Ribosome 18S m^6A methyltransferase METTL5 promotes pancreatic cancer progression by modulating c-Myc translation

HUA HUANG[1], HUAN LI[1], RUINING PAN[1], SIJIA WANG[1], AAMIR ALI KHAN[1], YUE ZHAO[2], HUIYU ZHU[3] and XINHUI LIU[1]

[1]Center of Excellence for Environmental Safety and Biological Effects, Beijing International Science and Technology Cooperation Base for Antiviral Drugs, Faculty of Environment and Life, Beijing University of Technology, Beijing 100124; [2]Intensive Care Unit, Beijing Tsinghua Changgung Hospital, Beijing 102218; [3]School of Pharmacy, Henan University of Chinese Medicine, Zhengzhou, Henan 450046, P.R. China

Received October 11, 2021; Accepted December 14, 2021

DOI: 10.3892/ijo.2021.5299

Abstract. Methyltransferase N6-adenosine (METTL5) is a methyltransferase that specifically catalyzes 18S rRNA N6 methylation at adenosine 1832 (m^6A_{1832}), which is located in a critical position in the decoding center, therefore suggesting its potential importance in the regulation of translation. However, the underlying mechanism of METTL5-mediated translation regulation of specific genes and its biological functions are largely undefined. To the best of our knowledge, the present study demonstrated for the first time that METTL5 was an oncogene that promoted cell proliferation, migration, invasion and tumorigenesis in pancreatic cancer. In addition, the oncogenic function of METTL5 may involve an increase in c-Myc translation, as evidenced by the fact that the oncogenic effect caused by METTL5 overexpression could be abolished by c-Myc knockdown. Notably, m^6A modifications at the 5' untranslated region (5'UTR) and coding DNA sequence region (near the 5'UTR) of c-Myc mRNA played a critical role in the specific translation regulation by METTL5. In addition, it was further demonstrated that METTL5 and its cofactor tRNA methyltransferase activator subunit 11-2 synergistically promote pancreatic cancer progression. These findings revealed important roles for METTL5 in the development of pancreatic cancer and present the METTL5/c-Myc axis as a novel therapeutic strategy for treatment.

Correspondence to: Dr Hua Huang or Dr Xinhui Liu, Center of Excellence for Environmental Safety and Biological Effects, Beijing International Science and Technology Cooperation Base for Antiviral Drugs, Faculty of Environment and Life, Beijing University of Technology, 100 Ping Le Yuan, Chaoyang, Beijing 100124, P.R. China
E-mail: huangh@bjut.edu.cn
E-mail: liuxinhui@bjut.edu.cn

Key words: pancreatic cancer, methyltransferase N6-adenosine, N6 methyladenosine, c-Myc, tRNA methyltransferase activator subunit 11-2

Introduction

RNA modifications have emerged as a crucial process in post-transcriptional gene regulation and are essential for RNA biogenesis and functions. As the most prevalent chemical modification of eukaryotic RNA, N6 methyladenosine (m^6A) modification of mRNA exerts a notable influence on mRNA stability, splicing, localization, transport and translation efficiency (1-4). m^6A modifications also occur on non-coding RNA, including ribosomal, transfer and small nuclear RNA (5). Ribosomal RNA (rRNA) is highly abundant in the total RNA of cells, and rRNA modifications play an important role in regulating ribosome structure and function (6). In humans, the m^6A modifications present in rRNA are located at two specific sites: 1832 on 18S rRNA (m^6A_{1832}) and 4220 on 28S rRNA (m^6A_{4220}) (7). The 28S rRNA m^6A_{4220} is catalyzed by rRNA N6-adenosine-methyltransferase ZCCHC4, which is essential for translation in ribosomes, cell proliferation and tumorigenesis (8-10).

Mammalian rRNA N6-adenosine-methyltransferase METTL5 (METTL5), a member of the conserved methyltransferase-like protein (METTL) family, was recently identified to methylate A1832 in 18S rRNA (11). The 18S rRNA m^6A_{1832} modification is located in a critical position in the decoding center, therefore suggesting its potential importance in translation regulation (10). Indeed, a recent study indicated that METTL5 promoted translation initiation, and the METTL5-mediated m^6A_{1832} modification may encourage the decoding center to interact with mRNA undergoing active translation (11). However, knowledge concerning the biological functions of METTL5 is limited at present. Several studies have revealed that METTL5 has an essential role in the pluripotency and differentiation of mouse embryonic stem cells, brain development and neural function in multiple species (12-15). However, the other biological functions of METTL5 are largely undefined, particularly in cancer. Therefore, in the present study, the potential oncogenic activity and the underlying mechanisms of METTL5 in pancreatic cancer were evaluated.

c-Myc, which is located at chromosome 8q24, is one of the three transcriptional activators of the Myc family

(c-, N- and L-Myc). c-Myc lies at a critical stage of a number of growth-promoting signal transduction pathways, including ERK, PI3K, AKT, MAPK and Wnt (16), and therefore is involved in a variety of biological activities, including ribosomal and mitochondrial biosynthesis, the cell cycle, cell survival, proliferation, apoptosis, cell competition, metabolic reprogramming and tumor progression (17-20). Aberrant expression of c-Myc has been reported to be involved in 70% of human cancers, including lung, colon, breast, prostate, colorectal and bladder cancer (20,21). Elevated expression of c-Myc has also been identified to be a key driver of pancreatic ductal adenocarcinoma (PDAC) through modulating multiple oncogenic pathways, such as enhancing the epithelial-mesenchymal transition and TGF-β signaling pathway (22). A variety of factors lead to the aberrant activation of c-Myc in cancer (23). The transcription of c-Myc is mediated by transcription factors, such as NF-κB and nuclear factor of activated T cells cytoplasmic 1, via the regulation of its promoter activity. Meanwhile, the aberrant downregulation of microRNAs (miRNAs/miRs), including Let7a, miR-145, miR-34a, miR-375, miR-494 and miR-148a, has also been reported to lead to the overactivation of c-Myc and further drive the progression of PDAC (24). In addition, post-translational modifications, such as acetylation and phosphorylation, have been shown to enhance the protein stability of c-Myc (25). Recent studies showed that the mRNA m^6A methyltransferase METTL3 enhanced c-Myc expression by increasing mRNA m^6A levels in prostate and lung cancer (19,26), suggesting that c-Myc mRNA modification plays a key role in c-Myc-centered oncogenic pathway regulation. Accordingly, intervention of c-Myc expression through epigenetic pathways may provide a potential therapeutic strategy for pancreatic cancer.

In the present study, the potential oncogenic activity and the underlying mechanisms of METTL5 in pancreatic cancer were investigated. It was demonstrated that METTL5 enhanced c-Myc translation and the mechanism via which METTL5 could promote specific selective translation was explored. It was hypothesized that the METTL5-mediated upregulation of 18S rRNA m^6A_{1832} modification affected ribosome structure, altering the affinity of ribosomes to mRNAs with specific structural features or modifications. In addition, it was verified that METTL5 and multifunctional methyltransferase subunit TRMT112-like protein (TRMT112) may function together in pancreatic cancer. Collectively, it may be proposed that the METTL5-mediated increase in c-Myc translation facilitates tumorigenesis and could represent a novel therapeutic strategy.

Materials and methods

Cell culture. Human pancreatic cancer cell lines, including PANC-1 (cat. no. CRL-1469), ASPC-1 (cat. no. CRL-1682) and BXPC-3 (cat. no. CRL-1687), and the human pancreatic ductal epithelial cell line HPDE6-C7 (cat. no. CVCL_0P38) were obtained from the American Type Culture Collection. The cells were cultured in DMEM (cat. no. 11995040; Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (cat. no. 10099141; Gibco; Thermo Fisher Scientific, Inc.), 100 ng/ml streptomycin reagent (HyClone; Cytiva) and 100 U/ml penicillin (HyClone; Cytiva). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37˚C.

