

## Original Article

# N6-methyladenosine methyltransferase METTL3 promotes colorectal cancer cell proliferation through enhancing MYC expression

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**Abstract:** N6-methyladenosine (m6A) modification is the most common chemical modification in eukaryotic mRNA, which plays a crucial role in regulating mRNA stability, splicing, and translation. METTL3 (methyltransferase like 3), a major RNA N6-adenosine methyltransferase, has been reported to participate in the progression of many cancers. However, its function in colorectal cancer (CRC) remains largely unknown. In this study, we revealed that METTL3 played an oncogenic role in CRC. We found that METTL3 was significantly upregulated in CRC, using quantitative real-time PCR, western blotting, and immunohistochemical staining, and upregulation of METTL3 was associated with clinicopathological features. Functionally, knockdown of METTL3 suppressed CRC cell proliferation *in vitro* and *in vivo*. In contrast, overexpression of METTL3 promoted the growth of CRC cells both *in vitro* and *in vivo*. Mechanistically, METTL3 exerted its function through enhancing MYC expression, at least partially in an m6A-IGF2BP1-dependent manner. In conclusion, we found that METTL3 was frequently upregulated in human CRC and promoted CRC progression through enhancing MYC expression. This provided new insights into the molecular mechanisms underlying the development of colorectal cancer.

**Keywords:** Colorectal cancer, METTL3, MYC, N6-methyladenosine (m6A), cell proliferation

## Introduction

Colorectal cancer (CRC) is one of the most common tumors of the human digestive tract, which ranks third in cancer morbidity and second in cancer mortality, with more than 880,000 new deaths occurring in 2018 worldwide [1]. The morbidity and mortality rates of CRC have shown an upward trend in China, with more than 370,000 new cases and 190,000 deaths annually [2]. Despite improvements in screening and treatment strategies for CRC in recent years, the prognosis of advanced CRC is still poor, which is largely due to the lack of knowledge of the molecular mechanism of CRC development. Thus, better understanding of the molecular mechanism underlying the development of CRC is urgently needed to improve the prognosis of CRC.

N6-methyladenosine (m6A) modification, enriched in 3'-UTRs and near stop codons of mRNAs, plays an important role in mRNA sta-

bility, splicing, and translational control [3-7]. Evidence suggests that abnormal m6A modification is closely related to the development of cancer and its response to drugs [8]. The m6A modification is dynamic and reversible. The key enzyme involved in m6A modification synthesis is a highly conserved mRNA methyltransferase complex composed of METTL3, METTL4 and WTAP (Wilms tumor-associated protein), known as the m6A "writer" [9]. The demethylation-related enzymes mainly are obese-related protein and alkylation repair homologous protein 5, known as m6A "eraser" [10, 11]. The enzymes recognizing m6A modification and exerting downstream regulative function mainly include the YTH domain family proteins (YTHDFs, including YTHDF1/2/3) and the insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs, including IGF2BP1/2/3), known as m6A "reader" [12, 13].

Increasing evidence has shown that abnormal expression and dysfunction of METTL3 are clo-

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sely related to cancer progression. Mechanistically, METTL3 can modulate translation efficiency of many oncogenes and tumor suppressor genes. A previous study reported that METTL3 interacted with the eukaryotic translation initiation factor 3 subunit h to promote translation of a large number of m6A-modified oncogenic mRNAs in an mRNA looping manner, which promotes lung cancer cell proliferation and invasion [14]. Consistently, another study revealed that METTL3 facilitated the translation of MYC, BCL2, and PTEN mRNAs in human acute myeloid leukemia cell lines, consequently reducing the level of phosphorylated AKT, which functionally inhibits cell differentiation and increases cell proliferation [15]. Additionally, METTL3 can regulate mRNA stability via altering the level of mRNA m6A modification, which largely depends on the m6A-associated recognition proteins. For instance, one previous study proved that METTL3 promoted liver cancer progression through post-transcriptional silencing suppressors of cytokine signaling 2, which depended on its reader protein YTHDF2 [16]. In addition, there are many other m6A reader proteins related to the stability and translation efficiency of m6A-modified mRNA. For example, one recent report showed that IGF2BP proteins enhance mRNA stability and translation, including some m6A modified oncogenic mRNAs (such as MYC) [13]. Recently, one study proposed that METTL3 facilitated CRC progression in an m6A-IGF2BP2-dependent manner [17]. In contrast, another study reported that METTL3 inhibited CRC cell proliferation, migration, and invasion through the p38/ERK pathway [18]. Thus, the role of METTL3 in the development of colorectal cancer remains controversial, so further study of METTL3 in CRC progression is needed.

MYC is an oncogene encoding a nuclear phosphorylation protein, which plays an important role in cell proliferation, apoptosis, and cell transformation. It is activated in many kinds of human cancers and is usually associated with prognosis of cancer [19]. The MYC oncogene has been considered as a key target of the WNT pathway in CRC, which is required for activation of many downstream oncogenes in WNT pathways after APC loss, suggesting that MYC is a key signaling molecule in CRC progression [20, 21]. However, the reason why

MYC is amplified in many tumors is not completely clear. Recently, evidence has shown that the expression of the MYC gene is closely related to the m6A modification level of its mRNA [22-24]. However, whether the expression of MYC in CRC is regulated by m6A modification or its related enzymes remains to be confirmed.

### Materials and methods

#### *Cell culture and clinical samples*

Human CRC cell lines SW620, HCT116, SW480, HT-29, Lovo, Caco-2 and the human embryonic kidney cell line 293T (HEK-293T) were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). All the cells were cultured in Dulbecco modified Eagle medium (DMEM; Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS; ExCell Bio, Shanghai, China), 1% penicillin and streptomycin (Hyclone, Logan, UT, USA). Tissue samples were collected from the First Affiliated Hospital of Chongqing Medical University. The criteria for patients enrolled in the study were as follows: pathological diagnosis confirmed as colorectal cancer, no familial genetic syndrome (such as Lynch syndrome); no radiotherapy or chemotherapy prior to surgery.

The RNAseq gene expression (HiSeqV2, Illumina, San Diego, CA, USA) data containing 434 CRC samples was downloaded from TCGA hub, UCSC Xena (<https://xenabrowser.net/datapages/>) and then analyzed using the R package.

#### *Establishment of stable knockdown and over-expression cell lines*

For knockdown of METTL3, the shRNA expression vectors were generated by annealing two complementary shRNA oligonucleotide strands synthesized by TsingKe (Chongqing, China) and then inserting into the pGreen-Puro vector (GenePharma, Shanghai, China). The target sequences of shRNA are listed in [Table S1](#). For over-expression of METTL3, the cDNA sequence containing METTL3 ORF was synthesized by TsingKe (Chongqing, China) and cloned into the lentiviral vector pCDH-CMV-MCS-EF1-CopGFP-T2A-puro at *EcoRI* and *BamHI* sites. The plasmid encoding human MYC was obtained from GeneChem (Shanghai, China). Plasmids were

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transfected into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. For the generation of shRNA and overexpression plasmid lentivirus, HEK-293T cells were cotransfected with pGreen-Puro shRNA vectors or overexpression plasmid and packaging plasmids, psPAX2 and pMD2.G (Addgene, Cambridge, MA, USA). The virus supernatant was collected after 48 or 72 hours and then mixed with 5 µg/mL polybrene and directly added to HCT116 or SW620 cells for infection. The cells were then selected using 1 µg/mL puromycin (Sigma-Aldrich, St. Louis, MO, USA) for 5 days. The knockdown and overexpression efficiency of stable cell lines were analyzed by reverse transcription-quantitative PCR (RT-qPCR) and western blotting.

### *Reverse transcription-quantitative PCR (RT-qPCR)*

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Reverse transcription PCR and quantitative real-time PCR were performed using a PrimeScript RT kit (Takara, Dalian, China) and SYBR green I Master Mix (Bimake, Houston, TX, USA). PCR amplification reactions were performed in triplicates for each cDNA sample. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control, and all gene-specific primers used in qPCR analysis are listed in [Table S1](#).

### *Immunohistochemistry (IHC) staining*

The CRC specimens were collected and immediately fixed with 10% paraformaldehyde. The fixed tissues were embedded in paraffin and then sectioned for subsequent experiments. The paraffin sections were incubated at 55°C for 4 hours, then continuously soaked in fresh xylene solution for 20 minutes twice to remove paraffin. Sections were hydrated with 100%, 95%, 85%, and 70% ethanol and deionized water, respectively, then immersed in citrate buffer solution and heated 20 minutes for antigen retrieval. After washing three times with phosphate-buffered saline (PBS) solution, sections were incubated with 0.5% Triton X-100 for 30 minutes. IHC was then performed with a biotin-streptavidin horseradish peroxidase (HRP) detection kit (ZSGB, Beijing, China) according to the manufacturer's instructions.

Briefly, sections were treated with 3% H<sub>2</sub>O<sub>2</sub> for 8 minutes, blocked with 3% bovine serum albumin for 1 hour, and then incubated with primary antibody targeting METTL3 [1:300, ab19-5352; Abcam, Cambridge, UK, MYC (1:200), ab32072; Abcam] overnight at 4°C. The above sections were incubated with HRP-conjugated secondary antibody for 30 minutes at room temperature, developed in diaminobenzidine (brown) solution for 3-5 minutes, and then counterstained with hematoxylin (blue). Images were acquired in multiples at 100 × or 400 × using a Leica inverted microscope (Leica, Wetzlar, Germany).

