**Abstract**

**Background**: Dysregulation of N6-methyladenosine (m6A) RNA methylation plays a crucial role in colorectal cancer (CRC) development. Despite the established oncogenic role of methyltransferase-like 3 (METTL3) in CRC, the mechanisms behind its tumor-promoting functions are not fully understood.

**Methods**: Functional impacts were assessed by measuring cell viability, invasion, colony formation, migration, apoptosis, and M2 macrophage polarization *in vitro*, as well as *in vivo* tumorigenicity. The interaction between METTL3 and claudin 2 (CLDN2) was validated via RNA immunoprecipitation and luciferase assays. The PRKN/METTL3 interaction was analyzed using immunoprecipitation (IP) and Co-IP experiments. The stability of mRNA and protein levels was examined by treating cells with actinomycin D or cycloheximide.

**Results**: METTL3 and CLDN2 levels were upregulated in CRC samples and cells. Depletion of METTL3 or CLDN2 suppressed CRC cell proliferation, invasion, and migration, and inhibited M2 polarization of THP-1-derived macrophages. Mechanistically, METTL3 promoted m6A methylation and stability of CLDN2 mRNA via an insulin-like growth factor 2 binding protein 2 (IGF2BP2)-dependent manner. METTL3 silencing diminished CRC cell malignant phenotypes and M2 macrophage polarization via CLDN2 reduction. Moreover, PRKN enhanced METTL3 protein degradation through K63-linked polyubiquitination. The PRKN/METTL3 axis regulated malignant phenotypes of CRC cells. Additionally, METTL3 depletion repressed tumor growth and lung metastasis of xenografts *in vivo* via CLDN2.

**Conclusion**: Our work uncovers a previously unrecognized PRKN-METTL3-CLDN2 signaling network that orchestrates colorectal tumorigenesis, providing a compelling rationale for developing METTL3-targeted therapies against CRC.

**Keywords**: Colorectal cancer, epigenetic modifications, METTL3, m6A methylation, ubiquitination

## Introduction

With nearly 935,000 fatalities recorded in 2020, colorectal cancer (CRC) represents the

third most lethal cancer worldwide [1]. Key risk factors such as population aging, physical inactivity, and obesity contribute significantly to its incidence [2]. Current treatment options, although improved, show limited efficacy against advanced or metastatic CRC, which is largely incurable. The polarization of macrophages towards the M2 phenotype confers pro-tumoral capabilities, including angiogenesis induction, tissue remodeling, and suppression of antitumor immunity [3]. In CRC, elevated levels of M2 macrophage infiltration are linked to disease advancement, metastasis, and poorer survival rates [4, 5]. Understanding the drivers of this polarization is therefore a promising avenue for creating innovative immunotherapeutic interventions for CRC.

As an essential form of epigenetic modification, N6-methyladenosine (m6A) methylation is a unique way that decorates RNA to control RNA metabolism, such as translation, stability and degradation, and is mediated by a series of evolutionarily conserved enzymes [6]. The methyltransferase-like 3 (METTL3) is a well-known m6A "writer" that mainly catalyzes RNA m6A methylation, and it has emerged as a pivotal mediator for a broad array of biological processes and carcinogenesis [7]. METTL3 is frequently overexpressed in most aggressive tumors, and its high abundance forebodes worse survival [8]. Recent work has proved the oncogenic properties of METTL3 in cancer, and METTL3 inhibitors are believed to block its enzymatic activity to possess anti-cancer efficacy [9].

In CRC, METTL3 contributes to cancer cell growth and metastasis through modulation of the m6A-Crumbs3-Hippo cascade [10]. METTL3 targets the m6A-glucose transporter type 1 (GLUT1)-mechanistic target of rapamycin complex 1 (mTORC1) axis to enhance glucose metabolism, thereby exerting a promoting function in CRC development [11]. Through m6A modification, METTL3 has been shown to suppress anti-cancer immunity in CRC [8, 12]. Moreover, METTL3 can recruit M2 macrophages in CRC via an m6A-Snail- C-X-C motif chemokine ligand 2 signaling cascade, enhancing immunosuppression and pulmonary metastasis [13]. However, the role and mechanism of METTL3 in CRC macrophage polarization are still incompletely understood.

In an effort to study METTL3-interacting effectors in CRC, this study reveals the

tight junction protein claudin 2 (CLDN2), which is capable of promoting colorectal carcinogenesis [14]. The ubiquitination system has critical functions in various cellular processes and cancer through modulation of protein degradation [15]. A search for ubiquitin-related modifiers governing METTL3 stability led to the identification of Parkin (PRKN), an E3 ubiquitin ligase already implicated in CRC pathogenesis [16, 17]. This evidence led to the hypothesis that the PRKN/METTL3/CLDN2 axis might affect malignant progression in CRC.

## Materials and methods

### Bioinformatics

To identify METTL3-interacting proteins in CRC, the GSE130012 dataset (including METTL3-knockdown and wild-type HCT-116 cells) [18] was retrieved from the NCBI database https://www.ncbi.nlm.nih.gov/. Expression patterns in CRC were further examined using colon adenocarcinoma (COAD) data from the ENCORI portal (https://rnasysu.com/encori/), which contains 471 tumor and 41 normal samples, as well as from The Cancer Genome Atlas (TCGA) (https://www.cancer.gov/ccg/research/genome-sequencing/tcga), encompassing 286 primary tumors and 41 normal controls. The regulatory potential of METTL3 in the m6A methylation of CLDN2 mRNA was evaluated via the RM2target platform (http://rm2target.canceromics.org/#/home). Putative m6A modifications on CLDN2 mRNA were predicted using the RMbase (http://bioinformaticsscience.cn/rmbase/modgene.php) and RMVar (https://rmvar.renlab.cn/#/home?pid=6) databases, while potential m6A sites were forecasted with the Sequence-based RNA Adenosine Methylation site Predictor (SRAMP) tool (http://www.cuilab.cn/sramp/). Additionally, ubiquitination-related regulators of METTL3 were explored using the ubibrowser3.0 database (http://ubibrowser.bio-it.cn/ubibrowser_v30/).