Generation of stable cell lines. To generate stable cell lines transfected with METTL5-overexpression vector (OE), $METTL5^{mut}$-OE, TRMT112-OE, short hairpin RNA (shRNA/sh) METTL5, shTRMT112, METTL5-OE + TRMT112OE, METTL5-OE + shTRMT112 and TRMT112-OE + shMETTL5, the coding DNA sequences (CDSs) of METTL5 and TRMT112 were subcloned into the pCDH-EF1α-MCS-T2A-Puro lentivirus vector (cat. no. CD527A-1; System Biosciences, LLC) and the shRNA sequences of METTL5 and TRMT112 were cloned into the pLVX-shRNA2-BSD lentivirus vector (in which the ZsGreen gene sequence is replaced with a BSD resistance gene sequence) (cat. no. 632179; Takara Bio USA, Inc.). For the $METTL5^{mut}$ CDS, four amino acids (NPPF) from 126 to 129 were removed compared with the wild-type CDS. 293T cells were transfected with second-generation lentivirus packing system, consisting of the aforementioned purified 0.5 μg/μl lentiviral plasmids, 0.5 μg/μl pMD2.0, 0.5 μg/μl pspAX2 (the pMD2.0: pspAX2: lentivirus ratio was 1:3:4) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) for 72 h at 37˚C. The supernatant containing infectious lentivirus was then collected and transduced to PANC-1 cells for 48 h, at a multiplicity of infection of 10. Positive overexpression and knockdown cells were selected by treatment with 2 μg/ml puromycin or 5 μg/ml blasticidin S for 10 days. shMETTL5-3 and shTRMT112-2 were chosen for subsequent experiments due to their higher efficiency. The shRNA sequences for target gene knockdown are shown in Table SI.

Bioinformatics analysis. The genes of interest, which were differentially expressed in PDAC cells, were investigated in comparison with adjacent normal non-neoplastic tissues using TCGA (http://gepia.cancer-pku.cn/; http://tcga-data.nci.nih.gov/tcga/). Overall survival, METTL5 and TRMT112 expression analysis and correlation analysis were examined using GEPIA (http://gepia.cancer-pku.cn/detail.php). The m^6A modification sites of c-Myc were predicted using the website (https://www.cuilab.cn/sramp).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from PANC-1 cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RT was performed using the HiScript III 1st Strand cDNA Synthesis kit (Vazyme Biotech Co., Ltd.) according to the manufacturer's protocol. qPCR was performed using ChamQ SYBR Color qPCR Master mix (Vazyme Biotech Co., Ltd.) and run on the ABI Prism 7900 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling protocol included an initial denaturation step at 95˚C for 3 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 15 sec and extension at 72˚C for 30 sec. The $2^{-\Delta\Delta Cq}$ values were calculated to analyze the relative changes in gene expression according to the previous reported method (27). β-actin was used as an internal control. Primer sequences are shown in Table SII.

Western blotting. Proteins were extracted using cell lysis buffer RIPA (Beyotime Institute of Biotechnology) and a protease and phosphatase inhibitor cocktail (Beyotime Institute of Biotechnology). A total of 20 μg protein/per lane was separated

via 4-20% SDS-PAGE gels. The separated proteins were subsequently transferred onto a membrane and blocked with 5% skim milk (Difco; BD Biosciences) at room temperature for 2 h. The membranes were incubated with appropriate primary antibodies at 4˚C overnight. Following primary incubation, membranes were incubated with secondary antibodies for 2 h at room temperature (Table SIII). Protein bands were visualized using a BeyoECL Moon Supersensitivity Detection kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol.

Ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) analysis of mononucleotides. A total of 1 μg purified 18S RNA was mixed with 0.1 unit Nuclease P1 (FUJIFILM Wako Pure Chemical Corporation) and 1.0 unit calf intestinal phosphatase (New England BioLabs, Inc.), in a 30-μl reaction volume adjusted with water, and incubated at 37˚C for 8 h. The digested RNA solutions were filtered using ultra-filtration tubes (MW cutoff, 3 KDa; Pall Life Sciences), then 2 μl solution were injected into the UPLC-MS/MS system for detection of m^6A and A. The UPLC-MS/MS analysis was performed with an Agilent 1290 UHPLC system coupled with a G6460 triple quadrupole mass spectrometer (Agilent Technologies, Inc.). IF2 C18 column (100x2.1 mm I.D., 2.0 μm particle size; Shiseido Co., Ltd.) was used for mononucleotide separation. A linear gradient elution procedure was used for UPLC separation, elution program settings were as follows: 0-1.5 min, 5.0% B; 1.6-10 min, the proportion of B increased from 5 to 25%; 10.1-15.0 min, 100% B; 15.1-20 min, 5.0% B. Solvent A was an aqueous solution of 0.1% formic acid, and solvent B was 100% methanol. The electrospray ionization ion source was used for nucleotide ionization and positive ion mode was selected for charged ion detection. A multiple reaction monitoring mode was adopted: m/z 282.2-150.1 for m^6A (collision energy, 20 eV) and m/z 268.2-136.1 for A (10 eV). The nebulization nitrogen was set at 40 psi with source temperature set at 300˚C and the flow-rate of desolvation nitrogen was 9 l/min. Capillary voltage was set at 3,500 V. High purity nitrogen (99.999%) was used as collision gas.

Site-specific m^6A modification. A CRISPR-RfxCas13d-based fusion system was established by fusion of RNA demethylase ALKBH5 (ALKBH5), an m^6A demethylase, and $ALKBH5^{mut}$ (H204A) to the dCas13d protein (replaced the EGFP coding sequence of pHAGE-IRES-puro-NLS-dRfxCas13d-EGFP-NLS-3xFlag plasmid to ALKBH5 gene coding sequence) by using a GSG flexible connection linker (dCas13d-ALKBH5/$ALKBH5^{mut}$). The pHAGE-IRES-puro-NLS-dRfxCas13d-EGFP-NLS-3xFlag was a gift from Ling-Ling Chen (Addgene plasmid no. 132411). In total, four independent guide RNAs (gRNAs) were designed to lead dCas13d-ALKBH5 near the specific m^6A modification sites. The gRNA1 and gRNA2 were designed to lead dCas13d-ALKBH5 near the m^6A modification sites in the 5'untranslated region (UTR) and CDS region, and gRNA3 and gRNA4 were designed to lead dCas13d-ALKBH5 near the m^6A modification near the stop codon. Stably transfected cell lines were established to directly remove the m^6A modifications in the 5'UTR and CDS region (closer to the 5'UTR) of c-Myc mRNA [dCas13d-ALKBH5 + gRNAs (5'CDS)] and the m^6A near the stop codon of c-Myc was removed mRNA [dCas13d-ALKBH5 + gRNAs (SC)] in PANC-1 cells, respectively. The dCas13d-ALKBH5 + gRNAs (scramble) was used as the control. The stable cell lines were established by nucleofection with 4 μg dCas13d-ALKBH5 plasmids and 2 μg dRfxCas13d gRNA cloning backbone (a gift from Patrick Hsu; Addgene plasmid no. 109053) using a Nucleofector 2b Device (Nucleofection system; Lonza Group, Ltd.). After the program was finished, cells were transferred to 6-well plates with 1.5 ml culture medium. After 48 h, cells were harvested for protein extraction and western blot assay. The gRNAs sequences were designed using website: https://cas13design.nygenome.org/. The gRNA sequences for targeting c-Myc m^6A sites are listed in Table SIV.