### *Western blot analysis*

Protein was extracted using RIPA lysis buffer (Beyotime, Shanghai, China) containing protease inhibitors at 100:1 (Beijing Dingguo, Beijing, China). Equal amounts of protein (30-50 µg) were separated by 10% SDS-PAGE and transferred to a PVDF membrane. Membranes were then blocked with 5% skim milk-TBST for 1.5 hours at room temperature and incubated with the primary antibody overnight at 4°C. The corresponding primary antibodies used for western blot analysis were as follows: METTL3 (1:1,000; ab195352; Abcam), MYC (1:1,000; ab32072; Abcam), and GAPDH (1:5,000; 10494-1-AP; Proteintech, Wuhan, China). After incubating with HRP-conjugated anti-rabbit IgG secondary antibody (1:5,000; Proteintech), the protein signals were developed with an ECL chemiluminescence solution (Advansta, San Jose, CA, USA).

### *Cell cycle and apoptosis analysis, colony formation assay, CCK-8 assay*

Cell cycle and apoptosis analysis were performed using flow cytometry by Academy of Life Sciences (Chongqing Medical University, Chongqing, China). For the colony formation assay, 1,000 HCT116 cells or 2,000 SW620 cells were seeded in 6-well plates. After culturing for 10-12 days, cells were fixed with 4% paraformaldehyde for 30 minutes and stained with 0.1% Crystal Violet for 10 minutes. After washing three times with PBS and drying at room temperature, the numbers of colonies containing more than 50 cells were counted. Cell proliferation was further detected by using a CCK-8 kit (MCE, Monmouth Junction, NJ, USA).

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according to the manufacturer's instructions. Briefly, HCT116 or SW620 cells were seeded into 96-well plates at 2,000 cells/well as triplicates and incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After the cells were attached, 10 µL CCK-8 reagents were added to each well of the plate at the indicated times. After 2 hours of incubation, the absorbance at 450 nm of each well was measured using a microplate reader.

### *Migration and invasion assay*

For cell migration and invasion Transwell assays,  $5 \times 10^4$  HCT116 cells or  $1 \times 10^5$  SW620 cells in 200 µL serum-free medium were plated on the top chambers of non-coated membranes (pore size, 8 µm; Corning, NY, USA) for the migration assay and Matrigel-coated membranes (BD Biosciences, San Jose, CA, USA) for the invasion assay, while 500 µL culture media with 10% FBS was added to the bottom. After 24-48 hours incubation, the cells in the top chambers were carefully removed by a cotton swab. The cells migrating to the lower surface of the membrane were fixed with 4% paraformaldehyde for 30 minutes and stained with 0.1% Crystal Violet for 10 minutes. The migrated and invaded cells were counted with a microscope (200 ×).

### *Measurement of total m6A levels*

Total RNA m6A level was measured using a colorimetric ELISA assay with the m6A RNA methylation quantification kit (P-9005; Epigentek, Farmingdale, NY, USA) following the manufacturer's instructions. Measurements were conducted in triplicates for each RNA sample. A total of 200 ng RNA was used per well.

### *RNA immunoprecipitation (RIP)*

RNA Immunoprecipitation (RIP) was performed according to a previous report [25] with some changes. Briefly,  $10^7$  cells were collected by trypsinization, then lysed in 1 mL RIP buffer [(50 mM Tris, pH 7.4), 150 mM NaCl, and 1% NP-40, supplemented with 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 100 U/mL RNase inhibitor (Beyotime)] on ice for 10 minutes and then centrifuged at  $16,000 \times g$  for 15 minutes in a microcentrifuge at 4°C.

Two micrograms anti-IGF2BP1 antibody (84-82S; Cell Signaling Technology, Danvers, MA, USA) or normal rabbit IgG antibody (B900610; Proteintech) were incubated with 1 mL cell lysates at 4°C overnight with gentle rotation. After incubation, 50 µL precleared protein A/G beads (B23202; Bimake, Houston, TX, USA) were added to the above complexes and incubated for 4 hours at 4°C with gentle rotation. The beads were collected by a magnetic separator and washed three times in 500 µL RIP buffer. Next, the beads were resuspended in 1 mL of TRIzol and subjected to RNA extraction. Then, RT-qPCR was used to analyze the mRNA expression levels of corresponding genes using immunoprecipitated samples and input samples. The RNA levels in the immunoprecipitated samples were normalized to the input samples.

### *Tumorigenesis in nude mice*

BALB/c male nude mice (4-6 weeks old) were purchased from the Animal Experimental Laboratory of Chongqing Medical University and housed under special pathogen-free condition. METTL3 stable knockdown or overexpression HCT116 cells were collected and resuspended at a density of  $5 \times 10^6$  or  $3 \times 10^6$  cells per 150 µL PBS, respectively. The randomly grouped mice (five in each group) were then injected subcutaneously into two flanks with 150 µL of the cell suspensions within 1 hour after cell collection. From the 4th day after injection, tumors were measured every 4 days using a vernier caliper, and the tumor volume was calculated by the following formula: volume (mm<sup>3</sup>) =  $0.5 \times \text{length} \times \text{width}^2$ . After 24 days, the mice were euthanized and the tumors were isolated, photographed, and weighed.

### *Statistical analysis*

GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Data are presented as the mean ± SD. Two-tailed Student's *t*-test was used to compare differences between groups. Pearson's correlation test was used to measure the strength of association between two variables. The chi-square test was used to assess correlations with clinical pathologies. *P*-value < 0.05 was considered statistically significant. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

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## Results

### *Upregulation of METTL3 was associated with clinicopathological features in CRC*

To investigate the correlation between the expression of METTL3 and clinicopathological features in CRC, we first detected the expression of METTL3 mRNA in 20 pairs of CRC tumor tissues and adjacent normal tissues by RT-qPCR, and found that compared with adjacent tissues, the expression level of METTL3 mRNA was significantly upregulated in CRC tissues (Figure S1A). Next, IHC staining was used to detect the protein level of METTL3 in 45 CRC samples. The results showed that METTL3 was significantly upregulated in CRC, and predominantly localized in the nucleus of CRC cells (Figure 1A and 1B). Additionally, we also observed a negative correlation between the expression level of METTL3 and the differentiation status of CRC tissues (Figure 1A and 1C). Western blot analysis of 40 CRC samples from the cohort also showed that the expression level of METTL3 in CRC tumor tissues was significantly higher than that in adjacent normal tissues (Figure 1D). All these data showed that METTL3 was frequently upregulated in CRC. In addition, compared with other CRC cell lines, the expression of METTL3 was higher in HCT116 and SW620 cells (Figure 1E and 1F).

To further explore the clinical relevance of METTL3 in CRC, we divided the 45 CRC samples into two groups according to METTL3 IHC staining levels: the METTL3 positive expression group (n = 19, 42.2%) and the negative expression group (n = 26, 57.8%) (Figure S1B). By analyzing the correlation between the expression of METTL3 and clinicopathological features in 45 CRC patients, we found that METTL3 expression was significantly associated with tumor size (P = 0.041), stages (P = 0.019), and the degree of tumor differentiation (P = 0.040) (Table 1). Therefore, we concluded that METTL3 was frequently upregulated in human CRC and might facilitate the development of CRC by promoting cell proliferation and inhibiting differentiation.

### *METTL3 knockdown inhibited CRC cell proliferation in vitro*

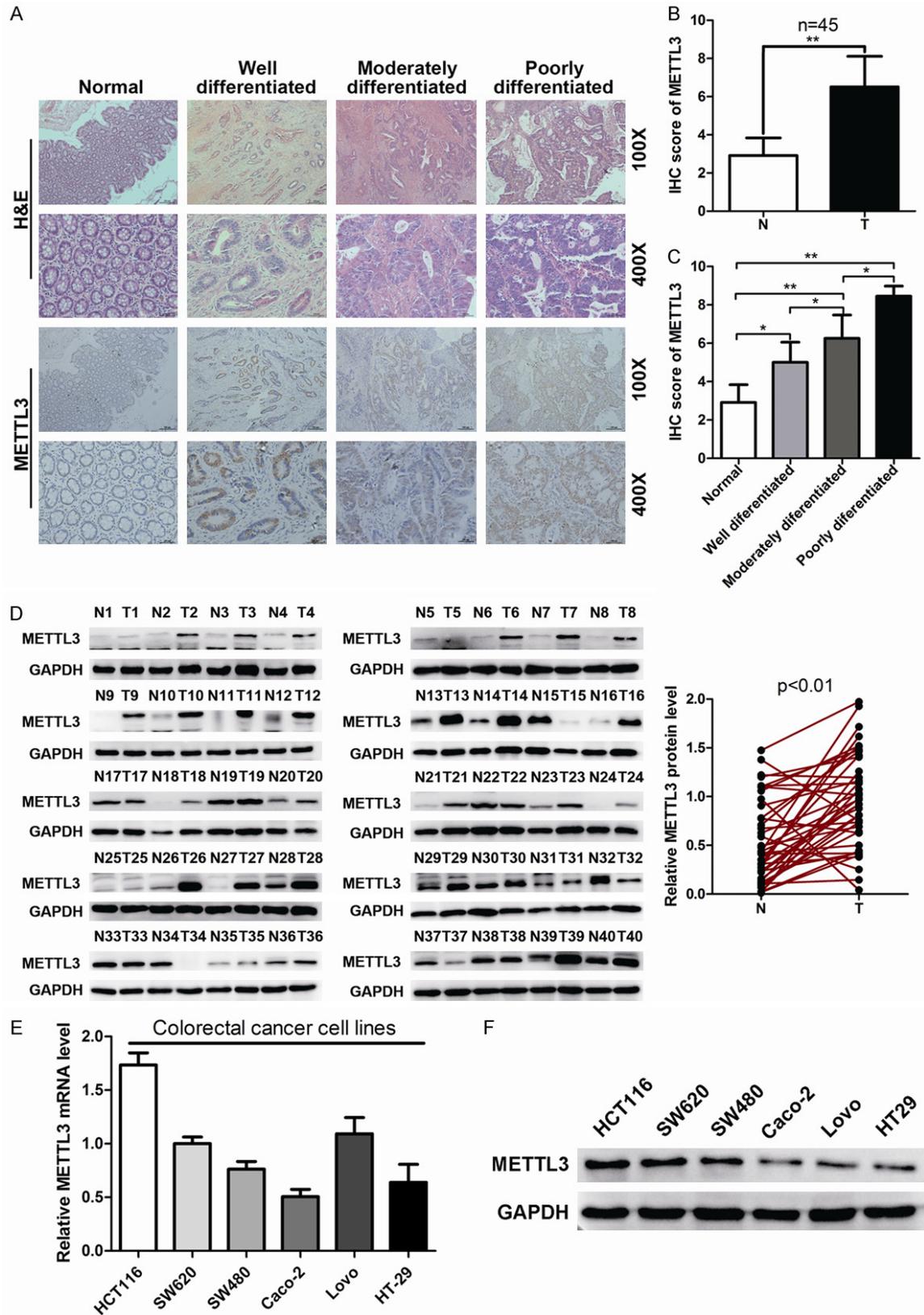
To investigate the function of METTL3 in CRC, we established stable METTL3 knock-