### Cell lines and culture conditions

This study employed normal (NCM460) and malignant (SW620, LoVo and HCT-116) colonic epithelial cell lines and THP-1 monocytes from Immocell (Xiamen, China).

The specific culture media included Dulbecco's Modified Eagle Medium (DMEM, Immocell) for NCM460, L15 (Immocell) for SW620, Ham's F-12K (Gibco, Schwerte, Germany) for LoVo, RPMI-1640 (Immocell) for THP-1 and McCoy's 5a (Procell, Wuhan, China) for HCT-116, all of which were enriched with 10% fetal bovine serum (FBS, Gibco). Human primary macrophages (Procell, Wuhan, China) were used for validation of a subset of key findings. All cell lines were used within 6 months of acquisition and maintained in a 5% $CO_2$ incubator at 37°C.

**Plasmids and transient transfections**

This study used constructs including pLV3-U6-METTL3(human)-shRNA-Puro (sh-METTL3), pLV3-U6-METTL3(human)-shRNA2-Puro (sh-METTL3#2), pcDNA-Flag-METTL3, pcDNA-PRKN(human)-3×FLAG, pLV3-U6-shRNA-NC (sh-NC) control and pcDNA-NC control, all from Miaoling Biology (Wuhan, China). CLDN2(human)-short hairpin RNA (shRNA) (sh-CLDN2), PRKN(human)-shRNA (sh-PRKN) and pcDNA-CLDN2(human)-FLAG plasmids were designed and made by Tsingke Biotech (Beijing, China). Si-NC, si-IGF2BP1, si-IGF2BP2, and si-IGF2BP3 were obtained from Tsingke Biotech. The expression alteration by transfection was carried out according to the vendor's instructions (Thermo Fisher Scientific, Milan, Italy). In brief, LoVo and HCT-116 CRC cells were maintained overnight before transfections with Lipofectamine 3000 mixed with plasmid(s). Transfected cells after 48 h were subjected to the assays described below.

**Patient samples**

The human cohort was recruited at Second Affiliated Hospital of Xi'an Jiaotong University and included 33 patients with CRC. After all subjects signed informed consent, primary CRC tumors (n=33) were collected at the time of surgical excision. Each tumor was matched with a neighboring normal colorectal tissue control from the same patient. The clinicopathologic features of patients were provided in Table 1. These fresh specimens were divided into two parts: one was fixed with 4% paraformaldehyde, and the other was stored in a Thermo Fisher Scientific refrigerator at -80°C. Protocols for human study were reviewed and approved by Second Affiliated Hospital of Xi'an Jiaotong University ethics committee (No.2025YS753).

**Lentivirus particles and stable transduction**

Lentiviral particles encoding sh-METTL3, sh-NC, CLDN2, PRKN, or a non-targeting scramble sequence were sourced from VectorBuilder (Guangzhou, China). HCT-116 cells were transduced in complete medium supplemented with 6 μg/mL polybrene (Beyotime, Shanghai, China). Following transduction for 16-20 h, the medium was refreshed. Stable cell pools were then selected using 2 μg/mL puromycin (Beyotime) over a period of 14-21 days to ensure a homogeneous population of transduced cells.

**Xenograft models**

Following the approved protocols by Second Affiliated Hospital of Xi'an Jiaotong University Animal Care and Use Committee (No.XJTUAE2024-2215), 50 male BALB/c nude mice aged two months (Gempharmatech, Nanjing, China) were divided into five groups: sh-METTL3 (n=5), sh-METTL3+CLDN2 (n=5), sh-METTL3+pcDNA (n=5), sh-NC+CLDN2 (n=5), or sh-NC (n=5). Both xenograft tumor growth and lung metastasis were evaluated in all groups. For generation of xenograft models, HCT-116 CRC cells transduced with sh-METTL3 lentivirus or sh-NC control were subcutaneously implanted into the right flank ($3 \times 10^6$ cells/mouse). Administration of lentivirus expressing CLDN2 or control virus was done by intratumoral injection ($5 \times 10^{10}$ TU/mouse) after 8 days of cell implantation every three days for a total of three times. Xenograft volume measurement began after 8 days and was done using the formula $\text{width}^2 \times \text{length} \times 0.5$. Thirty-three days post the implantation, xenografts were harvested for further analyses. For lung metastasis studies, HCT-116 CRC cells transduced with lentivirus expressing sh-NC or sh-METTL3 alone or along with CLDN2 or control sequence (vector) were injected into nude mice through tail vein injection ($1 \times 10^6$ cells/mouse). After 33 days, lung metastatic colonization was quantified by metastatic nodule number evaluation and H&E staining.