Cell proliferation assay. The cell proliferative rate was analyzed using Cell Counting Kit-8 (CCK-8) assay. PANC-1 cells were seeded in 96-well plate at a density of $2x10^3$ cells/well, and 10 μl of CCK-8 (Cell Counting Kit-8, Beijing Solarbio Science & Technology Co., Ltd.) reagent was added to each well. Following incubation at 37˚C for 2 h, the optical density was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Colony formation assay. PANC-1 cells were seeded in 6-well plates at a cell density of 300 cells/well and maintained in humidified air containing 5% CO_2 at 37˚C for 10-14 days. The cell seeding density for shMETTL5 and shCtrl groups was 600 cells/well. Cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min and stained with 0.5% crystal violet for 15 min at room temperature. After staining, images were captured and the number of colonies (containing cells >50 cells) was counted manually.

Wound healing assay. A total of $2.5x10^5$ PANC-1 cells were seeded and cultured in a twelve-well plate to create a confluent monolayer. When the confluency reached 70-80%, a horizontal scratch was made using a sterile 200-μl microliter pipette tip. The medium was removed and changed to fresh DMEM supplemented with 2% FBS. Images of the selected areas were obtained at 0 h and subsequently the culture was incubated at 37˚C with 5% CO_2. After 48 h, the selected wound area was imaged using a light microscope (Leica Microsystems, Inc.), and the remaining wound size was measured using ImageJ software (version 1.53e). Wound healing was quantified by calculating the percent of wound closure. The % wound closure=1-(wound surface area at the indicated time-point/initial wound surface area). The assay was performed in triplicate.

Transwell invasion assay. Cell invasion assays were performed in Transwell chambers pre-coated with Matrigel (8-μm Transwell inserts; BD Biosciences). Cells were seeded in the upper chamber at a cell density of $1x10^4$ cells in serum-free DMEM, and the lower chambers were filled with DMEM supplemented with 10% FBS as an attractant. The cells were incubated at 37˚C for 24 h. Then, the cells on the lower surface were fixed in 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet for 15 min at room temperature.

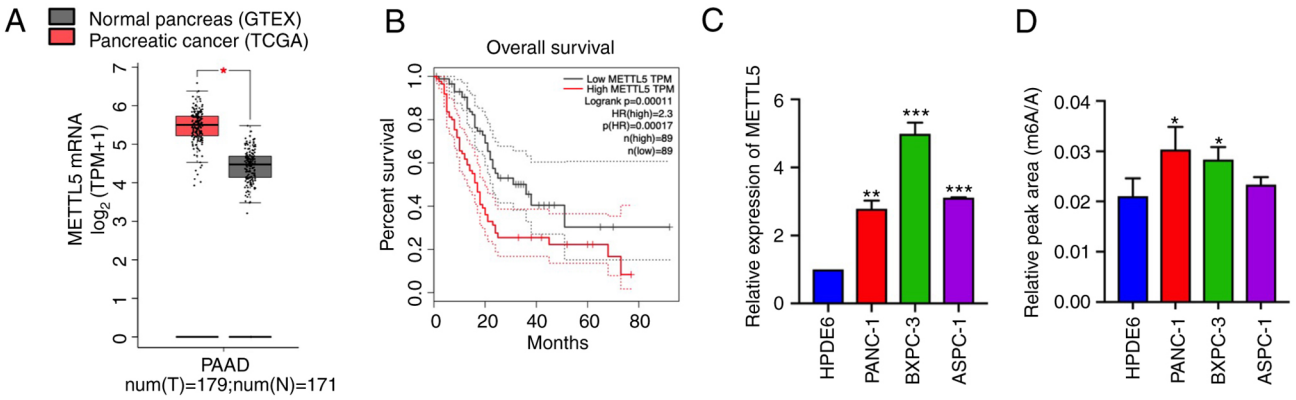

Figure 1. METTL5 is upregulated in human pancreatic cancer. (A) Expression of METTL5 in pancreatic cancer in TCGA. (B) The association between METTL5 expression and overall survival in pancreatic cancer was analyzed using TCGA. (C) The mRNA levels of METTL5 in cells were assessed by reverse transcription-quantitative PCR. (D) The 18S rRNA m^6A level in cells were measured by liquid chromatography tandem mass spectrometry and relative peak area (m^6A/A) was calculated. Data are shown as the mean ± SD (n=3). $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. METTL5, methyltransferase N6-adenosine; GTEX, Genotype-Tissue Expression; TCGA, The Cancer Genome Atlas; m^6A, N6 methyladenosine; PAAD, pancreatic adenocarcinoma.

Stained cells were visualized under a light microscope (Leica Microsystems, Inc.). For each insert, at least three selected fields were selected randomly and counted.

In vivo tumorigenicity assays. A total of 36 Female Balb/c nude mice (6-8 weeks of age; 20-25 g) were purchased from the Charles River Laboratories, Inc. and housed under 12-h light/dark conditions with a standard temperature of 18-23˚C with humidity of 40-60%, and access to food and water *ad libitum*. All animal experiments in the present study were approved (approval no. HS202105004) by the Ethics Committee of Beijing University of Technology (Beijing, China). METTL5-OE and the corresponding control cells ($2x10^6$ cells) in 200 μl PBS were injected subcutaneously into the right side of nude mice. At ~4 weeks after injection, mice were sacrificed after anesthesia with 1% pentobarbital sodium (45 mg/kg, intraperitoneally), then euthanized by cervical dislocation. The tumor weight was measured and the volume was calculated at the end point. The maximum tumor volume allowed by the Ethical Committee of the Beijing University of Technology was 1.5 cm diameter or 300 mm^3 per tumor.

Statistical analysis. The data are presented as the mean ± SD from at least three independent experiments. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc.). Significance levels between two groups were evaluated using a two-tailed paired Student's t-test. The differences between multiple groups were compared using a one-way or two-way ANOVA followed by Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

METTL5 is upregulated in human pancreatic cancer. First, the expression of METTL5 in PDAC was investigated. The Cancer Genome Atlas database showed that METTL5 expression was significantly elevated in pancreatic adenocarcinoma (PAAD) tissues (n=179) compared with adjacent non-tumor tissues (n=171; Fig. 1A). The increased mRNA expression level of METTL5 was confirmed in three PDAC cell lines (PANC-1, BxPC3 and ASPC1) and one pancreatic ductal epithelial cell line (HPDE6-C7; Fig. 1C). Meanwhile, the elevated 18S rRNA m^6A methylation was detected in PDAC cells via UPLC-MS/MS, indicating that increased rRNA methylation may affect tumorigenesis (Figs. 1D and S1A). Furthermore, a high level of METTL5 was associated with a poor overall survival rate (n=89; Fig. 1B). These results indicated that METTL5 was upregulated in pancreatic cancer and may play an important role in PDAC development.

METTL5 overexpression promotes pancreatic cancer growth and invasion. To directly address the biological role of METTL5 in PDAC, stable METTL5 overexpression and knockdown transfected cells were established (Fig. 2A and B), and a positive association between METTL5 and 18S rRNA m^6A level was confirmed in these cells (Figs. 2C and S1B). Cells overexpressing catalytically inactive METTL5 ($METTL5^{mut}$) showed no alteration in 18S rRNA m^6A compared with control cells (Fig. S1B). METTL5 overexpression significantly stimulated proliferation, colony-forming capacity, migration and invasion in PANC-1, ASPC-1 and BXPC-3 cells, whereas knockdown of METTL5 exerted the opposite effects (Figs. 2D-G and S2A-D). METTL5 overexpression enhanced tumor growth in xenotransplantation nude mice, specifically the volume and weight were significantly increased in the METTL5 overexpression group (Fig. 2H). Notably, $METTL5^{mut}$ cells had little effect on PDAC progression, indicating that the oncogenic function of METTL5 in PDAC was dependent on catalytic activity (Fig. 2I and J). Collectively, these results demonstrated that METTL5 promoted cell proliferation, migration, invasion and tumorigenesis in PDAC.