down HCT116 and SW620 cell lines with two different shRNA sequences (shMETTL3#1 and #2). Both mRNA and protein level analysis indicated that METTL3 was successfully knocked down (Figure 2A). The CCK-8 assay was used to examine the effect of METTL3 on cell proliferation. The results showed that METTL3 knockdown significantly inhibited cell proliferation, both in HCT116 and SW620 cells (Figure 2B). Colony formation assays were conducted to further evaluate the effect of METTL3 on cell proliferation. The results showed that METTL3 knockdown suppressed cell colony formation (Figure 2C). In addition, the cell cycle was analyzed by flow cytometry. The results showed that knockdown of METTL3 inhibited G0/G1 to S cell cycle progression (Figure 2D). Apoptosis analysis showed no significant changes in both stable knockdown HCT116 and SW620 cells (Figure S2A and S2B). Furthermore, Transwell assays were performed to test the effect of METTL3 on cell migration and invasion. The results showed that knockdown of METTL3 significantly suppressed cell migration and invasion in HCT116 cells but had no significant effect on SW620 cells (Figure S2C and S2D). Thus, whether METTL3 could affect the migration and invasion ability of colorectal cancer cells remains to be further confirmed. Taken together, these results indicated that METTL3 knockdown inhibited CRC cell proliferation by suppressing the cell cycle G0/G1 to S phase transition.

### *Overexpression of METTL3 promoted CRC cell proliferation in vitro*

To further characterize the role of METTL3 in CRC, we constructed stable cell lines overexpressing METTL3 in HCT116 and SW620 cells. Overexpression of METTL3 was verified at both mRNA and protein levels (Figure 3A). The CCK-8 assay was performed to test the effect of METTL3 overexpression on cell proliferation. Our results showed that overexpression of METTL3 significantly promoted cell proliferation compared with the empty vector control (Figure 3B). Consistently, the colony formation assay also demonstrated that overexpression of METTL3 promoted CRC cell proliferation (Figure 3C). Additionally, cell cycle analysis also showed that upregulation of METTL3 accelerated the G0/G1 to S phase transition both in

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**Figure 1.** The expression of METTL3 in CRC and its correlation with clinicopathological features. A. Hematoxylin and eosin (H&E) staining and IHC staining of METTL3 protein in normal, well differentiated, moderately differentiated, and poorly differentiated CRC tissues (Scale bar, 100  $\mu$ m). The lower the tumor differentiation, the higher

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the METTL3 expression and the expression of METTL3 in adjacent normal tissues is the lowest. B. Compared with adjacent normal tissues (N), the IHC staining score of METTL3 in CRC tumor tissues (T) is higher (n = 45, \*\*P < 0.01). C. IHC score of METTL3 in normal, well differentiated, moderately differentiated, and poorly differentiated CRC tissues, respectively. The lower the tumor differentiation, the higher the IHC score of METTL3 (\*P < 0.05; \*\*P < 0.01). D. Western blotting analysis of METTL3 in CRC tumor tissues (T) and adjacent normal tissues (N) (n = 40). Compared with adjacent normal tissues (N), the expression of METTL3 is up-regulated in CRC tumor tissues (T). GAPDH was used as a loading control. E, F. Relative mRNA level and protein level of METTL3 in six common CRC cell lines including SW480, SW620, HCT116, Caco-2, Lovo, HT-29. The expression level of METTL3 in HCT116 and SW620 is higher than the other four cell lines, so we chose HCT116 and SW620 for further study. GAPDH was used as a loading control.

**Table 1.** Clinicopathological characteristics of METTL3 expression in CRC patients

Characteristic	All (n = 45)	METTL3		X <sup>2</sup>	P-value
		Positive (%)	Negative (%)		
Gender				0.137	0.712
Men	27	12 (55.6)	15 (44.4)		
Women	18	7 (38.9)	11 (61.1)		
Age (Years)				0.205	0.651
> 55	34	15 (44.1)	19 (55.9)		
≤ 55	11	4 (36.4)	7 (63.6)		
Location				0.114	0.736
Colon	25	10 (40)	15 (60)		
Rectal	20	9 (45)	11 (55)		
Metastasis				0.320	0.572
Lymph nodes	20	12 (60)	8 (40)		
Distant	4	3 (75)	1 (25)		
Stages				5.472	0.019*
I-II	21	5 (23.8)	16 (76.2)		
III-IV	24	14 (58.3)	10 (41.7)		
Differentiation				6.440	0.040*
well	10	2 (20)	8 (80)		
Moderately	24	9 (37.5)	15 (62.5)		
Poorly	11	8 (72.7)	3 (27.3)		
Tumor size				4.185	0.041*
> 40 mm	16	10 (62.5)	6 (37.5)		
≤ 40 mm	29	9 (31.0)	20 (67.0)		

Abbreviations: CRC, colorectal cancer; METTL3, methyltransferase like 3. \*P < 0.05.

HCT116 and SW620 cells (**Figure 3D**). Taken together, these results showed that METTL3 overexpression promoted CRC cell proliferation by accelerating the cell cycle G0/G1 to S phase transition.

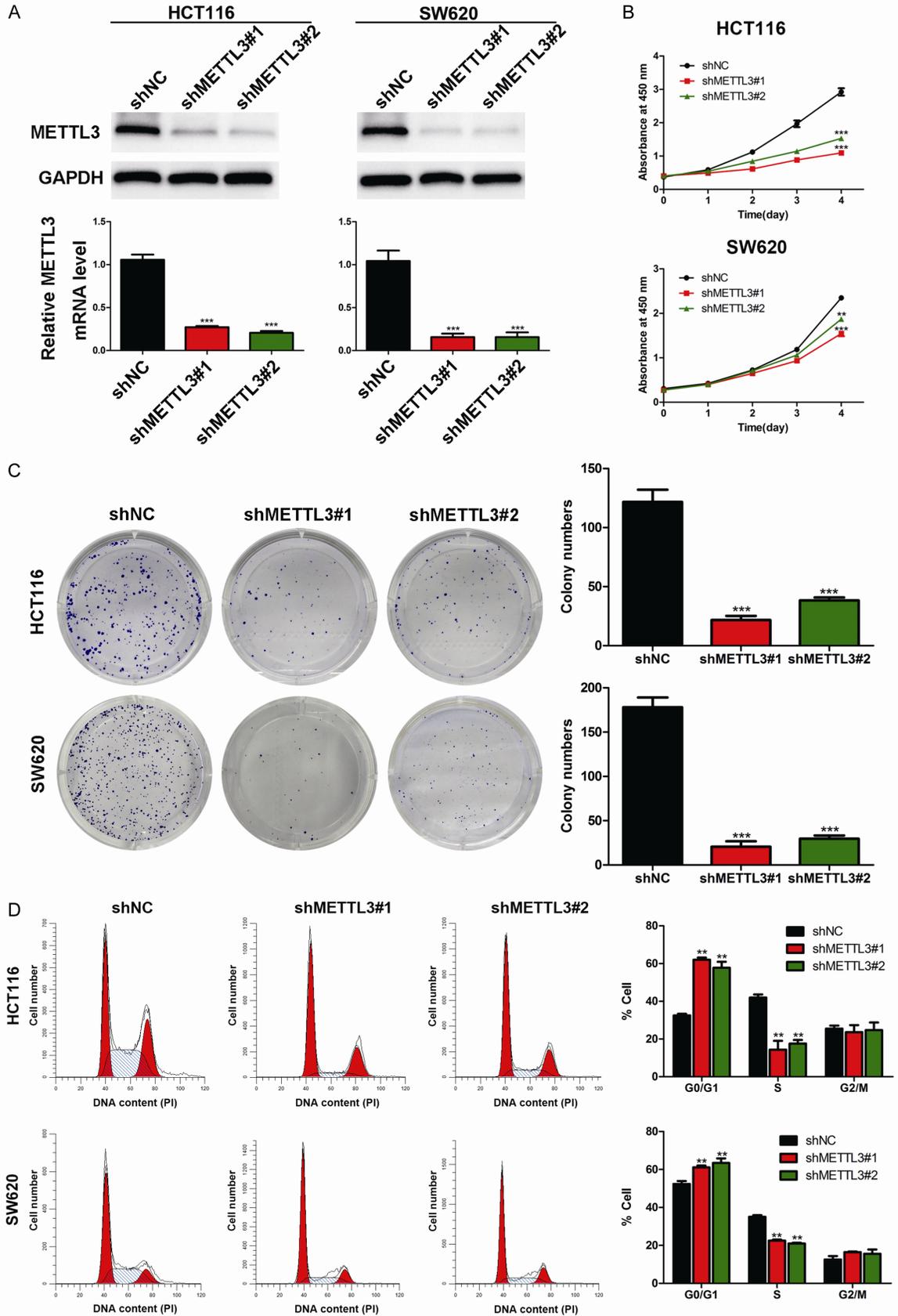
*METTL3 promoted MYC expression partially via an m6A-IGF2BP1 dependent manner in CRC cells*