**Immunohistochemistry (IHC)**

For IHC staining as described [19], slides (4 μm) from clinical CRC tumors and mouse xenografts, which were fixed and embedded in paraffin, were probed with anti-METTL3 (rabbit polyclonal (pAb), 1:1,500, Cat. No. 15073-1-AP, Proteintech, Wuhan,

China), anti-CLDN2 (rabbit monoclonal (mAb), 1:600, Cat. No. 18932, Cell Signaling, Danvers, MA, USA), anti-Ki67 (mouse mAb, 1:5,000, Cat. No. 9449, Cell Signaling), anti-PCNA (rabbit pAb, 1:3,500, Cat. No. 10205-2-AP, Proteintech), and anti-MMP9 (mouse mAb, 1:900, Cat. No. ab58803, Abcam, Cambridge, UK) antibodies. After protein visualization by 3,3'-diaminobenzidine (DAB) assay (Servicebio, Wuhan, China), slides were subjected to hematoxylin counterstaining.

**RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)**

Under the use of BeyoMag™ RNA Kit and accompanying regulations (Beyotime), total RNA was prepared from cultured CRC cells and patient samples. Synthesis of cDNA and SYBR-based qRT-PCR were set-up using PrimeScript™ RT Master (TaKaRa, Dalian, China) and SYBR Green kit (Vazyme, Nanjing, China), respectively, in accordance with the kit instructions. Oligonucleotide primer pairs (shown in Supplementary Table 1) were used for measurement of gene expression, which was determined by the formula $2^{-\Delta\Delta Ct}$ normalized to the expression of the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Antibodies and immunoblotting (IB)**

Protein preparation from cultured CRC cells or collected tissue specimens, concentration evaluation, and immunoblot analysis were conducted as reported elsewhere [20]. Antibodies used were: anti-METTL3 (1:2,000, Cat. No. 15073-1-AP, Proteintech), anti-CLDN2 (1:1,000, Cat. No. 18932, Cell Signaling), anti-PRKN (rabbit pAb, 1:2,000, Cat. No. 14060-1-AP, Proteintech), anti-ubiquitin (anti-Ub, mouse mAb, 1:1,000, Cat. No. 3936, Cell Signaling), and anti-GAPDH loading buffer (mouse mAb, 1:5,000, Cat. No. ab8245, Abcam). For development of blots, a chemiluminescent kit (Nakarai Tesque, Tokyo, Japan) was applied.

**Cell viability, invasion and migration assays**

For evaluation of viability, CRC cells were plated in 96-well sterile culture plates at 1,000 cells/well 12 h before plasmid transfection. Viable cells were quantified with Cell Counting Kit-8 (CCK-8) assay (Biotech, Nanjing, China) as described by the producer. For determination of invasive ability, researchers plated CRC cells after 48 h of plasmid transfection into Matrigel-coated 24-Tranwell inserts in serum-free medium at 100,000

cells/well. These cells were allowed to transfer towards 15% FBS media in the bottom chamber. Twenty-four hours later, invasive cells were counted after crystal violet (1%) staining. For assessment of migratory capacity, researchers plated CRC cells in 6-well sterile culture plates at 100,000 cells/well 12 h before plasmid transfection. A vertical wound was created on the cell monolayer. Pictures were obtained at 0 h and 24 h for determination of migration distance.

**Colony formation assay**

Approximately 300 CRC cells after 48 h of plasmid transfection were plated to 6-well sterile culture plates. After that, the incubation was done for 14 days at 37°C. After crystal violet (1%) staining, colonies (> 50 cells) were quantified.

**Flow cytometry analysis**

For cell apoptosis, CRC cells after 48 h of plasmid transfection were stained using fluorescein isothiocyanate (FITC)-Annexin V/propidium iodide (PI) Assay Kit as recommended by the vendor (Vazyme). For analysis of M2 macrophage polarization, the culture medium from transfected LoVo and HCT-116 CRC cells was harvested and used as the condition medium (CM). THP-1 cells were incubated for 24 h with the CM plus phorbol-12-myristate-13-acetate (PMA, Selleck, Shanghai, China) at a concentration of 100 ng/mL. Single-cell suspensions were labeled with FITC-CD206 antibody (Cat. No. FITC-65155, Proteintech), and followed by incubation with PC5.5-labeled 7AAD (Biolegend, San Diego, CA, USA) to eliminate dead cells. The apoptotic and $CD206^{+}$ cells were scored on LSR FORTESSA and CellQuest software (BD Biosciences, Stockholm, Sweden).

**RNA immunoprecipitation (RIP) assays**

Whole cellular lysates from sh-METTL3-transfected, sh-NC-introduced or untransfected LoVo and HCT-116 CRC cells were prepared using BeyoRIP™ RIP Kit and instructions (Beyotime). The bead-antibody complex of protein A/G agarose and anti-m6A (1:3000, Cat. No. 68055-1-Ig, Proteintech), isotype IgG (1:3000, Cat. No. 31903, Thermo Fisher Scientific), anti-IGF2BP2 (1:1000, Cat. No. 11601-1-AP, Proteintech), or anti-METTL3 (1:1500, Cat. No. 15073-1-AP, Proteintech) antibody was made and added into total lysates. After addition, a 4-h incubation was allowed at

4°C. The m6A- or METTL3-associating precipitates were harvested to purify RNA for assessment of CLDN2 mRNA enrichment by qRT-PCR.

**Luciferase assay**

To assess the modulation of METTL3 on CLDN2 mRNA, the fragment of CLDN2 mRNA harboring the wild (UGACU) or mutant (MUT, UGCCU) predicted m6A sites was inserted into the pmirGlO luciferase vector (Qiyunbio, Nanchang, China). CLDN2 luciferase reporter (1 μg) was introduced into LoVo and HCT-116 CRC cells ($1.0 \times 10^6$) with 1 μg of sh-METTL3, sh-NC, METTL3, or pcDNA plasmid. After cells were lysed 48 h post transfection, relative luciferase activity (the ratio of firefly/*Renilla*) was recorded using a plate reader (TriStar S LB 942, Berthold, Bad Wildbad, Germany).

