Am J Cancer Res 2023;13(7):3185-3202
www.ajcr.us /ISSN:2156-6976/ajcr0149870

Original Article

Mettl3 synergistically regulates TGF-β/SMAD2/3 to promote proliferation and metastasis of gastric cancer

Xiao-Ning Yuan[1*], Qin Liu[1*], You-Cheng Shao[1], Xiao-Qing Guan[1], Ze-Lin Yang[1], Meng-Fei Chu[2], Jing-Wei Zhang[3], Yi-Hao Tian[2], Lei Wei[1]

[1]Department of Pathology and Pathophysiology, Hubei Provincial Key Laboratory of Developmentally Originated Disease, TaiKang Medical School (School of Basic Medical Sciences), Wuhan University, Wuhan 430071, Hubei, P. R. China; [2]Department of Human Anatomy, TaiKang Medical School (School of Basic Medical Sciences), Wuhan University, Wuhan 430071, Hubei, P. R. China; [3]Department of Breast and Thyroid Surgery, Zhongnan Hospital of Wuhan University, Hubei Key Laboratory of Tumor Biological Behaviors, Hubei Cancer Clinical Study Center, Wuhan 430071, Hubei, P. R. China. [*]Equal contributors.

Received February 27, 2023; Accepted June 6, 2023; Epub July 15, 2023; Published July 30, 2023

Abstract: Transforming Growth factor-β (TGF-β)/Smad signaling is a complex regulatory network that both inhibits and promotes tumorigenesis. However, the mechanisms underlying the function of TGF-β/Smad signaling pathway remain to be fully elucidated. As a methyltransferase, METTL3 is closely related to tumor development, but the role of METTL3 in the proliferation and metastasis of TGF-β/Smad-activated gastric cancer (GC) is unclear. In this study, we identified TGF-β/Smad2/3 axis as an important carcinogenic pathway in GC, which significantly promoted the proliferation and metastasis of GC. Furthermore, we found that Smad3 mRNA could be modified by m6A, which was subsequently recognized and stabilized by IGF2BP2, thereby enhancing Smad3 protein expression and promoting the activation of TGF-β/Smad pathway. Importantly, we also found that METTL3 could combine with p-Smad3 to regulate the transcription of downstream target genes. Therefore, this study revealed a novel mechanism by which METTL3 synergistically regulates TGF-β/Smad2/3 signaling and provide a new potential therapeutic target for the treatment of GC.

Keywords: Gastric cancer, TGF-β/Smads, METTL3, interaction

Introduction

Gastric cancer (GC) is one of the leading causes of death globally, as well as the fifth most common cancer type and the fourth leading cause of cancer death in 2020. Most patients with GC are diagnosed at an advanced stage with poor prognosis. Current GC treatments include endoscopic detection in adjuvant or neoadjuvant settings, gastrectomy, and chemotherapy or chemoradiotherapy [1]. Therefore, it is urgent to identify novel biomarkers and effective therapeutic targets for the early diagnosis and treatment of GC.

Transforming growth factor-β (TGF-β)/Smad signaling pathway is significantly over-activated in various tumors. TGF-β binds to its membrane receptors which then phosphorylates and activates Smad2/3 protein to transmit signals to the nucleus [2]. Smad2/3 regulates the transcription of genes that are involved in various cellular processes such as cell adhesion, cell cycle and extracellular matrix remodeling, thereby playing important roles in cancer cell proliferation, apoptosis, differentiation, invasion and metastasis, angiogenesis, and epithelial-mesenchymal transformation (EMT) [3]. Furthermore, TGF-β/Smad2/3 signaling pathway inhibits E-cadherin transcription in several tumors including GC, breast cancer, and glioma by inducing the expression of EMT-related transcription factors Snail1, Slug, Zeb1/2, Twist, etc., which further promotes the occurrence of EMT [4-6]. We have also previously shown that phosphorylated Smad2/3 protein level was significantly higher in GC cell line MGC803 than in normal epithelial cells. Additionally, the growth and metastasis ability of MGC803 cells were significantly reduced by the treatment of

TGF/Smad2/3 inhibitor SB431542 but were significantly enhanced by TGF-β treatment, suggesting that the TGF-β/Smad2/3 pathway is indeed activated in GC cells and is critical for the development and metastasis of GC cells.

Epigenetic factors are important links between genetic and environmental factors. There are more than 170 types of RNA modifications, among which N6-methyladenine (m6A) methylation is the most common epigenetic modification on RNA molecules. The m6A methylation process is catalyzed by RNA methyltransferase (writers), METTL3, METTL14 and WTAP, while the reversible demethylation is catalyzed by the demethylase (erasers) FTO and ALKBH5. The m6A modified sites can be recognized and bound by m6A binding protein (readers), which regulates RNA metabolism, including translation, splicing, translocation, degradation, and processing [7-9]. Hence, the aberrant m6A methylation affects the mRNA processing, degradation, and translation, leading to the activation of oncogenes and the inactivation of tumor suppressor genes, which is closely related to the occurrence, development, and drug resistance of malignant tumors including hematomas and solid tumors. Among the enzymes involved in m6A methylation, METTL3 is the catalytic core enzyme. In fact, a study published in Nature in 20217 by Kouzarides et al. has reported that METTL3 plays a key role in the development and maintenance of AML and that targeting the enzymatic activity of METTL3 may be a therapeutic strategy for acute myeloid leukemia (AML) [10]. Subsequently, METTL3 was also found to be involved in the occurrence and development of multiple tumors [11-13]. Meanwhile, our team confirmed that the upregulation of METTL3 in GC was positively correlated with the poor prognosis of patients. In addition, we have shown that METTL3 promotes tumor growth and metastasis as knocking down METTL3 significantly attenuates the growth of xenograft tumor in a mouse model. Intriguingly, knocking down METTL3 can significantly inhibit TGF-β-induced proliferation and metastasis of GC. Conversely, overexpression of METTL3 can enhance the effect of TGF-β/Smads pathway inhibitor SB431542 on the malignant phenotype of MGC803 cells. However, the interplay between METTL3 and TGF-β/Smad pathway in GC remains to be determined. Therefore, in this study, we explored whether METTL3 participates in TGF-β/Smads pathway regulation and could serve as a therapeutic target for the treatment of patients with GC.