To identify METTL3 downstream targets in CRC, RT-qPCR was performed to detect the expres-

sion change of genes associated with cell proliferation in METTL3 stable knockdown cells, such as IGF2, EGFR, CTNNB1, P21, P16, CDK1, CDK2, CDK4, CDK6, CCND1, CDC25A, GADD45A, and MYC. The results showed that no other genes than MYC among these genes represented consistent and significant downregulation of both HCT116 and SW620 METTL3 in the stable knockdown cells (**Figures 4A** and **S3A**). Next, we examined the expression change in protein level of MYC by western blot analysis and found that MYC also was downregulated in both HCT116 and SW620 METTL3 stable knockdown cells compared with their controls (**Figure 4B**). To further study the relationship between METTL3 and MYC, RT-qPCR and western blot analysis were performed to detect mRNA and protein levels of METTL3 and MYC in six CRC cell lines, respectively. The result showed that there was a significantly positive correlation between METTL3 and MYC for both mRNA and protein expression levels in the six CRC cell lines (**Figure 4C**

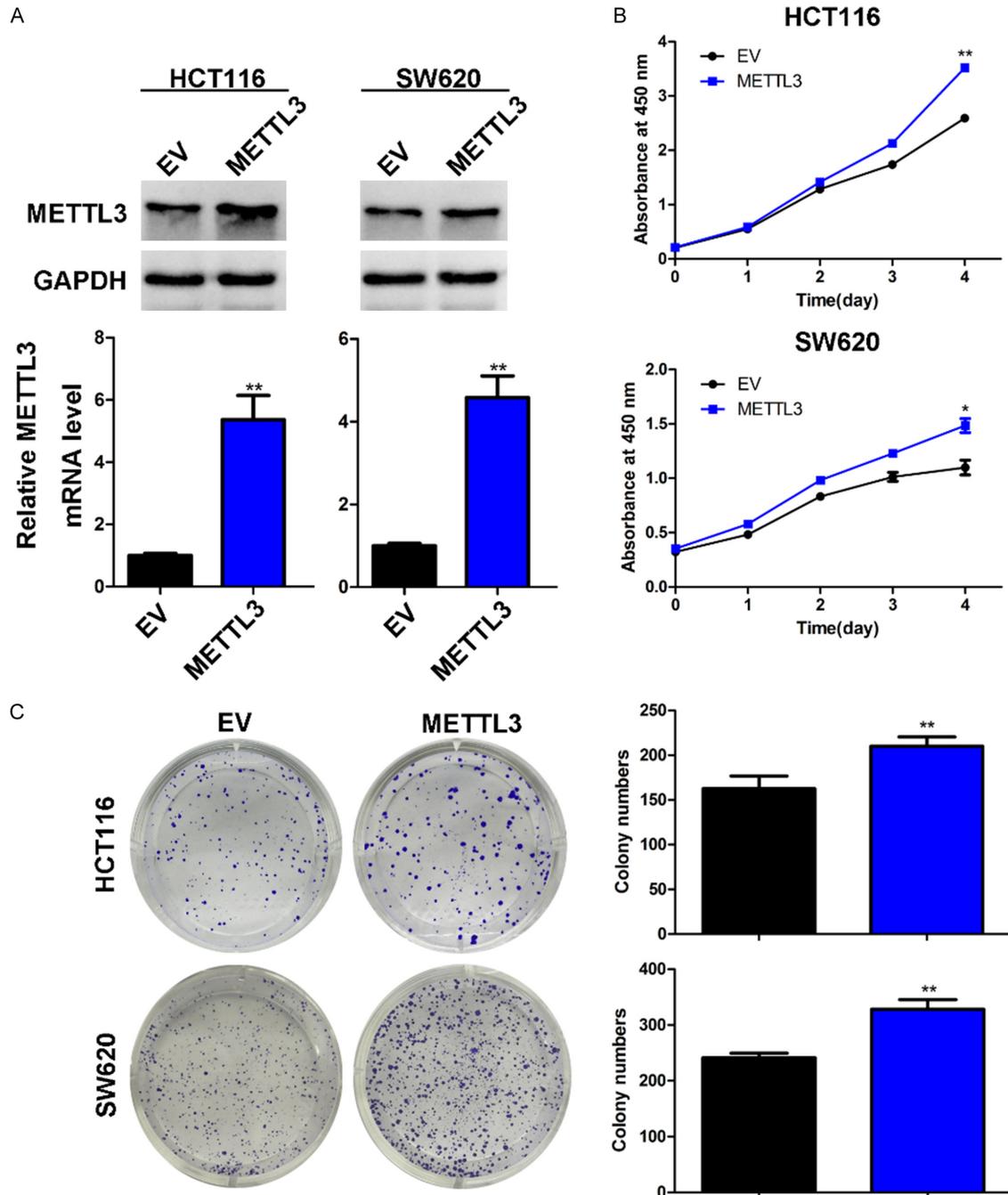
and **4D**). Additionally, we analyzed the correlation of METTL3 and MYC expression levels using RNA-seq gene expression data from TCGA, which contained 434 CRC samples. The results showed that the expression of MYC mRNA was also significantly positively correlated with the expression of METTL3 (**Figure S3B**). These results suggested that METTL3 enhanced the expression of MYC in CRC cells. To further verify the modulatory role of METTL3 on MYC, we performed MYC rescue experiments and found that overexpression of

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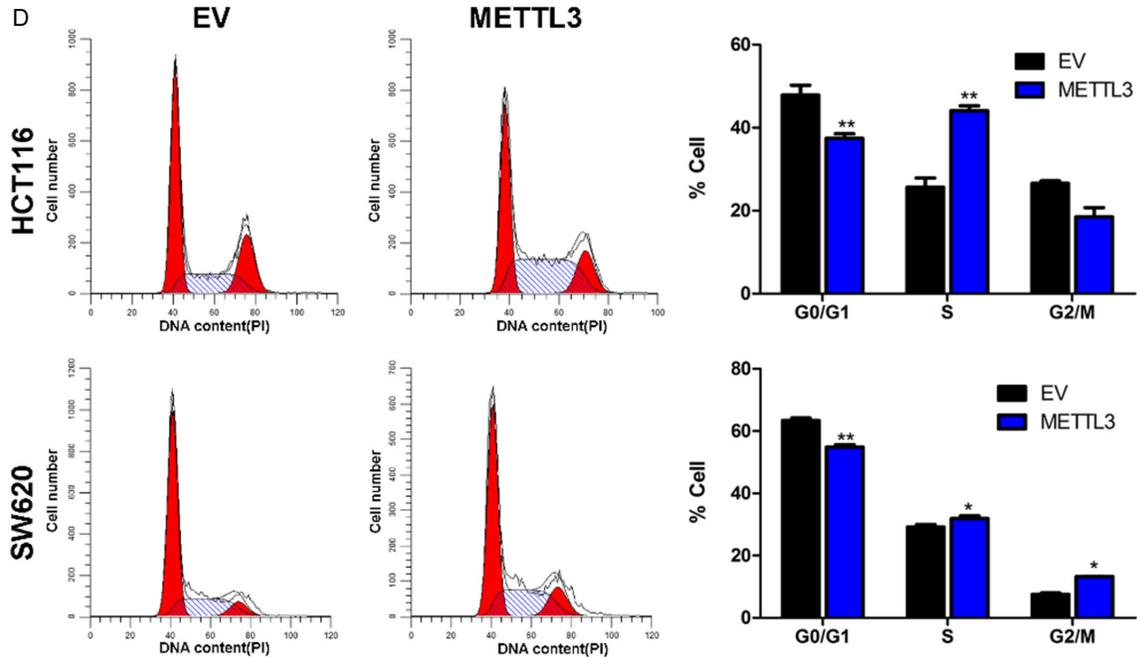


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**Figure 2.** Effect of METTL3 knockdown on CRC cell proliferation and cell cycle progression *in vitro*. A. Stable knock-down of METTL3 in HCT116 and SW620 cell lines by two different shRNA sequences (shMETTL3#1 and #2). Compared with the shNC control group, both mRNA and protein level were significantly decreased in shMETTL3#1 and #2 of HCT116 and SW620 cell lines ( $***P < 0.001$ ). B. CCK-8 assay demonstrated that the proliferation rate in HCT116 and SW620 METTL3 stable knockdown cell lines were significantly decreased compared with the control group ( $**P < 0.01$ ;  $***P < 0.001$ ). C. Colony formation assay showed that colony formation ability in HCT116 and SW620 METTL3 stable knockdown cell lines were decreased compared with the control group ( $***P < 0.001$ ). D. Flow cytometry analysis showed that the proportion of G0/G1 phase cells in HCT116 and SW620 METTL3 stable knockdown cell line were more than that in the control group, while the proportion of S phase cells were less than the control, which revealed that METTL3 knockdown significantly inhibited CRC cell cycle G0/G1 to S phase transition ( $**P < 0.01$ ).



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**Figure 3.** Effect of METTL3 overexpression on CRC cell proliferation and cell cycle progression *in vitro*. A. Overexpression of METTL3 in HCT116 and SW620 cells were achieved by the lentiviral vector pCDH plasmid. RT-qPCR and Western blotting analysis showed that the mRNA and protein level in both HCT116 and SW620 METTL3 overexpression cell lines were significantly higher than the EV control group ( $*P < 0.05$ ;  $**P < 0.01$ ). B. CCK-8 assay demonstrated that up-regulation of METTL3 moderately but significantly increased the proliferation rate of HCT116 and SW620 cells compared with the control group ( $*P < 0.05$ ;  $**P < 0.01$ ). C. Colony formation assay showed that colony formation ability in both HCT116 and SW620 METTL3 overexpression cell lines were increased compared with the EV control group ( $**P < 0.01$ ). D. Flow cytometry analysis showed that the proportion of G0/G1 phase cells in METTL3 overexpressing HCT116 and SW620 cell line were less than that in the control group, while the proportion of S phase cells were more than the control, which revealed that up-regulation of METTL3 facilitated CRC cell cycle G0/G1 to S phase transition ( $*P < 0.05$ ;  $**P < 0.01$ ). EV, PCDH empty vector; METTL3, PCDH overexpression vector containing METTL3 coding sequences.