**RNA pull-down assay**

To identify proteins binding to CLDN2 mRNA, an RNA pull-down was carried out with the PureBinding™ Kit (Geneseed, Guangzhou, China) employing biotin-labeled CLDN2-specific and negative control (NC) probes. Cell lysates from LoVo and HCT-116 lines were prepared, after which they were incubated with probe-conjugated streptavidin magnetic beads for 1 h at 4°C. Following incubation, proteins were eluted from the magnetically isolated beads for METTL3 immunoblot analysis.

**Immunoprecipitation (IP) and Co-IP experiments**

For IP experiment, whole cellular lysates from untransfected CRC cells and cells transfected with pc-PRKN+Flag-METTL3, pcDNA+Flag-METTL3, Ub-WT+Flag-METTL3, Ub-WT+Flag-METTL3+pc-PRKN, K48-Ub mutant+Flag-METTL3, K48-Ub mutant+Flag-METTL3+pc-PRKN, K63-Ub mutant+Flag-METTL3, K63-Ub mutant+Flag-METTL3+pc-PRKN, were obtained as described [21]. 2 mg of protein lysates were mixed with 1 μg of anti-Flag (rabbit pAb, Cat. No. 12076-1-AP, Proteintech), anti-PRKN (Cat. No. 14060-1-AP, Proteintech), anti-METTL3 (Cat. No. 15073-1-AP, Proteintech), or isotype IgG control (Cat. No. 30000-0-AP, Proteintech) antibody overnight at 4°C, which was followed by addition of Protein A/G agarose for 3 h. Protein in the immunoprecipitates was harvested for enrichment detection of ubiquitinated METTL3, METTL3 and PRKN by immunoblot analysis.

**Evaluation of mRNA and protein stability**

To evaluate the impact of METTL3 or IGF2BP2 on CLDN2 mRNA stability, transfected LoVo and HCT-116 cells were maintained for 24 h in media plus actinomycin D (Act D, 50 μg/mL, Selleck). RNA was used to quantify CLDN2 mRNA levels at the indicated time points.

To assess the impact of PRKN on METTL3 protein stability, CRC cells after 48 h of transfection with pcDNA or pc-PRKN were treated with 20 ng/mL cycloheximide (CHX, Selleck) for 0, 3, 6, 9 and 12 h, followed by immunoblot analysis to detect METTL3 protein levels.

**Statistical analysis**

Comparisons of two groups were conducted with Mann-Whitney *U* test or unpaired *t*-test and comparisons of three or more groups with analysis of variance (ANOVA) before use of Tukey's or Sidak's post-test. Results were expressed as mean ± standard deviation (SD), with a $P$-value < 0.05 considered significant. For expression association analysis, Pearson's correlation coefficient (r) was utilized.

## Results

### METTL3 and CLDN2 are upregulated in human CRC

To search METTL3-interacting proteins in CRC, this study used the GSE130012 dataset to compare gene expression in METTL3-downregulated CRC. Compared to unaltered CRC cells, CLDN2, a potent driver in colorectal carcinogenesis [14], exhibited the most significant change (smallest $P$ value) in METTL3-downregulated CRC cells (Fig. 1A and Supplementary Fig. 1), suggesting the potential association of METTL3 with CLDN2. To demonstrate this, METTL3 was silenced in LoVo and HCT-116 CRC cells with a sh-METTL3 plasmid (Fig. 1B). METTL3 depletion significantly reduced CLDN2 protein expression (Fig. 1C). Additionally, to rule out off-target effects, researchers used a second shRNA (sh-METTL3#2) and confirmed that METTL3 depletion also reduced CLDN2 mRNA expression in CRC cells (Supplementary Fig. 2A and 2B). This study then used COAD-TCGA and COAD-ENCORI datasets to reveal the upregulation of METTL3 and CLDN2 in CRC (Fig. 1D-1G), which was further confirmed by our IHC and qRT-PCR assays with human clinical samples (Fig.

1H-1K). Survival analysis was conducted to assess prognostic relevance, demonstrating that high levels of METTL3 or CLDN2 were significantly associated with inferior survival outcomes in these CRC patients (Fig. 1L and 1M). METTL3 expression was significantly associated with tumor TNM stage and lymph node metastasis (Table 1). Furthermore, in clinical CRC tumors, the positive expression association of METTL3 with CLDN2 was observed (Fig. 1N). Moreover, METTL3 and CLDN2 protein levels tended to be higher in CRC tumors and cell lines (Fig. 1O-1R). Due to the pronounced upregulation of METTL3 and CLDN2 in LoVo and HCT-116 cells, researchers selected these two cell lines for subsequent analyses.