It has been known that METTL3 initiates the methylation of m6A mRNA, resulting in a decreased or increased expression of target mRNA, depending on the recognition of m6A binding proteins such as YTH domain family proteins (YTHDFs) and protein insulin-like growth factor 2 mRNA binding proteins (IGF2BPs) [14, 15]. For example, IGF2BPs recognize as well as bind to the conserved GG (m6A) C sequence on mRNA and enhance the stability of mRNA under normal and stress conditions. In this study, we not only explored whether METTL3 functioned as a methyltransferase to regulate TGF-β/Smad pathway related gene expression, but also assessed if there was a direct interaction between METTL3 and Smads. Our results demonstrated that METTL3 combined with Smads to promote the development of GC via regulating the expression of downstream genes.

In conclusion, in this study, we identified TGF-β/Smad as an important cancer-promoting pathway in GC. Furthermore, we found that METTL3 could synergize with TGF-β/Smad pathway to promote the growth and metastasis of GC cells. Mechanistically, METTL3 enhanced Smad3 protein expression by modifying Smad3 mRNA with m6A and recognizing stable Smad3 mRNA by IGF2BP2. Significantly, we also found that METTL3 could interact with p-Smad3 to regulate the transcription of downstream target genes. Hence, this study has revealed a new mechanism by which METTL3 regulates TGF-β/Smad3 signaling and may serves as a new potential therapeutic target for the treatment of GC.

Materials and methods

Cell culture

Human embryonic kidney cell line 293T and GC cell lines GES-1, MGC803, MNK45, and SGC7901 were obtained from ATCC. MGC803, MNK45 and SGC7901 cells were cultured in RPMI1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin, while GES-1 and 293T cells were cultured in high-glucose DMEM medium supplemented with 10% FBS and with 1% penicillin-streptomycin. All cells were maintained in a 37°C humidified incubator with 5% CO_2.

CCK-8 assay

Briefly, cells (3×10^3 cells/well) were plated in a 96-well plate. At the indicated time, 10 ul of CCK-8 solution in 90 μl of medium was added to each well. After 2 h incubation, the optical density (OD) value at 450 nm was measured with a microplate reader.

Transwell assay

Transwell assay was used to determine the migration of cells. Briefly, cells (8000-10000 cells/well) were starved in serum-free medium for 8 h and seeded into the upper chamber of transwell in serum free medium. Complete medium containing 20% fetal bovine serum was added to the lower well. After overnight culture, the cells migrated to the lower side of the membrane were fixed, stained with crystal violet solution, and counted under microscope.

Colony formation assay

Briefly, 1000 cells per well were seeded in six-well plates and cultured for about two weeks. When cell colonies reached about 50 cells/per colony, the cell colonies were fixed, stained with crystal violet solution, and counted under microscope.

Western blot

Standard western blotting protocol was followed. Briefly, protein samples were separated by SDS-PAGE, transferred onto PVDF membrane and blocked with 5% skim milk powder. The membrane was then incubated with indicated primary antibody followed by incubation with corresponding HRP-conjugated secondary antibody (anti-Rabbit or -Mouse, 1:5000). ECL solution was used to detect the protein signal. Primary antibodies against the following proteins were used in this study: GAPDH (from proteintech), METTL3 (from proteintech), TGF-β (from wanlei), Smad2 (from Zen Bio), Smad3 (from Zen Bio), p-Smad2 (from Abconal), and p-Smad3 (from CST).

Mouse xenograft tumor model

Six-week-old/female nude mice were used in our in vivo tumor growth study. To develop xenograft tumors, appropriate 1×10^8 cells (MGC803 knockdown mettl3 stable cell line) and control were injected (subcutaneous injection) into nude mice. Tumor growth was monitored, and the tumor size was measured three times a week for 3 weeks. At the end of the experiments, the tumors were dissected for further analysis.

Nucleoplasmic separation

Nucleoplasmic separation assay was conducted using Nuclear protein and cytoplasmic protein extraction kit (Beyotime, China) according to the manufacturer's instruction.

Co-immunoprecipitation (Co-IP)

Co-IP was performed to determine the interaction between p-Smad3 and METTL3. It is a classical method used for investigating protein interactions based on the specific interaction between antibodies and antigens. It is an effective technique for determining the rational interaction of two proteins within an intact cell. Context The design concept of immunocoprecipitation is that if a known protein is a component member of a large protein complex, it is possible to "pull" the whole protein complex from the solution by using the specific antibody of this protein and the proteinA/G beads that can bind to the antibody, so as to achieve the purpose of enriching the protein complex. Western Blot was then used to verify whether the known protein was bound to the predicted protein.

RNA binding protein immunoprecipitation (RIP)-qPCR

The binding of RNA to proteins in cells was determined using RNA Immunoprecipitation (RIP) kit (BersinBio, China) following the manufacturer's protocol. Briefly, the RNA-protein complex is precipitated by antibodies against the target protein. After extensive washing, the RNAs that bound to the complex were examined by qPCR. In this study, RNA-binding proteins in the nucleus or cytoplasm were captured by the anti-IGF2BP2 antibody (proteintech, China).

Methylated RNA immunoprecipitation (meRIP)-qPCR

Briefly, anti-m6A RNA methylation antibodies (Abclonal, America) were used to purify RNA methylation modified fragments, and qPCR was then used to quantify the enriched RNA levels (BersinBio, China).