MYC partially recovered the proliferation ability in METTL3 stable knockdown HCT116 and SW620 cells (Figures 4E and S3C), which further indicated that MYC was a downstream target gene regulated by METTL3 in CRC.

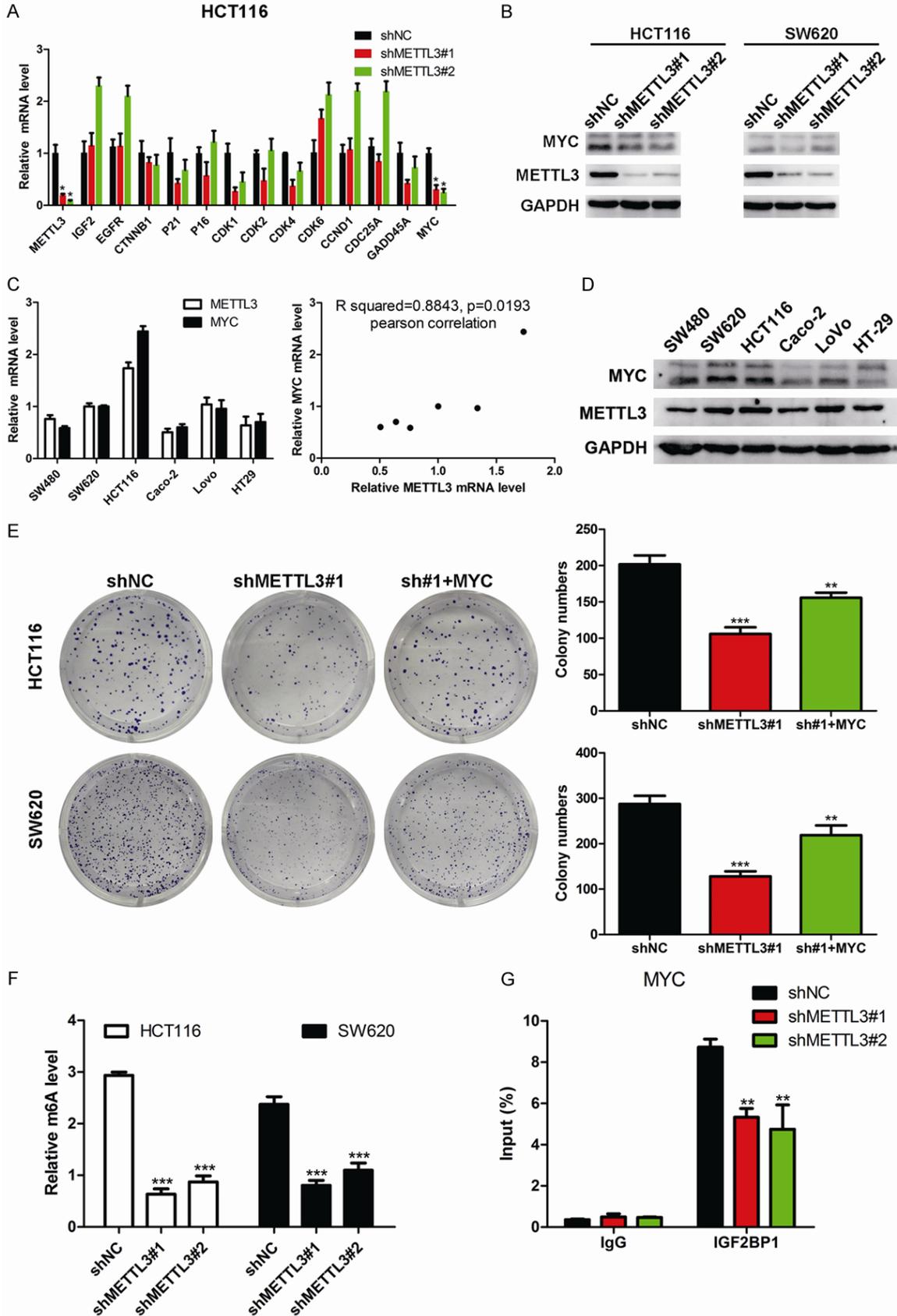
The human MYC gene is an oncogene, encoding a nuclear phosphoprotein that plays an essential role in cell cycle progression, apoptosis, and cellular transformation, which is a well-known target of IGF2BP1 [26, 27]. A previous study reported that m6A modifications were enriched in a coding region instability determinant of MYC, which could be recognized by IGF2BP proteins and enhanced its mRNA stability and translation. To investigate whether METTL3 regulated the expression of MYC in the manner of m6A-IGF2BP1-dependent in colorectal cancer, we examined the total RNA m6A modification level changes in both METTL3 stable knockdown HCT116 and SW620

cells using m6A colorimetric ELISA assays. The result showed that the total RNA m6A levels were significantly decreased upon knockdown of METTL3 both in HCT116 and SW620 cells (Figure 4F). Furthermore, IGF2BP1-RIP experiments were performed in METTL3 stable knockdown HCT116 cells and its corresponding controls. The results showed that MYC mRNA binding to IGF2BP1 significantly decreased upon METTL3 knockdown (Figure 4G), which indicated that m6A modification might facilitate the binding of IGF2BP1 to MYC mRNA. Taken together, we concluded that MYC was a downstream target of METTL3 in CRC cells, which might be regulated, at least partially in an m6A-IGF2BP1 dependent manner.

*METTL3 facilitated CRC tumorigenesis by enhancing the expression of MYC in vivo*

To further confirm the oncogenic role of METTL3 in CRC, subcutaneous tumor formation experi-

METTL3 promotes CRC proliferation through enhancing MYC expression



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**Figure 4.** METTL3 promoted MYC expression at least partially via an m6A-IGF2BP1 dependent manner in CRC cells. A. RT-qPCR was performed to test the mRNA expression of proliferation-related genes in METTL3 stable knockdown HCT116 cells and corresponding shNC controls. MYC mRNA levels were significantly decreased among these genes in HCT116 shMETTL3#1 and shMETTL3#2 cells compared with their shNC controls. GAPDH was used as a loading control ( $*P < 0.05$ ). B. Western blotting showed that the protein expression levels of MYC were significantly reduced upon knockdown of METTL3 in HCT116 and SW620 cells. C. RT-qPCR analysis showed that MYC mRNA expression level was relatively higher in six common CRC cell lines with higher METTL3 expression. Pearson correlation analysis indicated that MYC mRNA expression level was positively correlated with METTL3 mRNA expression level in CRC cells (Person's test;  $R = 0.8843$ ;  $P = 0.0193$ ). D. Western blotting analysis also showed that MYC protein expression level was positively correlated with METTL3 protein expression level in six CRC cell lines. E. MYC rescue experiments showed that after knocking down METTL3 expression in HCT116 and SW620 cells, the colony forming ability of the cells decreased, and when METTL3 was overexpressed in the knockdown cells, the cell colony forming ability increased compared with their shNC control groups, which indicated that overexpression of MYC in HCT116 and SW620 METTL3 stable knockdown cells partially rescued cell proliferation ( $**P < 0.01$ ;  $***P < 0.001$ ). F. m6A ELISA assays showed that the RNA m6A levels in HCT116 and SW620 shMETTL3#1 and shMETTL3#2 cells were significantly lower than their shNC controls, which revealed that knockdown of METTL3 significantly reduced the RNA m6A level in HCT116 and SW620 cells ( $***P < 0.001$ ). G. RIP-qPCR assay showed that compared with HCT116 shNC cells, IGF2BP1 protein bound to MYC mRNA in HCT116 shMETTL3#1 and shMETTL3#2 cells were both significantly decreased, which revealed that knockdown of METTL3 significantly attenuated the binding of IGF2BP1 to MYC mRNA in HCT116 cell ( $**P < 0.01$ ).

ments were performed to detect the effect of METTL3 overexpression or knockdown on tumorigenesis in nude mice. During tumor formation, the volume of the tumor was measured every 4 days from the 4th day after injection. Mice were euthanized at the 24th day and the tumors were removed, photographed, and weighed (**Figure 5A** and **5C**). The results showed that tumors generated by METTL3 overexpression cells were significantly larger and heavier than controls (**Figure 5B**). In contrast, the METTL3 knockdown group showed a significant decrease of tumor size and weight compared with its control group (**Figure 5D**). These results indicated that METTL3 facilitated CRC tumorigenesis *in vivo*. To further confirm whether METTL3 promoted tumor growth *in vivo* by regulating the expression of MYC, proteins of the isolated tumors were extracted and subjected to western blot analysis. The results showed that overexpression of METTL3 promoted MYC expression, and coincidentally, knockdown of METTL3 inhibited the expression of MYC (**Figure 5E**). Consistent with western blot analysis, IHC staining of mouse tumor tissues also confirmed these results (**Figure 5F**). Taken together, our results suggested that METTL3 promoted CRC cell proliferation by enhancing the expression of MYC *in vivo*.