**Depletion of METTL3 inhibits *in vitro* malignant phenotypes of CRC cells and represses M2 polarization of THP-1-derived macrophages**

This study next validated the precise action of METTL3 in CRC cell biological functions. Depletion of METTL3 by sh-METTL3 introduction markedly decreased viability and colony formation number of LoVo and HCT-116 CRC cells when compared to sh-NC mock (Fig. 2A and 2B and Supplementary Fig. 2C). Conversely, METTL3 depletion markedly accelerated the apoptosis rate of CRC cells (Fig. 2C and Supplementary Fig. 2D). Furthermore, reduced METTL3 expression clearly hindered CRC cell invasion (Fig. 2D and Supplementary Fig. 2E). Wound healing assay confirmed that METTL3 depletion decreased the motility distance of CRC cells *in vitro* (Fig. 2E). The M2 polarization of macrophages has profound positive effects on CRC metastasis and progression [22]. By incubating THP-1-differentiated macrophages with the CM of transfected CRC cells, our data revealed that downregulation of METTL3 significantly decreased the ratio of the $CD206^+$ cells (Fig. 2F and 2G and Supplementary Fig. 2F) and decreased the mRNA expression of M2 markers CD163 and arginase 1 (ARG1) (Fig. 2H and 2I), indicating that METTL3 depletion could repress M2 polarization of THP-1-derived macrophages.

**Reduced CLDN2 expression results in suppressed malignant phenotypes in CRC cells**

CLDN2 supports the metastasis of CRC and contributes to this disease aggressive progression [23]. Given the upregulation of CLDN2 in CRC, this study aimed to

analyze the consequences of silencing CLDN2. Efficient knockdown of CLDN2 expression was achieved in LoVo and HCT-116 cell lines using a specific shRNA plasmid (Fig. 3A). As summarized in Fig. 3B and 3C, CLDN2 reduction diminished CRC cell viability and colony formation ability. Meanwhile, the apoptosis of CRC cells was dramatically enhanced by CLDN2 reduction (Fig. 3D). Fig. 3E revealed the suppressed invasion ability of LoVo and HCT-116 CRC cells upon CLDN2 silencing. The migration distance of CLDN2-reduced cells was significantly shorter than that in sh-NC controls (Fig. 3F). Furthermore, reduced CLDN2 expression resulted in a distinct inhibition in M2 polarization of THP-1-differentiated macrophages (Fig. 3G-3I).

**METTL3 contributes to m6A methylation of CLDN2 mRNA via an IGF2BP2-dependent manner**

Our aforementioned data indicated that depletion of METTL3 resulted in reduced levels of CLDN2 protein in CRC cells (Fig. 1C). Intriguingly, the RM2target online tool predicted that METTL3 could mediate the m6A methylation of CLDN2 mRNA (Fig. 4A). The RMbase and RMVar databases predicted the potential m6A modification within CLDN2 mRNA (Fig. 4B and 4C). MeRIP and qRT-PCR assays showed that CLDN2 mRNA was substantially enriched in m6A antibody immunoprecipitates (Fig. 4D), indicating that m6A modification of CLDN2 mRNA occurs in CRC cells. RIP assays with specific antibody to METTL3 showed the enrichment of CLDN2 mRNA in METTL3-containing complex (Fig. 4E). Moreover, depletion of METTL3 significantly decreased the levels of m6A-modified CLDN2 mRNA in LoVo and HCT-116 CRC cells (Fig. 4F). Immunoblot analysis confirmed that elevated expression of METTL3 by a pcDNA plasmid approach, shown in Fig. 4G, resulted in enhanced levels of CLDN2 protein in the two CRC cell lines (Fig. 4H). Fig. 4I also showed the potential m6A sites of CLDN2 mRNA, predicted by SRAMP [24]. Next, researchers determined the modulation of METTL3 in CLDN2 mRNA by luciferase assay based on wild and mutant (MUT) CLDN2 reporters, which carried the predicted m6A sites (UGACU) or mutated m6A sequence (UGCCU), respectively. The luciferase activity of CRC cells with CLDN2 reporter introduction was significantly decreased by depletion of

METTL3, and it was conversely enhanced by elevated expression of METTL3 (Fig. 4J and 4K). In contrast, METTL3 alteration did not affect the luciferase of CLDN2-MUT reporter-transfected CRC cells (Fig. 4J and 4K). To confirm the regulation of METTL3 in CLDN2 mRNA stability, researchers treated CRC cells after transfection with Act D, a repressor of de novo transcription, and found that the stability of CLDN2 mRNA was impaired by METTL3 depletion and conversely enhanced upon METTL3 augmentation (Fig. 4L and 4M). The physical association between METTL3 and CLDN2 mRNA was further verified by RNA pull-down experiments with biotin-labeled CLDN2 mRNA probes (Fig. 4N). Together, these data demonstrate that METTL3 promotes m6A methylation and stability of CLDN2 mRNA.

Our study then investigated the m6A readers involved in METTL3-mediated CLDN2 mRNA m6A modification. The IGF2BP family recognizes m6A marks on target mRNAs to enhance their stability, thereby promoting oncogenesis [25]. The knockdown efficiencies of si-IGF2BP1, si-IGF2BP2, and si-IGF2BP3 were verified by immunoblotting (Fig. 5A-5C). Knockdown of IGF2BP2, but not IGF2BP1 or IGF2BP3, specifically reduced CLDN2 mRNA expression (Fig. 5D). This specific effect, consistent with IGF2BP2's known function as an m6A-dependent stabilizer of oncogenic RNAs in CRC [26, 27], prompted its selection for further analysis. Consistently, IGF2BP2 silencing reduced the protein levels of CLDN2 (Fig. 5E). Moreover, METTL3 depletion diminished the binding between IGF2BP2 and CLDN2 mRNA (Fig. 5F and 5G). Additionally, IGF2BP2 silencing reduced the stability of CLDN2 mRNA in LoVo and HCT-116 cells (Fig. 5H and 5I). Furthermore, IGF2BP2 overexpression by an IGF2BP2 plasmid (Fig. 5J) significantly reversed METTL3 depletion-mediated CLDN2 expression reduction (Fig. 5K). All these data indicate that METTL3 stabilizes CLDN2 mRNA via an IGF2BP2-m6A-dependent manner.