Statistical analysis

Data were analyzed using GraphPad Prism 7, and all experiments were independently repeated three times. The data were expressed as mean ± standard deviation. Difference between two groups of experimental data was compared by t-test, and $P<0.05$ was considered statistically significant.

Results

Upregulation of TGF-β/Smad pathway in GC

To evaluate the expression of TGF-β/Smad signaling pathway in GC, we compared the transcriptome of RNA-seq data between GC and the corresponding normal tissues in TCGA and GTEx databases through GEPIA website and found that, compared with normal tissues, the mRNA levels of TGF-β and Smad3 were significantly increased in GC tissues (**Figure 1A**). In addition, the analysis of GC data in TCGA database by UALCAN web portal revealed that the expression of TGF-β, Smad2 and Smad3 was closely related to the clinical stage of GC (**Figure 1B**). Furthermore, Kaplan-Meier survival analysis showed that the increased expression of TGF-β, Smad2 and Smad3 was positively correlated with the poor prognosis of patients (**Figure 1C**). Moreover, our qPCR results showed that the mRNA levels of TGF-β, Smad2 and Smad3 were significantly higher in GC cell lines than in normal GC epithelial cells (**Figure 1D**). Consistently, western blot analysis showed that the protein levels of TGF-β, Smad3 and p-Smad2/3 were significantly increased in GC cell lines (**Figure 1E**).

The activation of TGF-β/Smad2/3 pathway was crucial for the development of GC

To further explore the role of TGF-β/Smad2/3 pathway in the development of GC, we treated MGC803 cells with TGF-β and found that the morphology of MGC803 cells was significantly changed to spindle shape by 72 h of TGF-β treatment (**Figure 2A**). In line with this observation, transwell assay also showed an enhanced cell migration by TGF-β treatment (**Figure 2B**). In addition, colony formation assay demonstrated that TGF-β treatment promoted the clonogenic ability of MGC803 cells (**Figure 2C**). Since SB-431542 is a highly selective ALK5/TGF-β Type I receptor inhibitor that can significantly inhibit the phosphorylation of Smad2/3, we treated GC cells with different concentrations of SB-431542, and the protein level of phosphorylated Smad2/3 was assessed. The results showed that 10 μM SB-431542 could significantly inhibit the level of phosphorylated Smad2/3 in MGC803 cells (**Figure 2D**). Notably, SB431542 not only inhibited the proliferation of MGC803 cells in a time- and dose-dependent manner, as determined by CCK-8 assay and colony formation assay (**Figure 2E**, **2F**), but also attenuated the migration of MGC803 cell in transwell assay (**Figure 2G**). Consistently, the qPCR results showed that the mRNA levels of vimentin and fibronectin (FN), which is the indicator of metastasis, were decreased (**Figure 2H**). Together, these results indicated the regulation of GC progression by TGF-β/Smad2/3 pathway.

Upregulation of METTL3 in GC cells promoted GC growth and metastasis

"R" analysis on the data of GC samples from TCGA database suggested that the expression of the 16 m6A-related "writers", "eraser" and "reader" was upregulated in GC cell lines (**Figure 3A**, **3B**). Kaplan-Meier plotter survival analysis further showed that the expression of METTL3, METTL14 and WTAP was significantly elevated in GC tissues and was positively correlated with the poor prognosis of patients (**Figure 3C**). These findings were also experimentally verified, as western blot analysis showed that protein levels of METTL3 and METTL14 were significantly higher in GC cells than in normal GC epithelial GES-1 cells (**Figure 3D**). qPCR demonstrated the upregulation of transcription (**Figure 3E**). At the same time, Dot blot results showed that compared with normal gastric carcinoma epithelial cells GES-1, the overall mRNA modification level of gastric carcinoma cell lines was significantly higher (**Figure 3F**). To further explore the role of abnormal m6A modification in GC, we took the approach of METTL3 knockdown or overexpression in MGC803 cells, which was confirmed by western blot (**Figure 3G**). We then injected the stable shMETTL3-expressing MGC803 cells into nude mice and observed that the tumor growth was significantly inhibited by METTL3 knockdown (**Figure 3H**).

In addition to the in vivo tumor growth study, we also found that METTL3 knockdown significantly inhibited the migration of GC cells (**Figure 4A**), while METTL3 overexpression showed the opposite effect (**Figure 4B**). Furthermore, the colony formation ability of GC cells was also

