112, 2019.
29. Deng R, Cheng YK, Ye SB, Zhang J, Huang R, Li P, Liu H, Deng Q, Wu X, Lan P and Deng Y: m(6)A methyltransferase METTL3 suppresses colorectal cancer proliferation and migration through p38/ERK pathways. *Onco Targets Ther* 12: 4391-4402, 2019.



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