METTL5 overexpression enhances the translation of c-Myc. METTL5-mediated 18S rRNA m^6A modification has been suggested to play an important role in promoting translation (11). It was hypothesized in the present study that the oncogenic function of METTL5 in PDAC may be closely associated with its positive effect on the translation of key

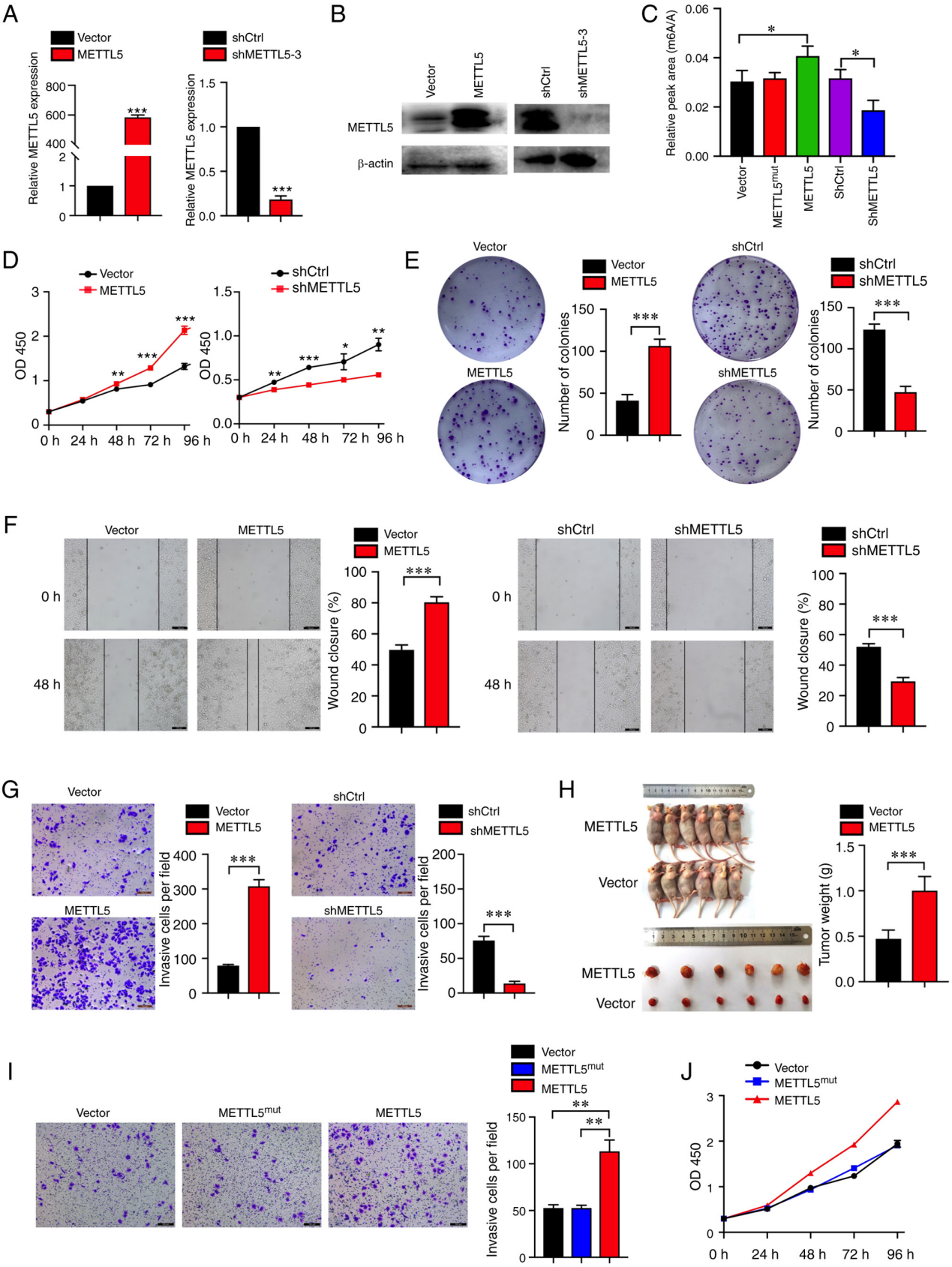

Figure 2. Elevated METTL5 promotes pancreatic cancer growth and invasion. (A) mRNA and (B) protein expression level of METTL5 in PANC-1 cells expressing vector, exogenous METTL5, shCtrl or shMETTL5. (C) The 18S rRNA m^6A level in cells was measured via liquid chromatography tandem mass spectrometry and relative peak area (m^6A/A) was calculated. (D) Proliferation of PANC-1 cells transfected with vector, exogenous METTL5, shCtrl or shMETTL5. (E) Representative images of the colony formation assay in indicated cells. (F) Wound healing and (G) Transwell assays were used to evaluate the migratory and invasive potentials of METTL5 overexpression, knockdown and corresponding control cells (Scale bar, 100 μm). (H) The volume and weight analysis of subcutaneous tumors from the indicated groups. (I) Transwell assays of PANC-1 cells transfected with vector, $METTL5^{mut}$ and METTL5. (J) Proliferation of PANC-1 cells transfected with vector, $METTL5^{mut}$ and METTL5. Data are shown as the mean ± SD (n=3). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. METTL5, methyltransferase N6-adenosine; sh, short hairpin RNA; m^6A, N6 methyladenosine; ctrl, control; mut, mutant.