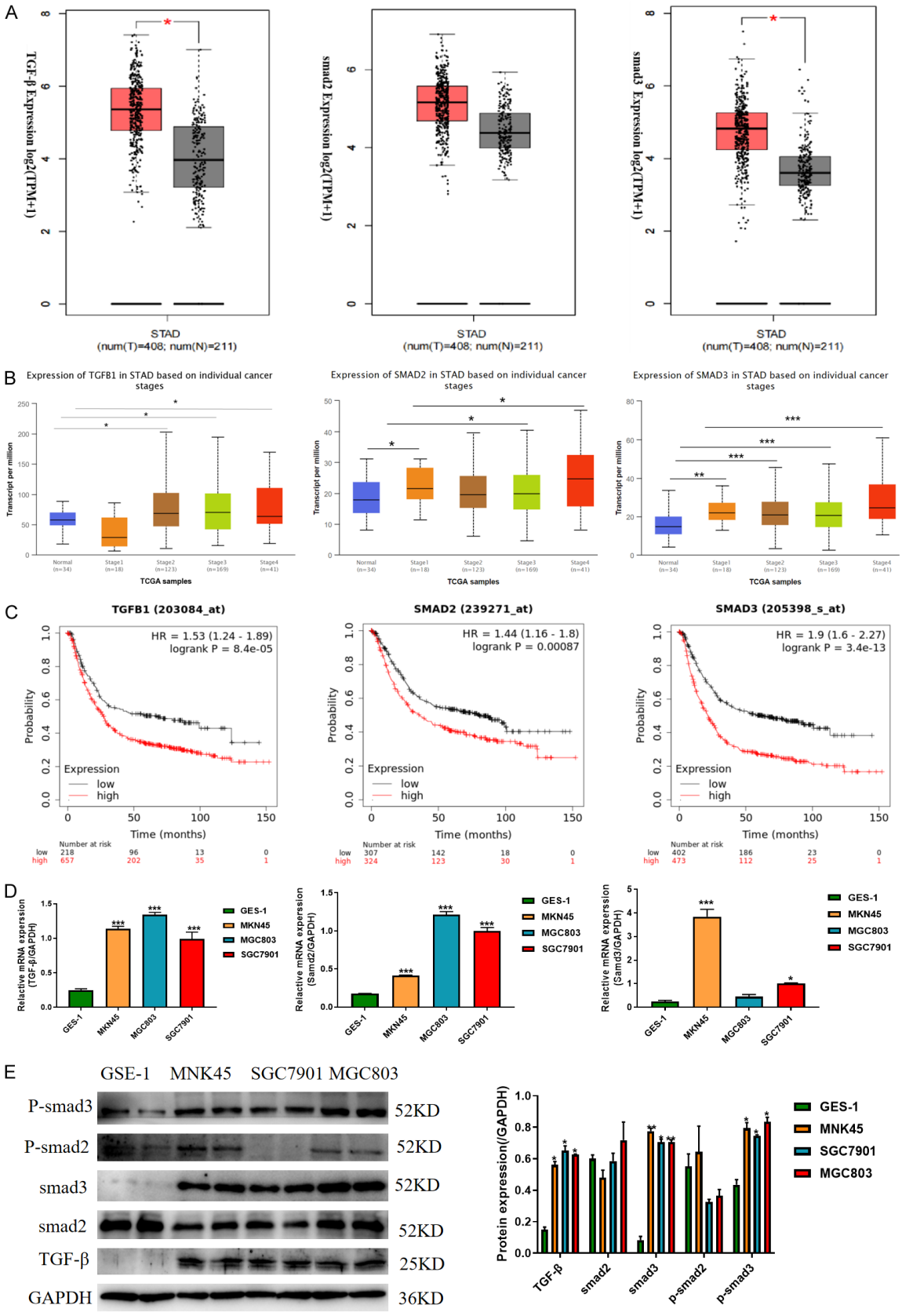
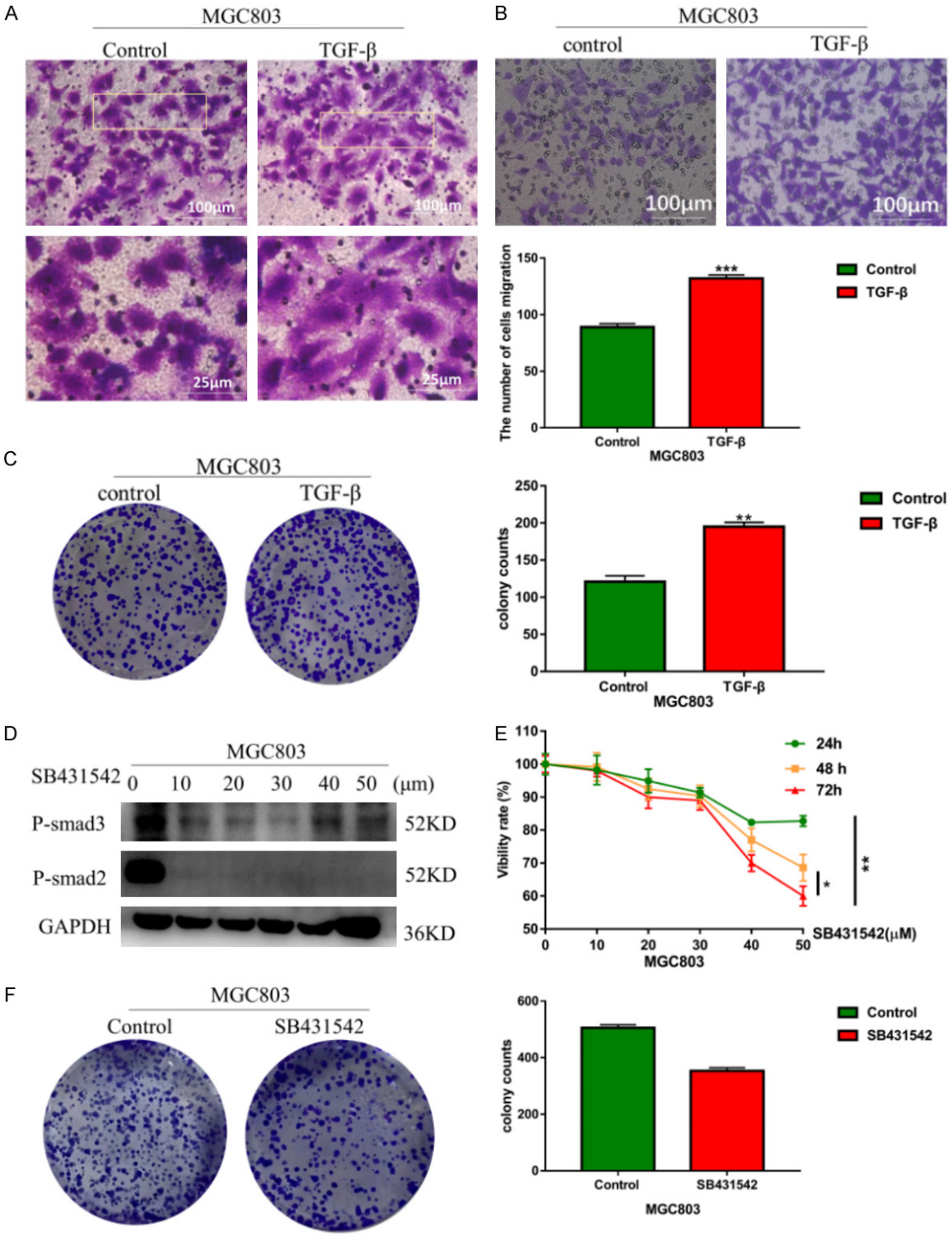