**METTL3 depletion inhibits malignant progression of CRC cells via CLDN2 reduction**

To gain more insights into the regulation of the METTL3/CLDN2 axis in CRC development, CLDN2 expression was increased at both mRNA and protein levels in LoVo and HCT-116 cells with CLDN2 reduction induced by sh-METTL3 *in vitro* (Fig.

6A and 6B). CLDN2 restoration enhanced cell viability and colony formation, which were decreased by sh-METTL3 (Fig. 6C and 6D). Restoration of CLDN2 partially but significantly abrogated sh-METTL3-induction of apoptosis of these cells (Fig. 6E). In addition, CLDN2 restoration exerted an obvious counteracting impact on sh-METTL3-driven invasion and motility defects in LoVo and HCT-116 cells (Fig. 6F and 6G). Meanwhile, CLDN2 expression partially abolished sh-METTL3-mediated inhibition in macrophage M2 polarization and reduction in CD163 and ARG1 mRNA expression in THP-1-derived macrophages and primary human macrophages (Fig. 6H-6K and Supplementary Fig. 3A-3C).

Male nude mice were subcutaneously inoculated in the right flank with either HCT-116 control cells (sh-NC lentivirus infection) or METTL3 depletion cells (sh-METTL3 lentivirus transduction), with or without intratumoral administration of CLDN2 lentivirus or control virus. The growth of HCT-116 xenografts was significantly diminished in mice inoculated with sh-METTL3-transduced cells compared to mice inoculated with sh-NC control cells; however, administration of a CLDN2 plasmid significantly promoted tumor growth; moreover, the CLDN2 plasmid abated the growth repression mediated by sh-METTL3 (Fig. 7A and 7B). Immunoblot assays confirmed the low expression of CLDN2 protein in sh-METTL3 HCT-116 xenografts, which was remarkably reversed by the CLDN2 plasmid (Fig. 7C). IHC results showed sh-METTL3 xenografts had fewer cells stained for Ki67 (a cell cycle marker) and PCNA (a regulator of cell proliferation) and exhibited lower levels of MMP9 (a metastasis-related factor) and CLDN2 than sh-NC controls, while CLDN2 restoration strongly abolished these influences of sh-METTL3 (Fig. 7D). Importantly, in lung colonization assays, METTL3 depletion suppressed lung metastasis of HCT-116 xenografts; CLDN2 overexpression enhanced tumor lung metastasis; whereas, CLDN2 expression restoration also reversed METTL3 depletion-mediated suppression in tumor lung metastasis (Fig. 7E and 7F). All these data indicate that METTL3 correlates with CRC development via CLDN2.

**PRKN diminishes METTL3 protein stability via ubiquitination**

Based on the dysfunction of METTL3 in CRC, this study then identified ubiquitin-

related modifiers that drive aberrant expression of METTL3. PRKN is an E3 ubiquitin ligase with documented tumor-suppressive roles in CRC [16]. Notably, PRKN mRNA expression was significantly downregulated and inversely associated with METTL3 expression in clinical CRC samples (Supplementary Fig. 4A and 4B). Intriguingly, the ubibrowser database predicted PRKN as a potential regulator of METTL3 stability (Fig. 8A). PRKN expression was then manipulated to determine the regulation of PRKN in METTL3. Alteration of PRKN expression by sh-PRKN and a PRKN plasmid was confirmed by immunoblot analysis (Fig. 8B). IP experiments validated the interaction of PRKN with METTL3 in CRC cells (Fig. 8C). Overexpression of PRKN clearly enhanced the abundance of ubiquitinated Flag-METTL3 protein in HCT-116 CRC cells transfected with the Flag-METTL3 plasmid (Fig. 8D). Moreover, PRKN increased the ubiquitination of METTL3 in the presence of WT-Ub and the K63-Ub mutant but not the K48-Ub mutant, indicating that PRKN mainly adds the K63-linked polyubiquitination chains on METTL3 protein (Supplementary Fig. 5). Although altered PRKN expression did not cause METTL3 mRNA changes, it did, however, negatively affected the levels of METTL3 protein in LoVo and HCT-116 cells (Fig. 8E and 8F), indicating that PRKN promotes the degradation of METTL3 protein. In support of this finding, researchers found that the MG132, a well-known suppressor of ubiquitin-proteasome [28], strikingly abated PRKN increase-driven METTL3 downregulation (Fig. 8G). In the presence of cycloheximide (CHX), increased PRKN expression could diminish the stability of METTL3 protein, as evidenced by shorter half-life of METTL3 protein upon transfection of the PRKN plasmid (Fig. 8H and 8I). Taken together, these data demonstrate that PRKN diminishes METTL3 protein stability via ubiquitination.

**The PRKN/METTL3 axis affects malignant progression of CRC cells**

In order to determine the implication of the PRKN in CRC through METTL3, researchers conducted rescue experiments in both CRC cell lines. Co-introduction of a METTL3 plasmid partially abated pc-PRKN-driven METTL3 downregulation in these cells (Fig. 9A). Increase of PRKN partially but greatly retarded the growth of LoVo and HCT-116 cells, while METTL3 re-expression partially abrogated this repression (Fig.

9B and 9C). Increase of PRKN also partially induced cell apoptosis and retarded cell invasive ability and motility; these impacts were reversed by METTL3 re-expression (Fig. 9D-9G). Furthermore, PRKN increase partially impeded M2 polarization of THP-1-derived macrophages, and re-expression of METTL3 markedly counteracted pc-PRKN-driven the suppression (Fig. 9H-9K). These findings further indicate the involvement of PRKN in CRC through METTL3.