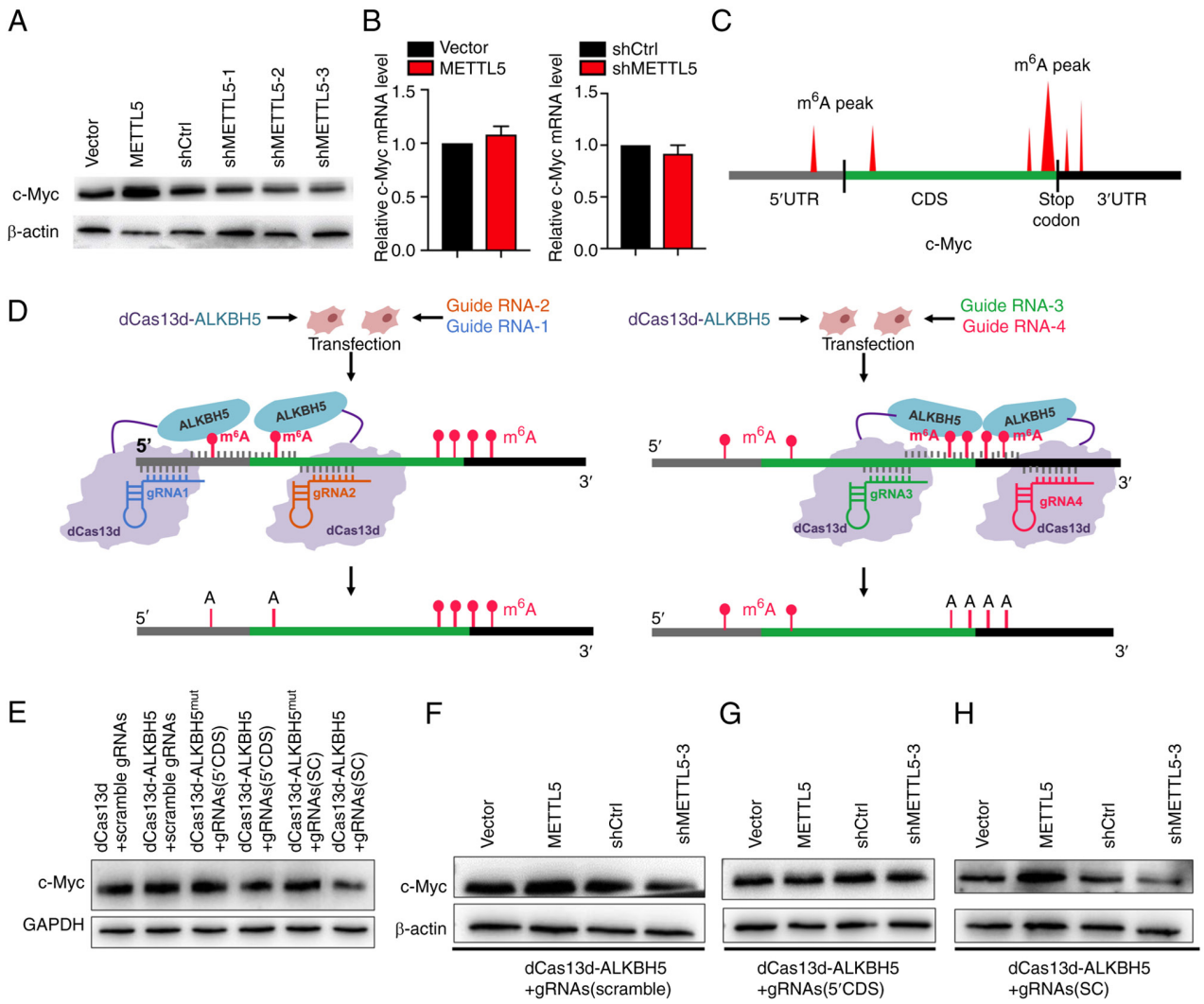

Figure 3. METTL5-induced translation of c-Myc is associated with m^{6}A on c-Myc mRNA. (A) Protein expression level of c-Myc in PANC-1 cells transfected with vector, exogenous METTL5, shCtrl or shMETTL5. (B) The relative mRNA expression levels of c-Myc in PANC-1 cells transfected with vector, exogenous METTL5, shCtrl or shMETTL5. (C) Predicted m^{6}A modification sites on c-Myc mRNA. (D) Schematic representation of the site-specific m^{6}A modification removal. Stably transfected cell lines were established to remove the m^{6}A modifications in the 5'UTR and CDS region (closer to the 5'UTR) of c-Myc mRNA [dCas13d-ALKBH5 + gRNAs (5'CDS)] and the m^{6}A near the stop codon of c-Myc mRNA was removed [dCas13d-ALKBH5 + gRNAs (SC)] in PANC-1 cells, respectively. The gRNA1 and gRNA2 were designed to lead dCas13d-ALKBH5 near the m^{6}A modification sites in the 5'UTR and CDS region (left), and gRNA3 and gRNA4 were designed to lead dCas13d-ALKBH5 near the m^{6}A modification near the stop codon (right). (E) c-Myc protein expression in stably transfected dCas13d-ALKBH5 + gRNAs (5'CDS), dCas13d-ALKBH5 + gRNAs (SC), dCas13d-ALKBH5^{mut} + gRNAs (5'CDS), dCas13d-ALKBH5^{mut} + gRNAs (SC), dCas13d-ALKBH5 + gRNAs (scramble) and dCas13d + gRNAs (scramble) cells. c-Myc protein expression in (F) dCas13d-ALKBH5 + gRNAs (scramble) cells, (G) dCas13d-ALKBH5 + gRNAs (5'CDS) cells and (H) dCas13d-ALKBH5 + gRNAs (SC) cells transfected with vector, exogenous METTL5, shCtrl or shMETTL5. Data are shown as the mean ± SD (n=3). METTL5, methyltransferase N6-adenosine; sh, short hairpin RNA; m^{6}A, N6 methyladenosine; CDS, coding DNA sequence; gRNA, guide RNA; ALKBH5, RNA demethylase ALKBH5; UTR, untranslated region; mut, mutant; SC, stop codon.

target genes. Thus, the changes in protein levels of several crucial oncogenes after METTL5 overexpression or knockdown were investigated to explore the potential candidates. The results indicated that the expression of c-Myc was positively regulated by METTL5 in PANC-1 cells (Fig. 3A). Notably, no significant differences in the mRNA expression level of c-Myc were found between METTL5 overexpression, knockdown and corresponding controls (Fig. 3B). This indicated that the regulation of METTL5 on c-Myc was mainly at the translation stage. Elevated c-Myc has been identified to be a key driver of PDAC (22). Therefore, it was hypothesized that the oncogenic effect of METTL5 in PDAC may be associated with the regulation of c-Myc translation. Thus, the functional association between METTL5 and c-Myc was further examined in PDAC, and it was demonstrated that the oncogenic effects of METTL5 overexpression could be abolished by c-Myc knockdown (Fig. S2E and F). These data supported the hypothesis that METTL5 promoted pancreatic cancer progression by enhancing the translation efficiency of c-Myc mRNA. Additionally, this supported the notion that the m^{6}A modification plays an important role in mRNA translation.

However, according to the present data, not all mRNA translations were regulated by METTL5 (Fig. S3). Previous studies have also mentioned that METTL5-mediated 18S rRNA m^{6}A may fine-tune the translation of a particular subset of mRNA (14,28), instead of influencing the basal translation activity of ribosomes. Therefore, the underlying mechanism