Figure 1. TGF-β/Smad pathway activation in GC. A: GEPIA website analyzes the mRNA expression of TGF-β/Smad signal pathway related genes in GC and corresponding normal tissues in TCGA and GTEx databases; B: UALCAN

website analyzes the correlation of clinical stage with the expression of TGF-β, Smad2 and Smad3 in GC using GC data in TCGA database; C: Kaplan-Meier plotter displayed the correlation between the expression of TGF-β/Smad signal pathway related genes and the poor prognosis of patients; D: The mRNA levels of TGF-β/Smad signal pathway related genes in normal gastric epithelial and GC cells, as determined by qPCR; E: Western blot analysis of the protein levels of TGF-β/Smad signal pathway related genes in normal gastric epithelial and GC cells (**P<0.05, **P<0.01, ***P<0.001*).



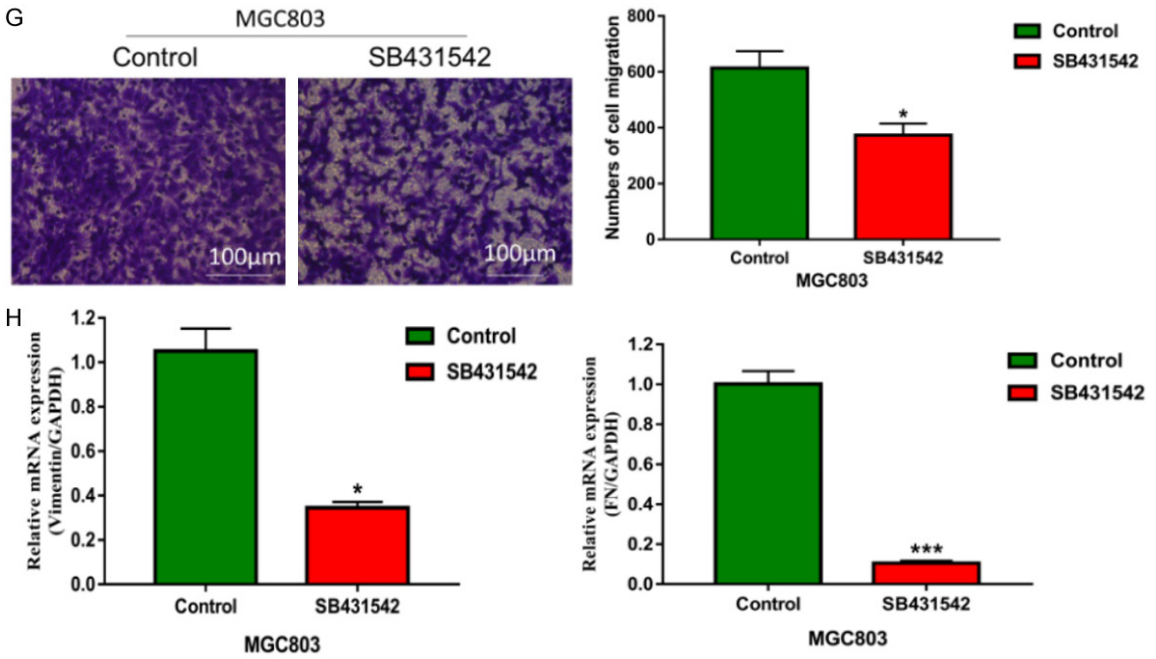


Figure 2. The activation of TGF-β/Smad2/3 pathway is crucial in the development of GC. A: Morphology changes in TGF-β-treated MGC803 cells; B: TGF-β promoted the migration of MGC803 cells, as determined by transwell assay; C: TGF-β promoted the colony formation of MGC803 cells; D: Inhibitor SB-431542 suppressed phosphor-Smad2/3 levels in MGC803 cells; E: The proliferation of MGC803 cells was detected by CCK-8; F: SB431542 inhibited the colony formation of MGC803 cells; G: The migration of MGC803 cells, as determined by transwell assay; H: The mRNA levels of Vimentin and FN was detected by qPCR (**P<0.05, **P<0.01, ***P<0.001*).

inhibited by METTL3 knockdown (**Figure 4C**), whereas METTL3 overexpression promoted this ability (**Figure 4D**). Moreover, flow cytometry showed that METTL3 knockdown blocked G0/G1 phase to S phase progression of cell cycle. Conversely, METTL3 overexpression caused the opposite effect (**Figure 4E**). Collectively, these results indicate that METTL3 is essential for the growth and metastasis of GC cells.

METTL3 knockdown suppressed the TGF-β-induced proliferation and migration of GC cells and improved the sensitivity of cells to SB431542

Studies have shown that TGF-β/Smad2/3 signaling is critical in promoting the proliferation and migration of GC cells. Therefore, we explored whether METTL3 promoted TGF-β-induced GC cell proliferation and migration by using METTL3 knockdown MGC803 cells. Transwell assay showed that METTL3 knockdown inhibited TGF-β-induced GC cell migration (**Figure 5A**). Notably, when we treated METTL3 knockdown MGC803 cells with SB-421542, METTL3 knockdown could enhance the inhibitory effect of SB431542 on GC cell migration (**Figure 5B**). Similar results were also observed in colony formation assay, in which METTL3 was indeed involved in the proliferation of GC cells by affecting the TGF-β/Smad pathway (**Figure 5C**, **5D**).