**Discussion**

In this report, our data demonstrate METTL3's upregulation and pro-tumorigenic role in CRC, in line with previous work [11, 12, 29]. Through epigenetic modifications, this study unveils the critical modulation of the PRKN/METTL3/CLDN2 cascade in CRC development. Our observations reinforce the potential of METTL3 to prevent or treat CRC [9].

Our results indicate the upregulation of METTL3 in CRC, in agreement with recent reports [10, 30], suggesting its diagnostic potential. Functional depletion studies have demonstrated that METTL3 knockdown suppresses CRC cell growth, invasion, and motility, supporting its pro-tumorigenic role [10]. The M2 polarization of tumor-associated macrophages (TAMs) has been increasingly recognized as a key driver of CRC progression and metastasis [22, 31]. While emerging evidence has established METTL3 as a critical regulator of M2 macrophage polarization in CRC, the underlying mechanisms appear context-dependent and, in some cases, contradictory. For instance, METTL3 has been shown to promote pulmonary metastasis by modifying SNAIL mRNA, thereby enhancing CXCL2 secretion and subsequent recruitment of M2-type immunosuppressive macrophages [13]. Similarly, M2-polarized TAMs induce oxaliplatin resistance through upregulation of METTL3-mediated m6A modification [32]. In contrast, another study reported that METTL3 suppresses M2 polarization by inhibiting SNAIL protein translation in an m6A-IGF2BP3-dependent manner, thereby attenuating tumor growth [33]. Distinct from prior works that primarily characterized METTL3's cell-autonomous effects or its unidirectional influence on immune recruitment, our study uniquely elucidates the bidirectional crosstalk within the

immune microenvironment. We provide novel evidence that METTL3 not only dictates the functional polarization of TAMs but also serves as a critical molecular bridge linking tumor-intrinsic m6A modifications to the broader immunosuppressive landscape, thereby offering a more integrated perspective on how METTL3 orchestrates CRC progression through dynamic tumor-immune interactions. In addition, the conditioned medium of METTL3-depleted THP-1 macrophages (sh-METTL3-THP-1-CM) significantly repressed the invasion of CRC cells (Supplementary Fig. 6), suggesting a reciprocal influence whereby METTL3 depletion in macrophages alters the tumor microenvironment to restrain cancer progression.

Deregulation of CLDN2 has been unveiled to possess a causative function in gastrointestinal malignancies [34]. In CRC, CLDN2 functions as a pro-tumorigenic factor through enhancement of cancer cell metastasis and growth by diminishing the transcription of NDRG1 [14]. Interestingly, analysis of the GSE130012 dataset to identify METTL3-interacting proteins revealed CLDN2 as a potential downstream effector. Our results of CLDN2 expression in clinical CRC tumors are in agreement with the previous study where CLDN2 was presented at high levels in CRC samples [14]. Conversely, the underexpression of CLDN2 is found in breast cancer and osteosarcoma [35, 36]. These data suggest the potential dual role of CLDN2 in tumorigenesis by operating as a pro-tumorigenic driver or an anti-cancer factor. Our shRNA-knockdown experiments broaden the pro-tumorigenic activity of CLDN2 in CRC by examining its impact on M2 macrophage polarization. Our study establishes a previously unrecognized tumor-intrinsic mechanism wherein METTL3 drives colorectal carcinogenesis by enhancing the m6A methylation and stability of CLDN2 mRNA. Notably, while CLDN2 contributes to METTL3's oncogenic function, incomplete rescue effects suggest additional downstream targets warrant further exploration. In the context of the immune microenvironment, our findings offer a distinct contribution. While recent research has linked METTL3 to immune modulation—such as its role in promoting M2 macrophage polarization and CRC progression via m6A methylation of CDKN2B mRNA in an IGF2BP3-dependent manner [37], the present study uncovers a complementary axis centered on CLDN2.

Unlike pathways directly involved in immune cell recruitment or polarization, our work highlights how METTL3-mediated regulation of tight junction components like CLDN2 may influence tumor immunity indirectly by modulating epithelial integrity and barrier function. This offers a new perspective on how METTL3 shapes the tumor immune microenvironment, extending beyond classical immune-related targets and underscoring the multifaceted nature of METTL3-driven CRC pathogenesis.

PRKN (Parkin) is a crucial E3 ubiquitin ligase implicated in the regulation of protein degradation and mitochondrial quality control and has emerged as a significant player in human carcinogenesis [38, 39]. In CRC, aberrant expression of PRKN is linked to this disease pathogenesis [17]. Moreover, PRKN has been identified as a strong anti-cancer factor in CRC by mediating the ubiquitination of IQGAP3 [16]. Building on this established role, our study unveils a previously unknown facet of PRKN's tumor-suppressive function: the direct regulation of METTL3 protein stability via ubiquitination. While existing research on its interaction with IQGAP3 ubiquitination [16] has largely focused on PRKN-mediated degradation of classical signaling or structural proteins, we here demonstrate that PRKN also governs the epigenetic machinery in CRC. While our data demonstrate that METTL3 is a functionally significant downstream target mediating the tumor-suppressive effects of PRKN, the partial rescue of phenotypes suggests that PRKN likely operates through additional substrates, positioning METTL3 within a broader network of effectors rather than as the sole linear mediator. Although K63-linked polyubiquitination is traditionally associated with non-degradative signaling, emerging evidence suggests it can also serve as a distinct signal for selective autophagy or lysosomal degradation [40, 41], a mechanism frequently employed by PRKN in mitochondrial quality control. Our findings imply that PRKN destabilizes METTL3 not through the canonical K48-proteasome axis, but potentially by tagging it with K63 chains that recruit autophagic machinery, thereby offering a novel layer of post-translational regulation in CRC.