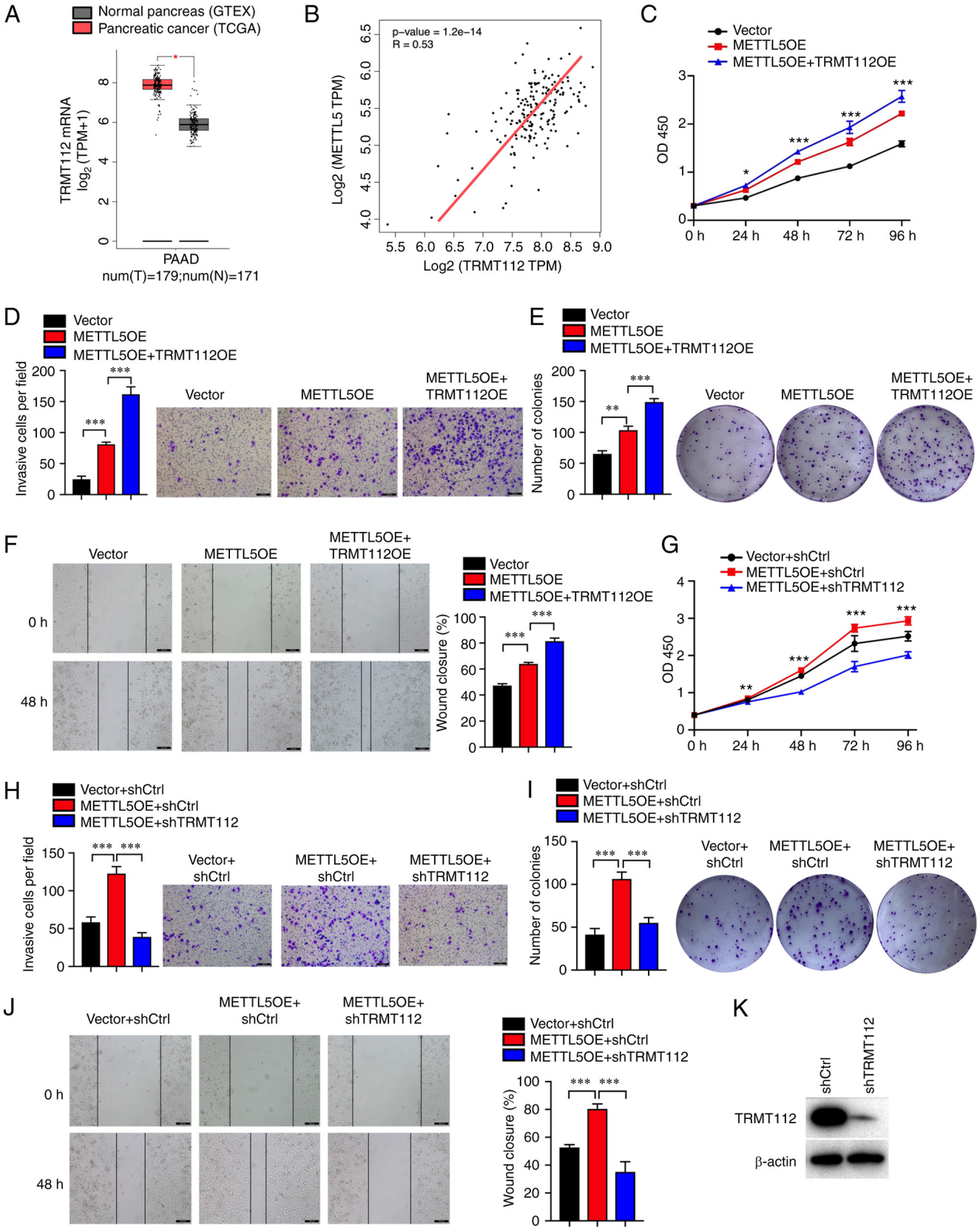

Figure 4. METTL5 and TRMT112 may function together in pancreatic cancer. (A) Expression of TRMT112 in pancreatic cancer according to TCGA database. (B) Analysis of the possible association between TRMT112 and METTL5 mRNA expression in pancreatic cancer tissues according to TCGA database. The combined overexpression of TRMT112 and METTL5 further promoted (C) cell proliferation, (E) colony formation capacity, and (F) migratory and (D) invasive abilities compared with METTL5 overexpression alone in PANC-1 cells. TRMT112 knockdown abolished the effect METTL5 overexpression on (G) proliferation, (I) colony formation capacity, (J) migration and (H) invasion of PANC-1 cells. (K) Protein level of TRMT112 in PANC-1 cells expressing shCtrl and shTRMT112. Data are shown as the mean ± SD (n=3). *P<0.05, **P<0.01 and ***P<0.001. METTL5, methyltransferase N6-adenosine; TRMT112, multifunctional methyltransferase subunit TRMT112-like protein; TCGA, The Cancer Genome Atlas; GTEX, Genotype-Tissue Expression; PAAD, pancreatic adenocarcinoma; ctrl, control; OE, overexpression; TPM, transcripts per million.

via which METTL5 regulates the translation of specific genes was further explored.

METTL5-mediated translation of c-Myc is associated with m^{6}A on c-Myc mRNA. m^{6}A is the most abundant RNA modification located on mRNA with a clear positioning bias (1,4). Thus, it was speculated whether METTL5-mediated translation regulation of specific mRNAs was related to m^{6}A modifications of these mRNAs, including the abundance and distribution characteristics. A previous study reported that m^{6}A peaks of most genes were predominantly localized near stop codons (29). EGFR, tafazzin, MAP kinase-activated protein kinase 2 and DNA (cytosine-5)-methyltransferase 3A have all been found to have one or more m^{6}A peaks near the stop codon, whereas c-Myc has more broadly distributed m^{6}A across multiple exons (29). Therefore, it was hypothesized in the present study that the m^{6}A modifications of c-Myc, in addition to the modification near the stop codon, may be associated with its specific regulation by METTL5. Accordingly, the predicted m^{6}A modification sites of c-Myc were investigated using bioinformatics. In addition to the four m^{6}A modification sites near the terminal region of the CDS, c-Myc was also found to have two distinct m^{6}A modification sites near the translation initiation site, one in the 5'UTR and another in the CDS region near the 5'UTR (Fig. 3C).

To gain insight into the involvement of m^{6}A modifications on c-Myc mRNA in METTL5-mediated c-Myc translation regulation, stably transfected cell lines were established by applying a CRISPR-dRfxCas13d-based fusion system to fuse dCas13d with an m^{6}A demethylase ALKBH5 (dCas13d-ALKBH5) (30-32), the m^{6}A modifications in the 5'UTR and CDS region (close to the 5'UTR) of c-Myc mRNA (named 5'CDS) were directly removed and the m^{6}A near the stop codon of c-Myc mRNA (named SC) was removed in PANC-1 cells, respectively, in the presence of a specific gRNA (Fig. 3D). Cells overexpressing dCas13d fused with a catalytically inactive ALKBH5 (dCas13d-ALKBH5^{mut}) were used as a negative control. c-Myc expression was decreased in both dCas13d-ALKBH5 + gRNAs (5'CDS) and dCas13d-ALKBH5 + gRNAs (SC) cells compared with control cells, indicating that the removal of m^{6}A on mRNA at these two regions affected the expression level of c-Myc (Fig. 3E), and also demonstrated that this system was successful in deleting the m^{6}A at target sites of c-Myc mRNA.

Subsequently, METTL5 was overexpressed or knocked down in dCas13d-ALKBH5 + gRNAs (5'CDS), dCas13d-ALKBH5 + gRNAs (SC) and dCas13d-ALKBH5 + gRNAs (scramble) cells, and the c-Myc protein levels were compared to evaluate the effects of these m^{6}A alterations on the METTL5-mediated c-Myc translation regulation. METTL5 overexpression and knockdown without m^{6}A intervention significantly mediated c-Myc translation levels (Fig. 3F). METTL5 overexpression and knockdown with the removal of m^{6}A near the translation initiation site failed to maintain translation increase and inhibition, indicating that METTL5-mediated regulation of c-Myc translation was greatly weakened by the loss of m^{6}A deposition at the 5'UTR and CDS region (near the 5'UTR) of c-Myc mRNA (Fig. 3G). On the other hand, METTL5-mediated c-Myc translation alteration was only slightly affected by the removal of the four m^{6}A modifications near the stop codon (Fig. 3H). It was suspected that the presence of m^{6}A on 18S rRNA caused subtle changes in the structure of rRNA that affect the translation efficiency of specific mRNAs, while the distribution characteristics of m^{6}A on mRNAs determined the significance of the influence. Collectively, the m^{6}A modifications of c-Myc mRNA played a critical role in specific selective translation increase of c-Myc by METTL5 in PANC-1 cells.

METTL5 and TRMT112 may function together in pancreatic cancer. TRMT112 acts as a cofactor for multiple methyltransferases and is involved in the modification of tRNA, rRNA and various proteins (33). METTL5 has been shown to form a METTL5-TRMT112 m^{6}A rRNA methyltransferase complex to gain metabolic stability in cells (10). In addition, as a coactivator of METTL5, TRMT112 is essential for the enzymatic activity of METTL5, and may synergistically catalyze 18S rRNA m^{6}A methylation (28). In summary, TRMT112 plays an important role in stabilizing METTL5 and METTL5 activity. Compared with normal tissues, the expression of TRMT112 in PAAD tissue was also significantly upregulated (Fig. 4A), and associated with the expression pattern of METTL5 in PDAC (Fig. 4B). Therefore, it was hypothesized that the function of METTL5 in PDAC may also be associated with the involvement of its coactivator TRMT112.