METTL3 positively controlled TGF-β/Smad2/3 signaling through the m6A modification of Smad3 mRNA in GC cells

In METTL3 knockdown GC cells, we found that Smad3 and p-Smad3 levels were significantly decreased (**Figure 6A**); they were significantly increased in METTL3-overexpressing cells (**Figure 6B**). Consistently, qPCR results showed that the mRNA level of Smad3 was decreased significantly in METTL3 knockdown GC cells (**Figure 6C**), suggesting that the expression of Smad3 might be regulated by m6A modification. Hence, we utilized SRAMP and identified 25 potential m6A modification sites near the 3'UTR of Smad3 mRNA (**Figure 6D**, **6E**). The top three putative modification sites, 6354, 6360 and 6406, closest to the 3'UTR region were

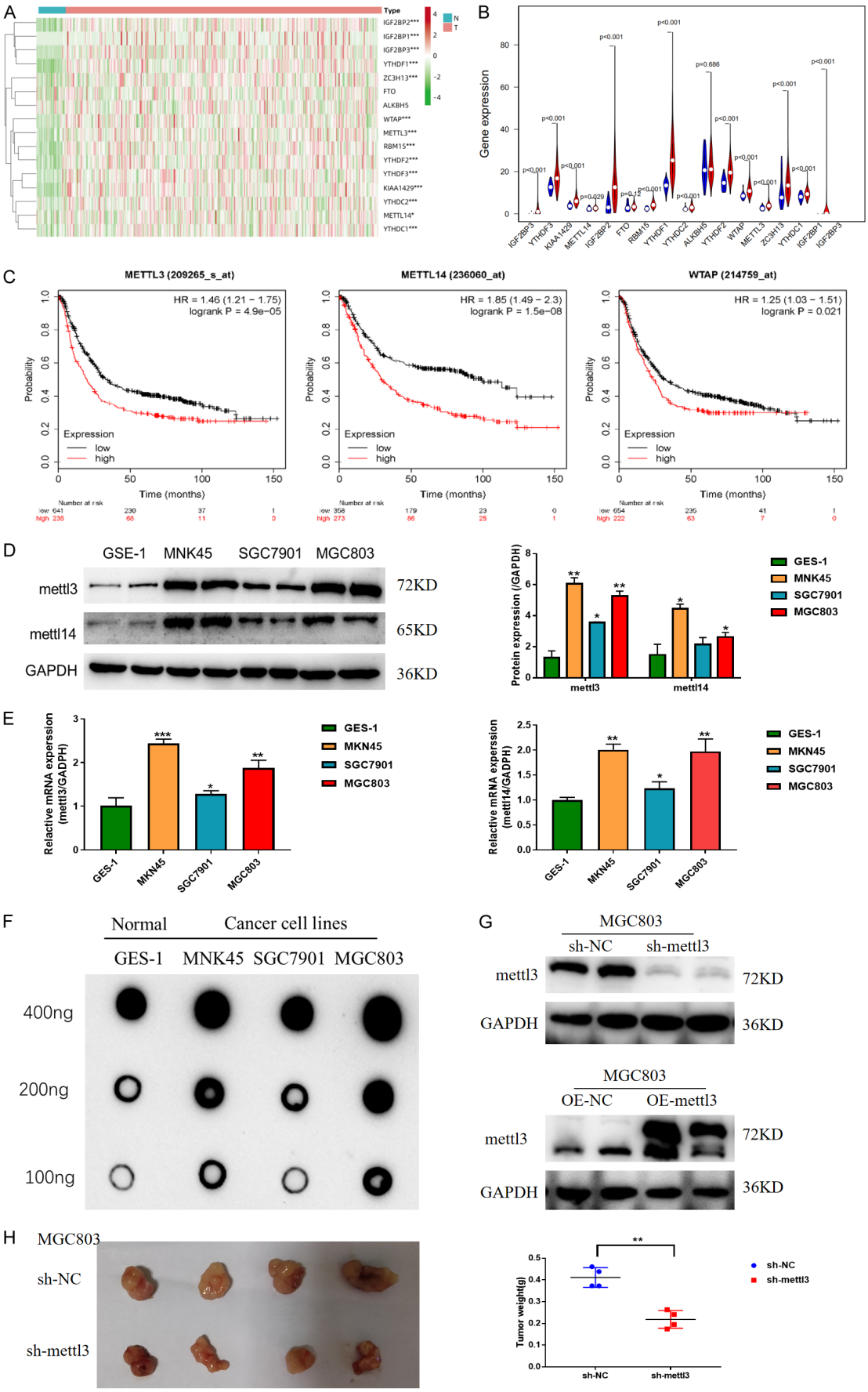
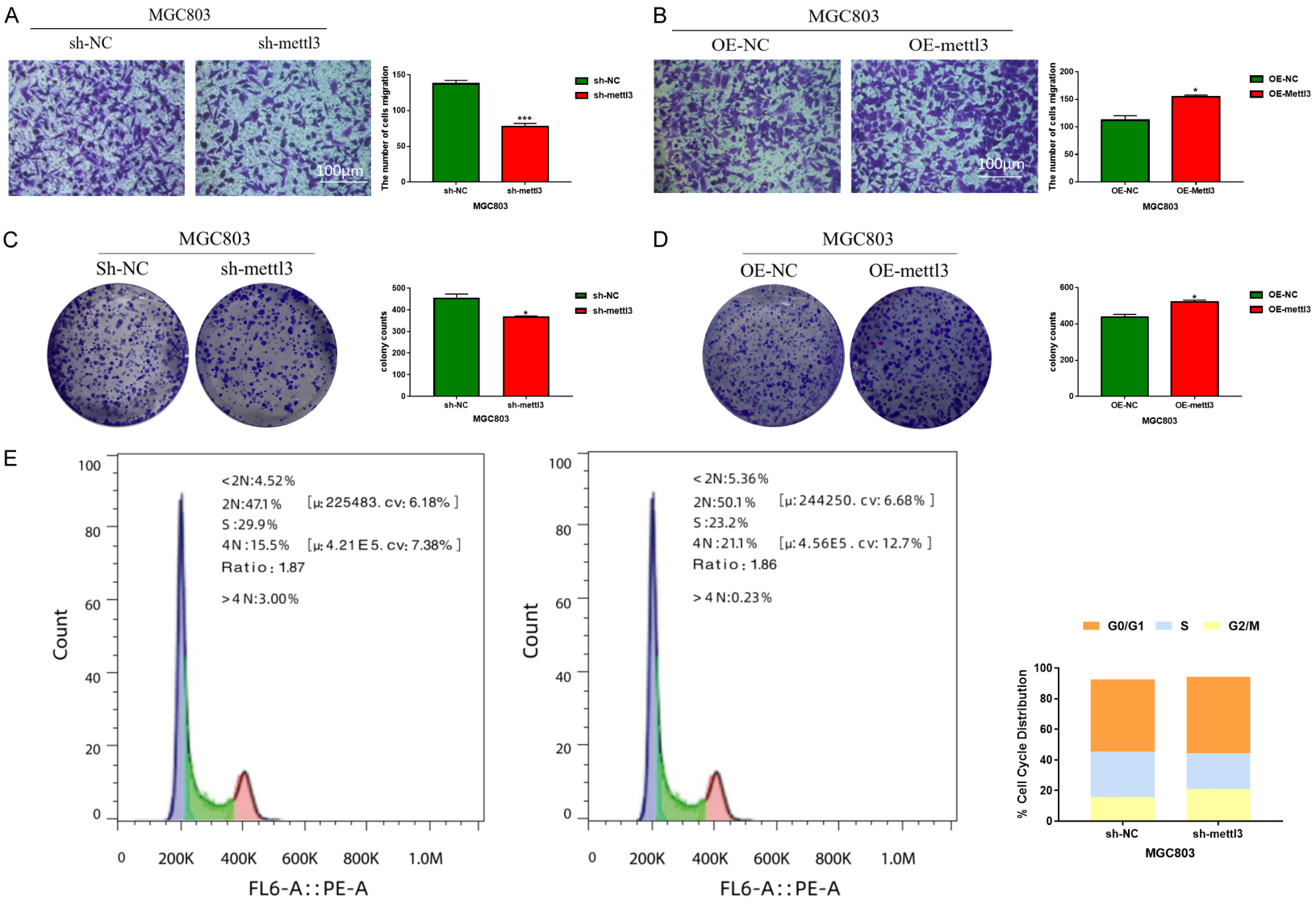