However, several limitations should be acknowledged. First, the *in vivo* experiments in this study were conducted using subcutaneous xenografts and node mouse models in nude mouse models. This approach, while informative, does not fully

recapitulate the complex immune microenvironment and spontaneous metastatic cascade of native human tumors. More importantly, due to the immunodeficient nature of nude mice, the role of macrophage polarization and its regulatory effects within a functional immune system could not be comprehensively evaluated. Future studies employing genetically engineered mouse models, patient-derived organoids, or immunocompetent and humanized mouse models will be essential to validate these observations within a more physiologically relevant context and to better understand the interplay between macrophages and the broader immune system. Second, while the present study establishes a post-transcriptional mechanism for CLDN2 regulation, the potential contribution of indirect effects, such as modulation at the transcriptional level, has not been conclusively excluded and warrants future investigation. Third, the precise molecular mechanisms by which CLDN2, a tight junction protein, orchestrates intracellular signaling to influence M2 macrophage polarization remain incompletely defined. Given its established role in maintaining epithelial barrier integrity, we hypothesize that CLDN2 may indirectly modulate macrophage polarization through several non-exclusive mechanisms: (1) by regulating paracellular permeability, thereby controlling the passage of microbial products or tumor-derived metabolites that educate tumor-associated macrophages; (2) by influencing the secretion of cytokines or chemokines from CRC cells through tight junction-associated signaling pathways; or (3) by directly participating in signal transduction complexes that modulate gene expression programs linked to the tumor immune microenvironment. Elucidating these possibilities—including the identification of potential binding partners or downstream effectors that mediate this non-canonical function—represents an important focus of our future investigations. Fourthly, although our preliminary data showed the inhibitory effect of PRKN overexpression on xenograft growth (Supplementary Fig. 7), a definitive investigation into the *in vivo* role of the PRKN/METTL3 axis in CRC tumorigenesis will be the focus of future studies. Additionally, it should be noted that the conclusion regarding macrophage modulation primarily relies on conditioned medium experiments. While our data indicate a clear phenotypic change, this approach does not fully rule out potential confounding factors such as differential cell growth or

variations in secretome concentration. Furthermore, the specific secretory factors downstream of the METTL3-CLDN2 axis responsible for this effect remain to be precisely identified through comprehensive proteomic profiling of the secretome. While our study delineates the critical function of the PRKN/METTL3/CLDN2 axis in preclinical models, its clinical translational value has yet to be systematically established. To enhance the clinical relevance of our findings, future investigations should focus on evaluating the potential of this molecular axis as a combined biomarker or therapeutic target. Specifically, it will be important to correlate the expression levels of PRKN, METTL3, and CLDN2 with detailed clinicopathological data in large, well-characterized cohorts of CRC patients. Analyses should examine whether the combined expression signature of this axis is associated with disease stage, metastatic potential, or patient survival outcomes. Furthermore, assessing its correlation with response to conventional therapies (e.g., 5-fluorouracil, oxaliplatin) or targeted agents could reveal its utility as a predictive biomarker. Such studies would provide critical insights into the translational potential of targeting or monitoring this axis in CRC management. Finally, it should be noted that our assessment of METTL3's impact on M2 macrophage polarization was performed using CM from CRC cells, which may not fully recapitulate the complex intercellular dynamics within the tumor microenvironment. This experimental approach cannot entirely exclude the possibility that the observed effects on macrophage polarization could be indirectly influenced by alterations in CRC cell proliferation or by quantitative changes in the secretome resulting from METTL3 manipulation. Future studies employing co-culture systems or *in vivo* models with lineage-specific tracing would provide more definitive evidence regarding the direct paracrine effects of METTL3-modified CRC cells on tumor-associated macrophage polarization.

Notably, several pharmacological inhibitors targeting the METTL3 methyltransferase activity are currently under preclinical investigation. These compounds (e.g., STM2457) show promising anti-tumor efficacy *in vitro* and *in vivo*, suggesting that pharmacological suppression of METTL3 could represent a viable strategy for a subset of CRC patients [42]. Conversely, strategies to activate PRKN are

also being explored, albeit with greater developmental challenges. While small-molecule PRKN activators remain in early stages, their potential to restore cellular homeostasis and suppress tumor growth offers a compelling therapeutic avenue. However, the translational application of both METTL3 inhibitors and PRKN activators faces considerable hurdles. A primary concern is the achievement of sufficient selectivity to avoid off-target effects, given the ubiquitous role of m6A modification in normal physiology and the pleiotropic functions of PRKN. Furthermore, potential toxicity profiles, particularly arising from chronic modulation of these fundamental pathways, need to be thoroughly evaluated in future studies.

In summary, our study elucidates a novel mechanism for METTL3's pro-tumorigenic role in CRC. Our findings identify PRKN as a regulator of METTL3 through a ubiquitination mechanism and CLDN2 as a downstream effector of METTL3 depending on METTL3-mediated m6A modification. The PRKN/METTL3/CLDN2 epigenetic cascade underlies CRC development (Fig. Graphical Abstract). Our study suggests that METTL3 inhibition might be an attractive strategy to prevent or treat CRC.

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