To further investigate the underlying mechanisms of METTL5 and TRMT112, their abilities to influence cell proliferation and invasion were compared by using established stable cell lines transfected with different combinations of vectors (METTL5-OE, TRMT112-OE, shMETTL5, shTRMT112 and relative controls). Transfection of PANC-1-shTRMT112 cells was confirmed by analyzing the relative protein level of TRMT112 (Fig. 4K). As expected, the oncogenic function of METTL5 in PDAC was significantly enhanced when TRMT112 was overexpressed simultaneously (Fig. 4C-F), and exchange with each other led to similar trends (Fig. S4). In addition, the oncogenic effects of METTL5 could be abolished by TRMT112 knockdown (Fig. 4G-J), and the oncogenic effects of TRMT112 could also be abolished by METTL5 knockdown (Fig. S5). These results suggested that METTL5 and TRMT112 may function together in pancreatic cancer.

Discussion

rRNA is highly abundant in the total RNA of cells, and rRNA modifications play an important role in regulating ribosome structure and functions (7). METTL5 is a methyltransferase that specifically catalyzes 18S rRNA m^{6}A$_{1832}$, which is located in a critical position in the decoding center, and therefore suggests its potential importance in translation regulation (10). However, the underlying mechanism via which METTL5-mediated translation regulation of specific genes, and its biological functions are largely undefined. To the best of our knowledge, the present study showed for the first time that METTL5 functioned as an oncogene, significantly promoting cell proliferation, migration, invasion and tumorigenesis in pancreatic cancer. The oncogenic function of METTL5 may involve an increase in c-Myc translation, while the oncogenic effect of METTL5 overexpression could be abolished by c-Myc knockdown. Mechanistically, it was suggested that

m^6A modifications at the 5'UTR and CDS region (near the 5'UTR) of c-Myc mRNA played a critical role in the specific selective translation regulation of c-Myc by METTL5. In addition, it was further validated that METTL5 and its cofactor TRMT112 may function together in pancreatic cancer. Thus, the METTL5/c-Myc pathway in PDAC was proposed, which may represent a potential therapeutic strategy for treatment.

The 18S rRNA m^6A_{1832} modification is located in a critical position in the decoding center, which indicates its importance in mRNA translation processes. A recent study indicated that METTL5 promotes translation initiation, and the METTL5-mediated m^6A_{1832} modification may encourage the decoding center to interact with mRNA undergoing active translation (11). In the present study, it was verified that METTL5 enhanced the translation of c-Myc, which supported the speculation that METTL5-mediated m^6A modification on rRNA plays an important role in translation regulation. However, the present study and previous study observed that not all mRNA translations were regulated by METTL5 (28). The mechanism via which METTL5 regulates the translation of a particular subset of mRNA is not clear. It was suggested in the current study that the METTL5-mediated upregulation of 18S rRNA m^6A_{1832} modification affected ribosome structure, altering the affinity of ribosomes to mRNAs with specific structural features or modifications. Thus, site-specific m^6A modification was directly removed on c-Myc to test whether METTL5-mediated translation regulation of c-Myc was related to m^6A modifications of c-Myc mRNAs, including the abundance and distribution characteristics. The results showed that METTL5-mediated c-Myc translation was associated with the m^6A modifications on c-Myc mRNA, in which m^6A modifications at the 5'UTR and CDS region (near the 5'UTR) of c-Myc mRNA played a critical role in the specific translation regulation by METTL5. Meanwhile, METTL5-mediated c-Myc translation alteration was only slightly affected by the removal of the four m^6A modifications near the stop codon. It was indicated that the METTL5-mediated upregulation of 18S rRNA m^6A_{1832} modification affected ribosome structure, altering the affinity of ribosomes to the mRNAs with specific structural features conferred by the m^6A modifications. In this case, the closer the m^6A modification site to the initial region of the translation on the mRNA, the more significant the impact will be. This may be the reason that METTL5-mediated c-Myc translation alteration was still significant after the removal of the four m^6A modifications near the stop codon. In conclusion, it was suggested that the presence of m^6A on 18S rRNA causes subtle changes in the structure of rRNA that affect the translation efficiency of specific mRNAs, while the distribution characteristics of m^6A on mRNAs determined the significance of the influence. Notably, the functions of METTL5 in the translation of c-Myc may be cell-type specific. The abundance of m^6A modification of c-Myc mRNA varies in different cells, which may lead to different regulatory effects of METTL5. Further research is required to verify whether this underlying mechanism is widely applicable to METTL5-mediated translation regulation in other genes.

As a coactivator of METTL5, TRMT112 is essential for the stabilization and enzymatic activity of METTL5 and may synergistically catalyze 18S rRNA m^6A methylation (10). In the present study, it was verified that the oncogenic function of METTL5 in PDAC was associated with the involvement of its coactivator TRMT112. Additionally, the significant effect of TRMT112 interference on the carcinogenic function of METTL5 suggested that the role of METTL5 in cancer may depend on its catalytic activity. However, it could not be excluded that METTL5 also promotes cancer progression through other mechanisms, such as interactions with key proteins. It will be interesting and important to further investigate the potential molecular mechanisms of METTL5 in cancer in the future.

To conclude, to the best of our knowledge, the present study revealed for the first time a distinct oncogenic role of METTL5 in pancreatic cancer. It was demonstrated that METTL5 promoted c-Myc translation, while the location and abundance of c-Myc mRNA m^6A modifications played a critical role in the specific translation regulation by METTL5. It was further validated that TRMT112, as a cofactor of METTL5, is required for the oncogenic functions of METTL5 in pancreatic cancer.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81702802) and the programs of the Beijing Municipal Education Commission (grant no. KM201910005005).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HH conceptualized the study. HH, HL, RP, AAK, HZ and SW performed the experiments. YZ performed the animal experiments. HL and YZ analyzed the data. HH and XL were involved in writing the manuscript. XL and HH supervised the overall project. All authors have read and agreed to the published version of the manuscript. HL and XL confirm the authenticity of the raw data.