A
Type
N
T
IGF2BP2***
IGF2BP1***
IGF2BP3***
YTHDF1***
ZC3H13***
FTO
ALKBH5
WTAP***
METTL3***
RBM15***
YTHDF2***
YTHDF3***
KIAA1429***
YTHDC2***
METTL14*
YTHDC1***
B
Gene expression
IGF2BP3
YTHDF3
KIAA1429
METTL14
IGF2BP2
FTO
RBM15
YTHDF1
YTHDC2
ALKBH5
YTHDF2
WTAP
METTL3
ZC3H13
YTHDC1
IGF2BP1
IGF2BP3
C
METTL3 (209265_s_at)
HR = 1.46 (1.21 − 1.75)
logrank P = 4.9e−05
METTL14 (236060_at)
HR = 1.85 (1.49 − 2.3)
logrank P = 1.5e−08
WTAP (214759_at)
HR = 1.25 (1.03 − 1.51)
logrank P = 0.021
Probability
Expression
low
high
Time (months)
Number at risk
D
GSE-1
MNK45
SGC7901
MGC803
mettl3
mettl14
GAPDH
72KD
65KD
36KD
Protein expression /(GAPDH)
E
Relactive mRNA expression (mettl3/GADPH)
Relactive mRNA expression (mettl14/GADPH)
GES-1
MKN45
SGC7901
MGC803
F
Normal
Cancer cell lines
400ng
200ng
100ng
G
MGC803
sh-NC
sh-mettl3
OE-NC
OE-mettl3
H
sh-NC
sh-mettl3
Tumor weight(g)

Figure 3. METTL3 expression was upregulated in GC cells. A, B: High expression of m6a related genes in TCGA database, as determined by "R" analysis; C: Kaplan-Meier plotter survival showed the correlation between the mRNA levels of METTL3, METTL14 and WTAP and the poor prognosis of GC patients; D: The protein levels of METTL3 and METTL14 were detected by Western blot; E: The mRNA levels of METTL3 and METTL14 in GC cells were detected by qPCR; F: The overall m6A modification level of cell mRNA was detected by Dot blot; G: The protein level of METTL3 was detected by Western blot; H: Tumor growth in nude mice (**P<0.05, **P<0.01, ***P<0.001*).