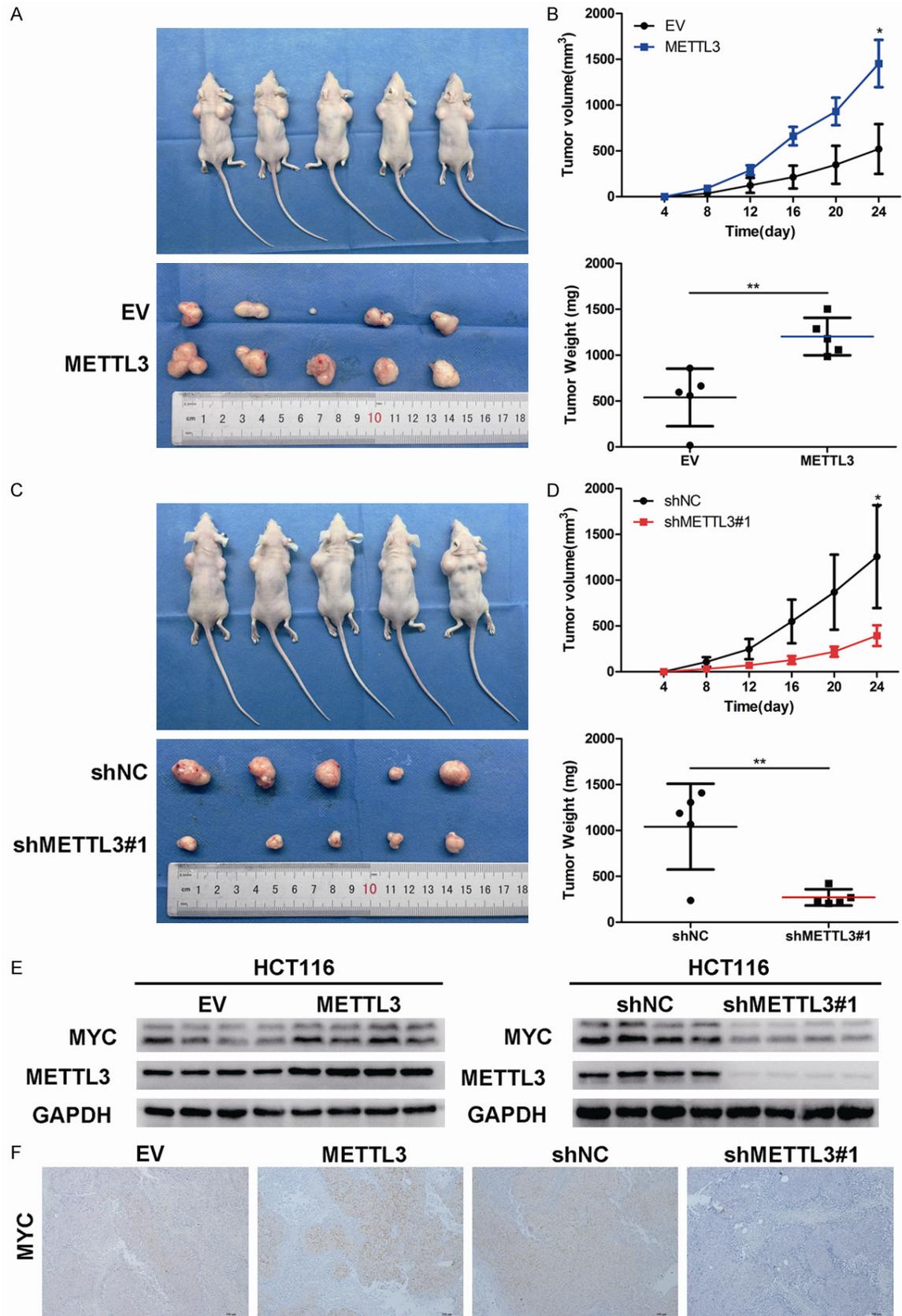
### Discussion

Evidence has indicated that METTL3 is abnormally expressed in many tumors and is closely related to the development of tumors [8]. In this

study, we found that METTL3 was significantly up-regulated in CRC, which was consistent with previous studies [16, 17]. Moreover, analysis of clinical relevance revealed that the expression level of METTL3 was significantly associated with tumor size, stage, and differentiation grade. These results suggested that METTL3 might be involved in the progression of CRC. Functionally, we demonstrated that the essential role of METTL3 in promoting CRC cell proliferation was by accelerating the cell cycle from the G0/G1 to S phase transition, both *in vitro* and *in vivo*. In addition, METTL3 showed higher expression in HCT116 and SW620 cells among six common CRC cell lines (**Figure 1E** and **1F**), which coincided with their proliferative capacity. Notably, we found a positive correlation between METTL3 and MYC expression in six common CRC cell lines, including mRNA and protein levels (**Figure 4C** and **4D**). Additionally, MYC rescue experiments showed that overexpression of MYC in both HCT116 and SW620 METTL3 stable knockdown cells partially compensated for the inhibition of METTL3 knockdown on cell proliferation (**Figures 4E** and **S3C**). These results indicated that METTL3 promoted CRC cell proliferation by enhancing MYC expression.

Increasing evidence has shown that m6A modification has an effect on RNA synthesis, processing, translation, and metabolism [6, 7, 28, 29] and contributes to the development of multiple tumors [30]. As the key component of a m6A methyltransferase complex, METTL3 reg-

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**Figure 5.** METTL3 facilitated CRC tumorigenesis by enhancing the expression of MYC in nude mice. A and C. Tumorigenesis assay was performed by subcutaneous injection of METTL3 stable overexpression or knockdown HCT116 cells into two flanks of BALB/c nude mice. The nude mice were harvested and tumors were removed and photographed at 24th day after injection. The result showed that the tumors in the METTL3 overexpression group were significantly larger than those in the EV control group, while the tumors of METTL3 knockdown group were smaller than the shNC control group. B and D. Tumor volume was measured periodically after injection and tumors were removed and weighed at 24th day after injection. The results showed that the tumor volume and weight were larger in the METTL3 overexpression group than in the EV control group, while the tumor volume and weight were smaller in the METTL3 knockdown group compared with the shNC control group, which demonstrated that METTL3 facilitated CRC cell proliferation *in vivo* (Student's t-test; \* $P < 0.05$ ; \*\* $P < 0.01$ ). E. Western blotting analysis of the protein expression level of METTL3 and MYC in the tumor tissues of the treatment group and control group (Remove the tumors with too much difference in tumor size in the METTL3 overexpression group and the tumors with no significant difference in METTL3 knockdown group). The results showed that the protein expression level of MYC in tumor tissues of METTL3 overexpression group was higher than that in EV control group, while the protein expression level of MYC in tumor tissues of knockdown group was lower than that in shNC control group, which demonstrated that the expression of MYC was positively correlated with METTL3 *in vivo*. F. IHC staining showed that the expression of MYC was upregulated in the METTL3 overexpression group compared with the EV control group, while the expression of MYC in the METTL3 knockdown group was decreased, compared with the shNC control group.

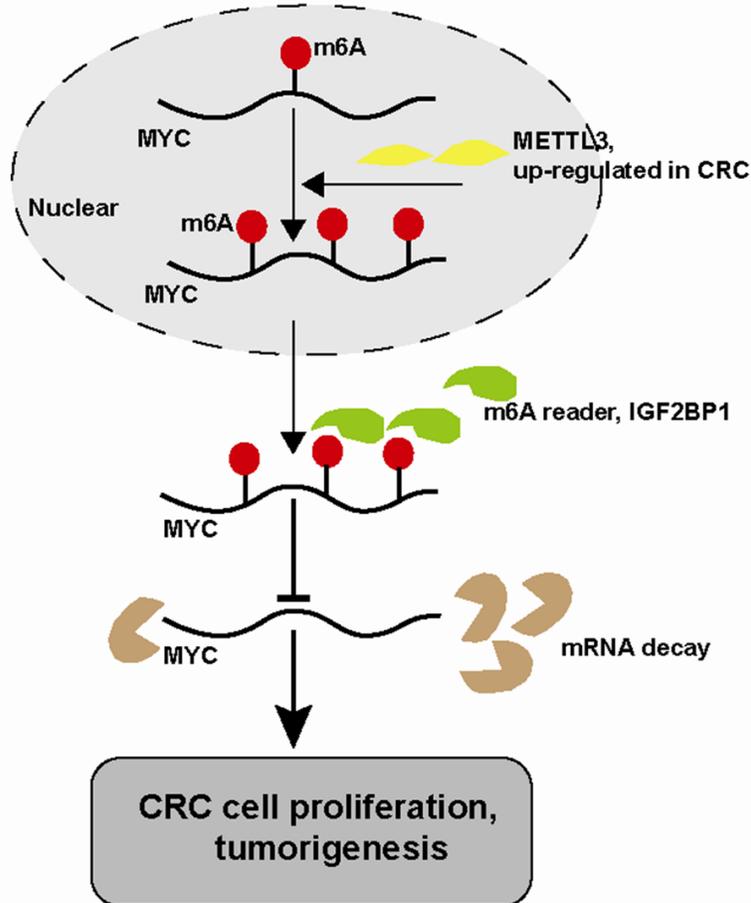
ulates tumor progression primarily through its m6A methyltransferase activity. Nonetheless, there are also some studies reporting that the tumor-promoting role of METTL3 is independent of its m6A modification function, which largely depends on its location in the cell. METTL3 located in the nucleus mainly exerts its function by regulating the status of m6A modification on mRNAs, which are subsequently recognized by the m6A “reader” protein. In contrast, METTL3 located in the cytoplasm promotes the translation of downstream mRNAs by recruiting the translation initiation factor eIF3, which does not depend on its m6A modification function [14, 31]. In our study, IHC analysis showed that METTL3 was basically located in the nucleus (Figure 1A). Thus, we hypothesized that METTL3 promoted CRC cell proliferation, depending on its m6A modification function. To verify our hypothesis, we detected the total RNA m6A level in both HCT116 and SW620 METTL3 stable knockdown cells. The results showed that the total RNA m6A level significantly decreased upon knockdown METTL3 (Figure 4F), which was consistent with changes in cell proliferative capacity. We therefore proposed that the cell proliferative potential of CRC cells might be positively related to the level of RNA m6A modification in cells. Consistent with our data, a previous study also reported that METTL3 promoted the expression of HBXIP by increasing its mRNA m6A modification level, consequently facilitating cell proliferation of breast cancer [32].