Ethics approval and consent to participate

All animal experiments in the present study were approved (approval no. HS202105004) by the Ethics Committee of Beijing University of Technology (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Wang X, Lu Z, Gomez A, Hon GC, Yue Y, Han D, Fu Y, Parisien M, Dai Q, Jia G, *et al*: N6-methyladenosine-dependent regulation of messenger RNA stability. Nature 505: 117-120, 2014.
2. Bartosovic M, Molares HC, Gregorova P, Hrossova D, Kudla G and Vanacova S: N6-methyladenosine demethylase FTO targets pre-mRNAs and regulates alternative splicing and 3'-end processing. Nucleic Acids Res 45: 11356-11370, 2017.
3. Meyer KD, Patil DP, Zhou J, Zinoviev A, Skabkin MA, Elemento O, Pestova TV, Qian SB and Jaffrey SR: 5' UTR m(6)A promotes cap-independent translation. Cell 163: 999-1010, 2015.
4. Liu N, Dai Q, Zheng G, He C, Parisien M and Pan T: N(6)-methyladenosine-dependent RNA structural switches regulate RNA-protein interactions. Nature 518: 560-564, 2015.
5. Roundtree IA, Evans ME, Pan T and He C: Dynamic RNA modifications in gene expression regulation. Cell 169: 1187-1200, 2017.
6. Natchiar SK, Myasnikov AG, Kratzat H, Hazemann I and Klaholz BP: Visualization of chemical modifications in the human 80S ribosome structure. Nature 551: 472-477, 2017.
7. Sergiev PV, Aleksashin NA, Chugunova AA, Polikanov YS and Dontsova OA: Structural and evolutionary insights into ribosomal RNA methylation. Nat Chem Biol 14: 226-235, 2018.
8. Ma H, Wang X, Cai J, Dai Q, Natchiar SK, Lv R, Chen K, Lu Z, Chen H, Shi YG, *et al*: N(6-)Methyladenosine methyltransferase ZCCHC4 mediates ribosomal RNA methylation. Nat Chem Biol 15: 88-94, 2019.
9. Pinto R, Vagbo CB, Jakobsson ME, Kim Y, Baltissen MP, O'Donohue MF, Guzmán UH, Małecki JM, Wu J, Kirpekar F, *et al*: The human methyltransferase ZCCHC4 catalyses N6-methyladenosine modification of 28S ribosomal RNA. Nucleic Acids Res 48: 830-846, 2020.
10. van Tran N, Ernst FGM, Hawley BR, Zorbas C, Ulryck N, Hackert P, Bohnsack KE, Bohnsack MT, Jaffrey SR, Graille M and Lafontaine DLJ: The human 18S rRNA m6A methyltransferase METTL5 is stabilized by TRMT112. Nucleic Acids Res 47: 7719-7733, 2019.
11. Rong B, Zhang Q, Wan J, Xing S, Dai R, Li Y, Cai J, Xie J, Song Y, Chen J, *et al*: Ribosome 18S m(6)A methyltransferase METTL5 promotes translation initiation and breast cancer cell growth. Cell Rep 33: 108544, 2020.
12. Ignatova VV, Stolz P, Kaiser S, Gustafsson TH, Lastres PR, Sanz-Moreno A, Cho YL, Amarie OV, Aguilar-Pimentel A, Klein-Rodewald T, *et al*: The rRNA m(6)A methyltransferase METTL5 is involved in pluripotency and developmental programs. Genes Dev 34: 715-729, 2020.
13. Wang L, Liang Y, Lin R, Xiong Q, Yu P, Ma J, Cheng M, Han H, Wang X, Wang G, *et al*: Mettl5 mediated 18S rRNA N6-methyladenosine (m6A) modification controls stem cell fate determination and neural function. Genes & Diseases 9: 268-274, 2022.
14. Xing M, Liu Q, Mao C, Zeng H, Zhang X, Zhao S, Chen L, Liu M, Shen B, Guo X, *et al*: The 18S rRNA m(6) A methyltransferase METTL5 promotes mouse embryonic stem cell differentiation. EMBO Rep 21: e49863, 2020.
15. Leismann J, Spagnuolo M, Pradhan M, Wacheul L, Vu MA, Musheev M, Mier P, Andrade-Navarro MA, Graille M, Niehrs C, *et al*: The 18S ribosomal RNA m(6) A methyltransferase Mettl5 is required for normal walking behavior in Drosophila. EMBO Rep 21: e49443, 2020.
16. Yoshida GJ: Emerging roles of Myc in stem cell biology and novel tumor therapies. J Exp Clin Cancer Res 37: 173, 2018.
17. AlSultan D, Kavanagh E, O'Grady S, Eustace AJ, Castell A, Larsson LG, Crown J, Madden SF and Duffy MJ: The novel low molecular weight MYC antagonist MYCMI-6 inhibits proliferation and induces apoptosis in breast cancer cells. Invest New Drugs 39: 587-594, 2021.
18. Destefanis F, Manara V and Bellosta P: Myc as a regulator of ribosome biogenesis and cell competition: A link to cancer. Int J Mol Sci 21: 4037, 2020.
19. Wu H, Li F and Zhu R: miR-338-5p inhibits cell growth and migration via inhibition of the METTL3/m6A/c-Myc pathway in lung cancer. Acta Biochim Biophys Sin (Shanghai) 53: 304-316, 2021.
20. Dang CV: MYC on the path to cancer. Cell 149: 22-35, 2012.
21. Shan H, Cao Y, Xiao X, Liu M, Wu Y, Zhu Q, Xu H, Lei H, Yao Z and Wu Y: YL064 activates proteasomal-dependent degradation of c-Myc and synergistically enhances the anti-tumor activity of ABT-199 in diffuse large B cell lymphoma. Signal Transduct Target Ther 5: 116, 2020.
22. Yang G, Xiong G, Feng M, Zhao F, Qiu J, Liu Y, Cao Z, Wang H, Yang J, You L, *et al*: OLR1 promotes pancreatic cancer metastasis via increased c-Myc expression and transcription of HMGA2. Mol Cancer Res 18: 685-697, 2020.
23. Zhong Y, Yang L, Xiong F, He Y, Tang Y, Shi L, Fan S, Li Z, Zhang S, Gong Z, *et al*: Long non-coding RNA AFAP1-AS1 accelerates lung cancer cells migration and invasion by interacting with SNIP1 to upregulate c-Myc. Signal Transduct Target Ther 6: 240, 2021.
24. Shams R, Aghdaei HA, Behmanesh A, Sadeghi A, Zali M, Salari S and Padrón JM: MicroRNAs targeting MYC expression: Trace of hope for pancreatic cancer therapy. A systematic review. Cancer Manag Res 12: 2393-2404, 2020.
25. Hessmann E, Schneider G, Ellenrieder V and Siveke JT: MYC in pancreatic cancer: Novel mechanistic insights and their translation into therapeutic strategies. Oncogene 35: 1609-1618, 2016.
26. Wang XS, He JR, Yu S and Yu J: Methyltransferase-like 3 promotes the proliferation of acute myeloid leukemia cells by regulating N6-methyladenosine Levels of MYC. Zhongguo Yi Xue Ke Xue Yuan Xue Bao 40: 308-314, 2018 (In Chinese).
27. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
28. Chen H, Liu Q, Yu D, Natchiar K, Zhou C, Hsu CH, Hsu PH, Zhang X, Klaholz B, Gregory RI, *et al*: METTL5, an 18S rRNA-specific m6A methyltransferase, modulates expression of stress response genes. bioRxiv: doi: 10.1101/2020.04.27.064162.
29. Lin S, Choe J, Du P, Triboulet R and Gregory RI: The m(6)A methyltransferase METTL3 promotes translation in human cancer cells. Mol Cell 62: 335-345, 2016.
30. Wilson C, Chen PJ, Miao Z and Liu DR: Programmable m(6) A modification of cellular RNAs with a Cas13-directed methyltransferase. Nat Biotechnol 38: 1431-1440, 2020.
31. Konermann S, Lotfy P, Brideau NJ, Oki J, Shokhirev MN and Hsu PD: Transcriptome engineering with RNA-targeting type VI-D CRISPR effectors. Cell 173: 665-676 e614, 2018.
32. Li S, Li X, Xue W, Zhang L, Yang LZ, Cao SM, Lei YN, Liu CX, Guo SK, Shan L, *et al*: Screening for functional circular RNAs using the CRISPR-Cas13 system. Nat Methods 18: 51-59, 2021.
33. Liger D, Mora L, Lazar N, Figaro S, Henri J, Scrima N, Buckingham RH, van Tilbeurgh H, Heurgué-Hamard V and Graille M: Mechanism of activation of methyltransferases involved in translation by the Trm112 'hub' protein. Nucleic Acids Res 44: 1482, 2016.