Recently, another study reported that METTL3 promoted CRC progression through an m6A-

IGF2BP2-dependent mechanism [17]. In their study, they identified SOX2 as the downstream target of METTL3. However, we did not find significant changes of SOX2 mRNA levels both in HCT116 and SW620 stable METTL3 knockdown cells (Figure S3D). In contrast, our study found that MYC gene expression was significantly decreased both in HCT116 and SW620 METTL3 stable knockdown cells compared with their controls (Figure 4A and 4B). Furthermore, we found that IGF2BP1 bound directly to MYC mRNA, using the RIP-qPCR assay (Figure 4G). Consistent with our findings, two early studies also reported that IGF2BP1 bound directly to MYC mRNA and regulated its stability [13, 27]. A reasonable explanation for this diversity between the two studies could be the differences in METTL3 knockdown efficiency and m6A modification status on individual mRNA. This phenomenon has also been reported by previous studies [3, 15, 33]. However, further investigations, such as by using a METTL3 knockout model and gene-specific m6A qPCR assay, will be required to better address this discrepancy.

The oncogene MYC encodes a transcription factor that regulates cell proliferation, apoptosis, and differentiation [34, 35]. Activation of MYC is one of the most common abnormalities in human tumors. The Cancer Genome Atlas Network Comprehensive analysis of molecular characterization in CRC revealed that nearly 100% of CRC tumors had changes in MYC transcriptional targets, including those promoted or inhibited by MYC, suggesting an important role for MYC in CRC [36]. MYC can promote tumor cell proliferation by accelerat-

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**Figure 6.** Proposed working model of METTL3 promoting CRC cell proliferation and tumorigenesis. METTL3 was frequently upregulated in CRC tissues and was mainly localized in the nucleus of tumor cells, which increased the m6A modification level on the proto-oncogene MYC mRNA in CRC cells. The m6A modification on MYC mRNA could be recognized by IGF2BP1, which further enhanced MYC mRNA stability, consequently promoting CRC cell growth and tumorigenesis.

ing cell cycle transition. Downregulation of MYC by JQ1, a selective small molecule bromodomain inhibitor, could inhibit multiple myeloma cell proliferation by prompting cell cycle arrest and cell senescence [37]. In our study, we found that the cell cycle G0/G1 to S phase transition was significantly inhibited when knocking down METTL3 (Figure 2D), and MYC was also coincidentally downregulated both in HCT116 and SW620 stable knockdown cells (Figure 4A and 4B). In a similar manner as the METTL3 knockdown models, our METTL3 overexpression models also acquired consistent results (Figures 3D, 5E and 5F). All this evidence indicated that METTL3 accelerated cell cycle transition by enhancing the expression of MYC in CRC.

MYC activation in human tumors can occur both directly and indirectly. Gene amplification can directly result in the dysregulation of MYC in a number of tumors and cell lines [38], but in most cases, the cause of the elevated MYC expression remains unclear. The other direct mechanism is mainly through enhancing MYC mRNA transcript stability [13, 27], whereas the indirect mechanisms are mainly through activation of upstream signaling pathways, including WNT, TGF- $\beta$  pathway, etc. [36]. Recently, evidence has suggested that the dysregulation of MYC was related to abnormal m6A modification in its mRNA [15, 22-24]. Notably, we also demonstrated that the mRNA level of MYC and the total RNA m6A level were significantly and simultaneously decreased in HCT116 and SW620 METTL3 stable knockdown cells compared with the controls, which indicated that expression of MYC might be correlated with RNA m6A modification levels in CRC cells; however, MYC gene-specific m6A qPCR assays would be needed to better confirm this

possibility in future studies. One recent study reported that m6A modification in MYC mRNA could be recognized by IGF2BPs and consequently could increase MYC mRNA stability and translation efficiency [13]. Consistent with the previous study, our RIP-qPCR assay also showed that IGF2BP1 bound directly to MYC mRNA in CRC cells, and the bound MYC mRNA was significantly decreased when METTL3 was knocked down in HCT116 cells (Figure 4G). Therefore, we concluded that METTL3 upregulated MYC expression in CRC, at least partially in an m6A-IGF2BP1-dependent manner.

In summary, our finding demonstrated that METTL3 was frequently upregulated in CRC and closely related to the clinicopathological

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features. Upregulated METTL3 promoted CRC cell proliferation and tumorigenesis by enhancing MYC expression, which might be in an m6A-IGF2BP1-dependent manner (Figure 6). Thus, we found that METTL3 played an oncogenic role as a m6A “writer” in CRC, which might provide a potential therapeutic target for CRC.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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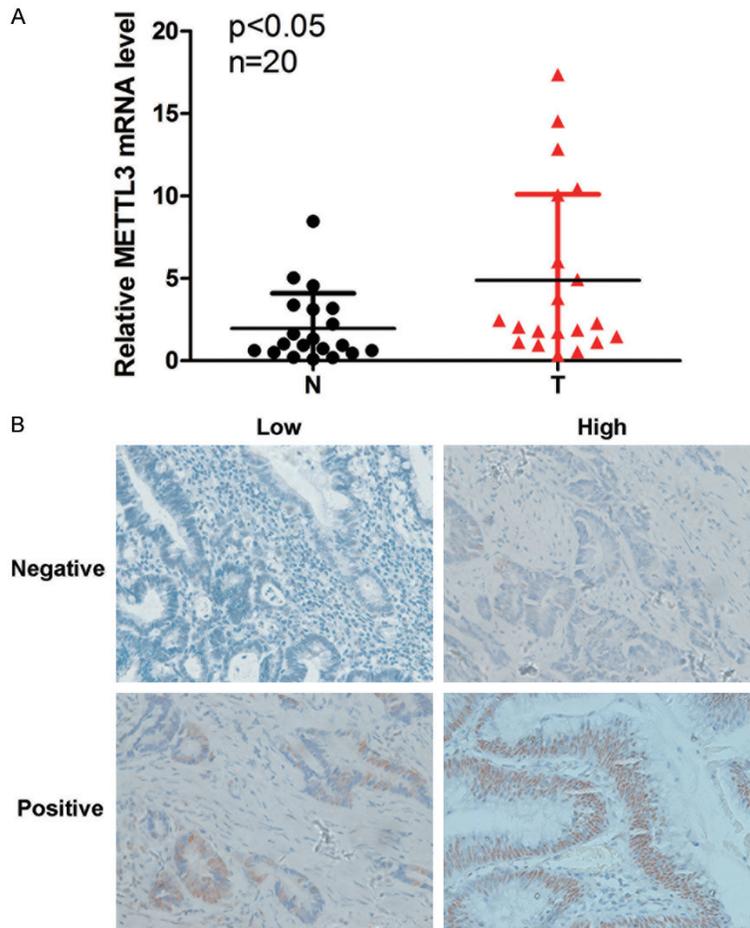
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**Table S1.** shRNA and RT-qPCR primer sequences

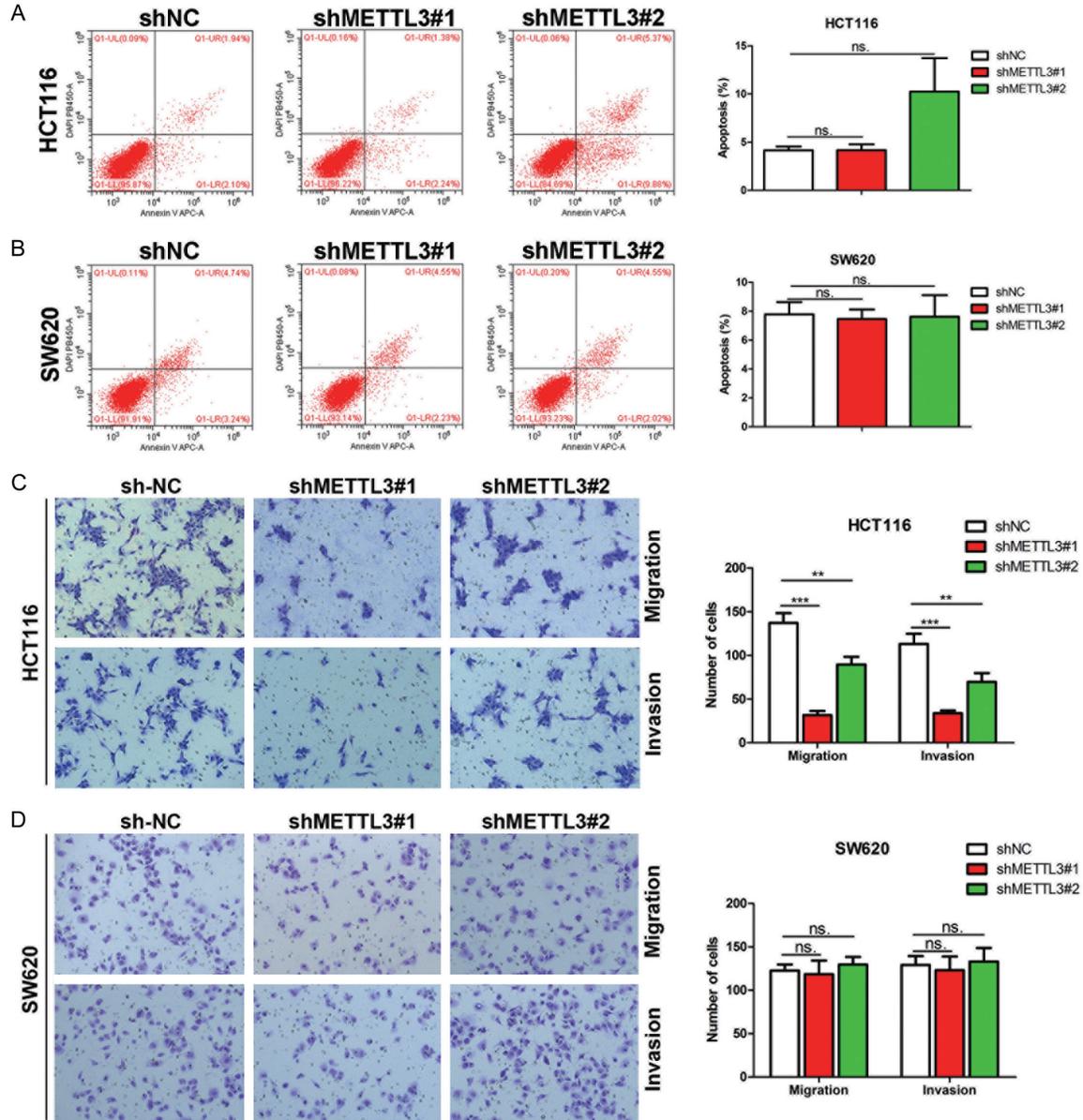
Name	sequences	Name	sequences
shMETTL3#1	GCAAGTATGTTCACTATGAAA	CDK1	F: ATGAAGTGTGGCCAGAAGTG R: TGACATGGGATGCTAGGCTT
shMETTL3#2	GCTGCACCTCAGACGAATTAT	CDK2	F: TCCGGATCTTCGGACTCTG R: ACAAGCTCCGTCACATTTCA
METTL3	F: GTCCATCTGTCTTGCCATCTC R: GAGACCTCGCTTTACCTCAATC	CDK4	F: CTCCCATCAGCACAGTTCCG R: GGGGTGCCTGTCCAGATAT
IGF2	F: ATGACACCTGGAAGCAGTCC R: GTCTTGGGTGGGTAGAGCAA	CDK6	F: CCAGGCAGGCTTTTCATTCA R: AGGCCTGGAAGTATGGGTG
EGFR	F: TCTACAACCCACCACGTAC R: TCGCACTTCTTACACTTGCG	CCND1	F: ACAGATCATCCGCAAAACAG R: GGCGGTAGTAGGACAGGAAG
CTNNB1	F: GAAACGGCTTTCAGTTGAGC R: CTGGCCATATCCACCAGAGT	CDC25A	F: CTA CTGATGGCAAGCGTGTC R: TCTCTCTCACATACCGGCAC
P21	F: TGTCTGTACCCTTGTGCCT R: GGCGTTTGGAGTGGTAGAAA	GADD45A	F: GCTGGTGACGAATCCACATT R: TCCATGTAGCGACTTTCCCG
P16	F: CGATGTCGCACGGTACCT R: GACCTCCGCGGCATCTAT	MYC	F: TTCGGGTAGTGGAACCAG R: AGTAGAAATACGGCTGCACC
SOX2	F: ATCAGGAGTTGTCAAGGCAGAG R: AGAGGCAAACCTGGAATCAGGA		

Abbreviations: shRNA: short hairpin RNA; RT-qPCR: Quantitative real-time Polymerase Chain Reaction; METTL3: Methyltransferase like 3; IGF2: Insulin-like growth factor 2; EGFR: Epidermal growth factor receptor; CTNNB1: Catenin beta-1; P21: Cyclin dependent kinase inhibitor 1A; P16: Cyclin dependent kinase inhibitor 2A; SOX2: SRY-box transcription factor 2; CDK1: Cyclin-dependent kinase 1; CDK2: Cyclin-dependent kinase 2; CDK4: Cyclin-dependent kinase 4; CDK6: Cyclin-dependent kinase 6; CCND1: Cyclin D1; CDC25A: Cell division cycle 25A; GADD45A: Growth arrest and DNA damage inducible alpha; MYC: MYC proto-oncogene, bHLH transcription factor.



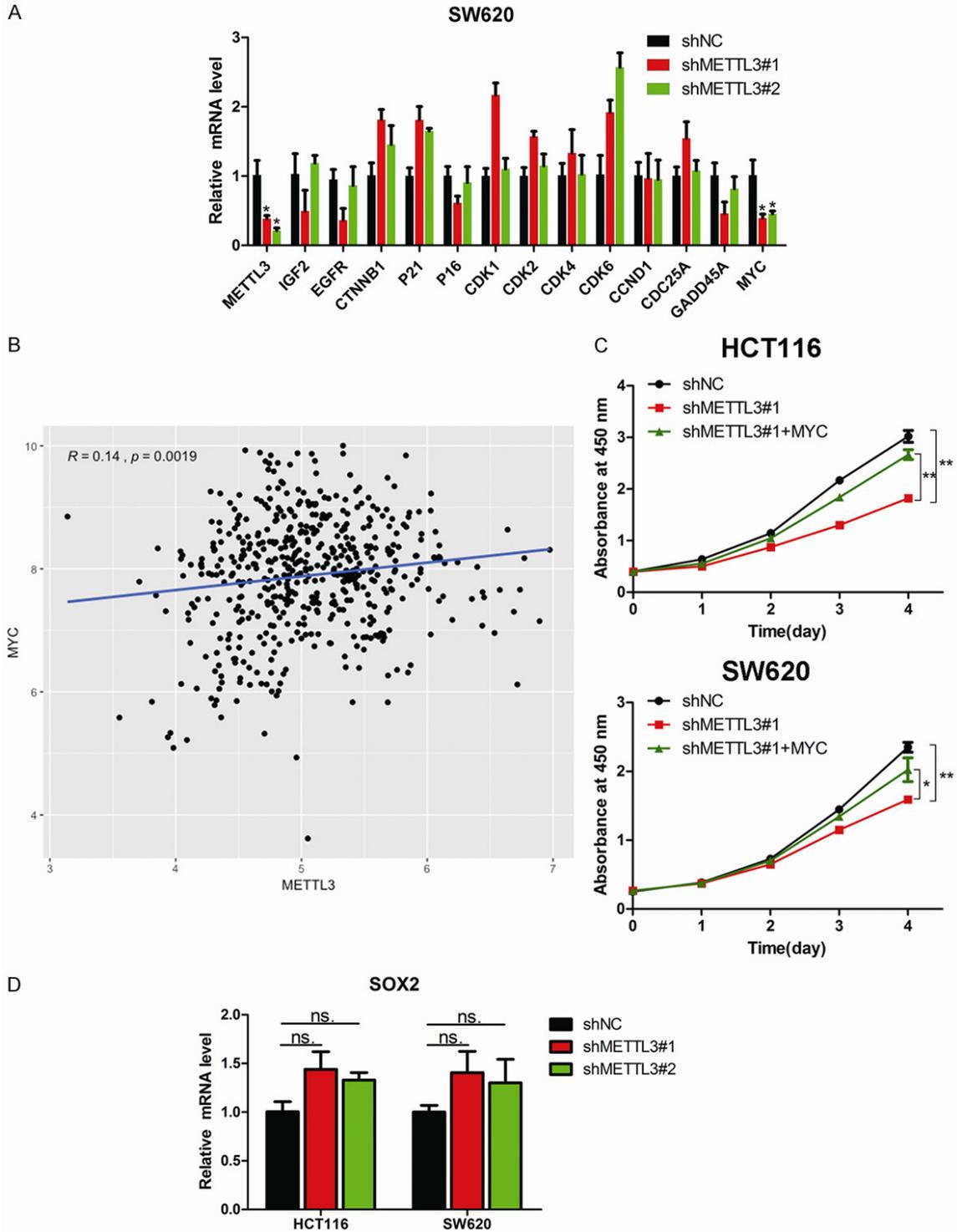
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**Figure S1.** A. RT-qPCR analysis showed that mRNA expression level of METTL3 in CRC tumor tissues was significantly higher than that in adjacent normal tissues ( $n = 20$ ,  $*P < 0.05$ ). B. Immunohistochemistry staining grade for METTL3. A total of 45 CRC samples were divided into two groups according to the immunostaining score of METTL3: a positive METTL3 staining group (more than 10% of the cells were stained) and a negative METTL3 staining group (less than 10% of the cells were stained). The positive staining group was further divided into 2 groups according to the intensity of staining: high METTL3 staining (> 50% of cells were intensely stained) and low METTL3 staining (< 50% of cells were intensely stained).



**Figure S2.** A and B. Flow cytometry analysis showed that compared with the shNC control group, the proportion of apoptotic cells in METTL3 stable knockdown HCT116 and SW620 cells had not significant changes. The result revealed that knockdown of METTL3 had no significantly effect on apoptosis in both HCT116 and SW620 cells. C. Transwell assays showed that the migration and invasion ability were significantly decreased in METTL3 stable knockdown HCT116 cells compared with the shNC control group. D. Transwell assays showed that there was no significant change of migration and invasion ability in METTL3 stable knockdown SW620 cells compared with the shNC control group (ns, nonsignificant).

# METTL3 promotes CRC proliferation through enhancing MYC expression



**Figure S3.** A. RT-qPCR was used to detect the mRNA expression of proliferation-related genes in METTL3 stable knockdown SW620 cells and shNC controls. MYC mRNA levels were significantly decreased among these genes in HCT116 shMETTL3#1 and shMETTL3#2 cells compared with their shNC controls. GAPDH was used as a loading control ( $*P < 0.05$ ). B. Correlation analysis of METTL3 and MYC mRNA expression in 434 samples from TCGA database showed that MYC mRNA expression was significantly positively associated with METTL3 mRNA expression (Person's test;  $R = 0.14$ ;  $P = 0.0019$ ). C. CCK-8 assays showed that overexpression of MYC in both HCT116 and SW620 METTL3 stable knockdown cells partially rescued cell proliferation ( $*P < 0.05$ ;  $**P < 0.01$ ). D. RT-qPCR analysis showed that there were no significant changes of the SOX2 mRNA level in both HCT116 and SW620 METTL3 stable knockdown cells compared with their corresponding shNC controls (ns, nonsignificant).