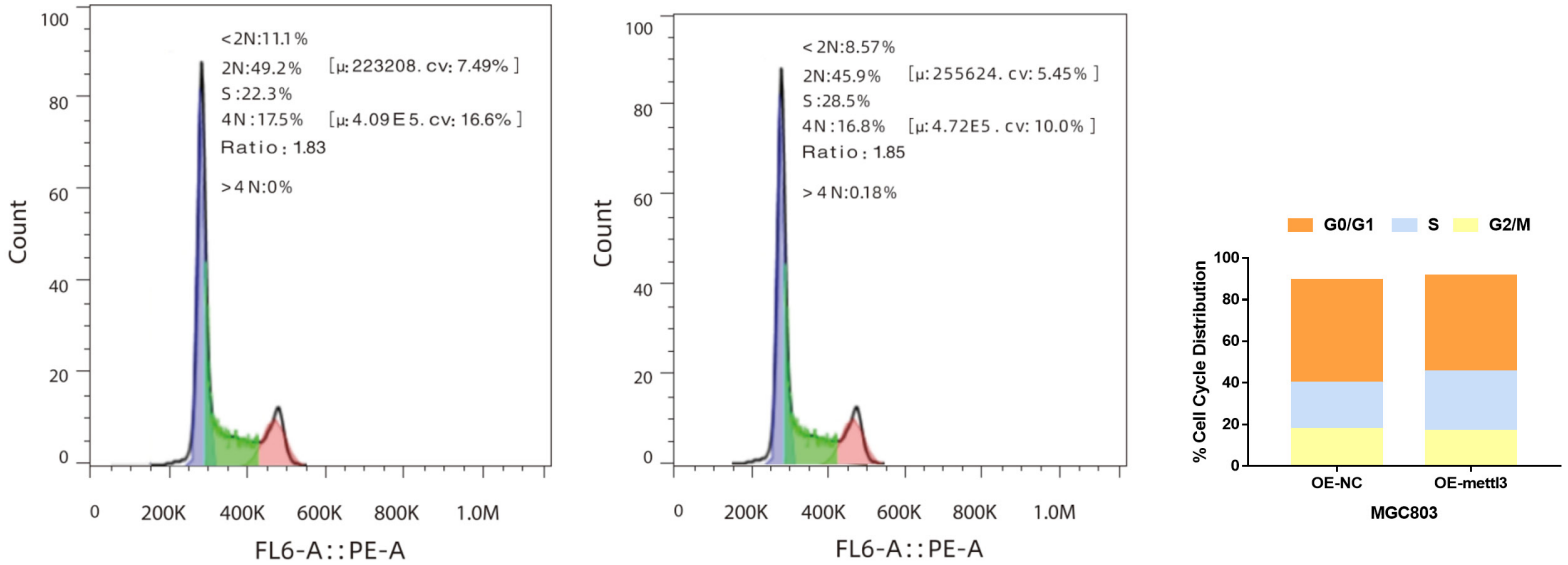


Figure 4. METTL3 promoted the growth and migration of GC cells. A, B: METTL3 knockdown attenuated the migration of GC cells, as determined by transwell assay; C, D: METTL3 overexpression enhanced the colony formation of GC cells; E: The cell cycle of GC cells was measured by flow cytometry (**P<0.05, **P<0.01, ***P<0.001*).

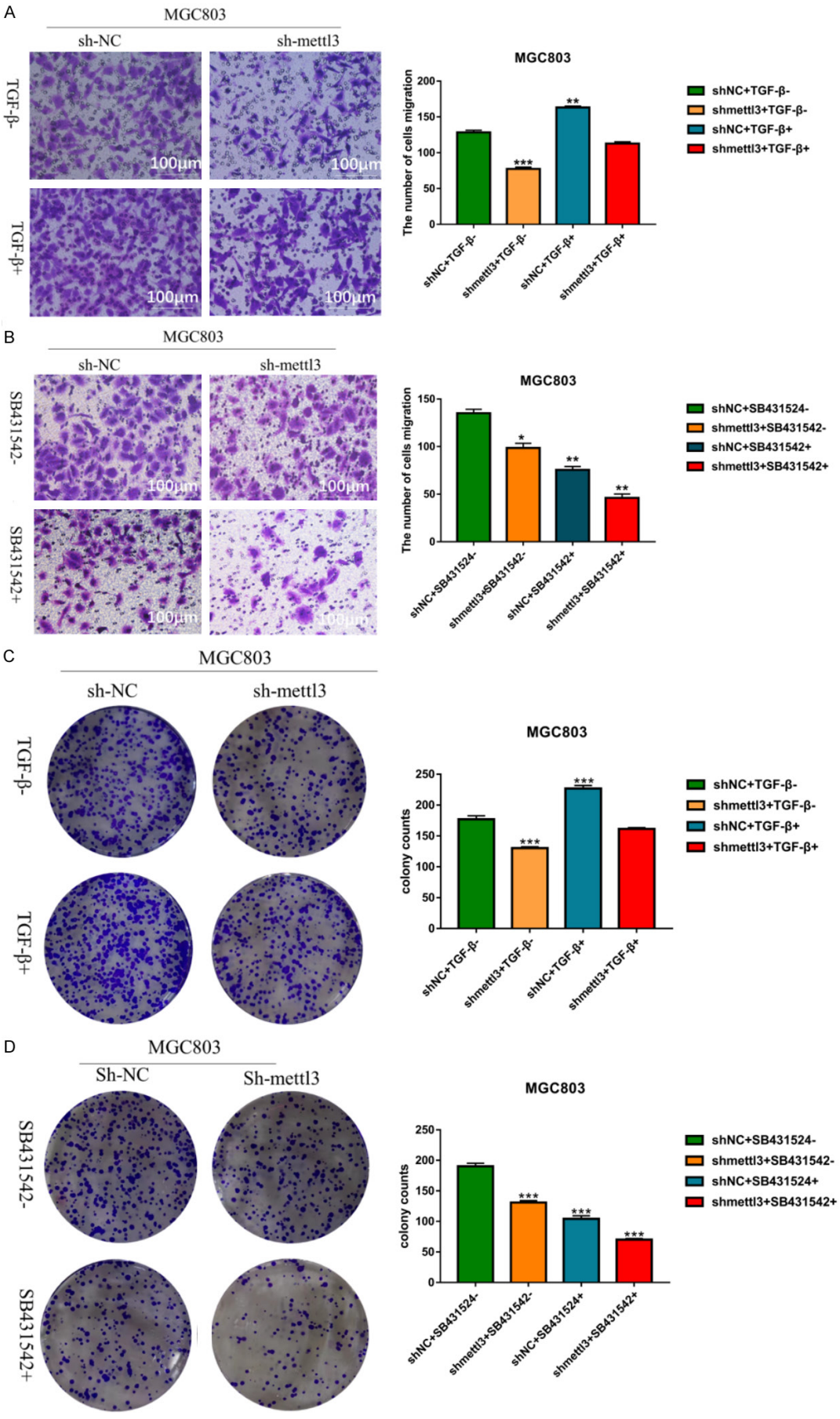
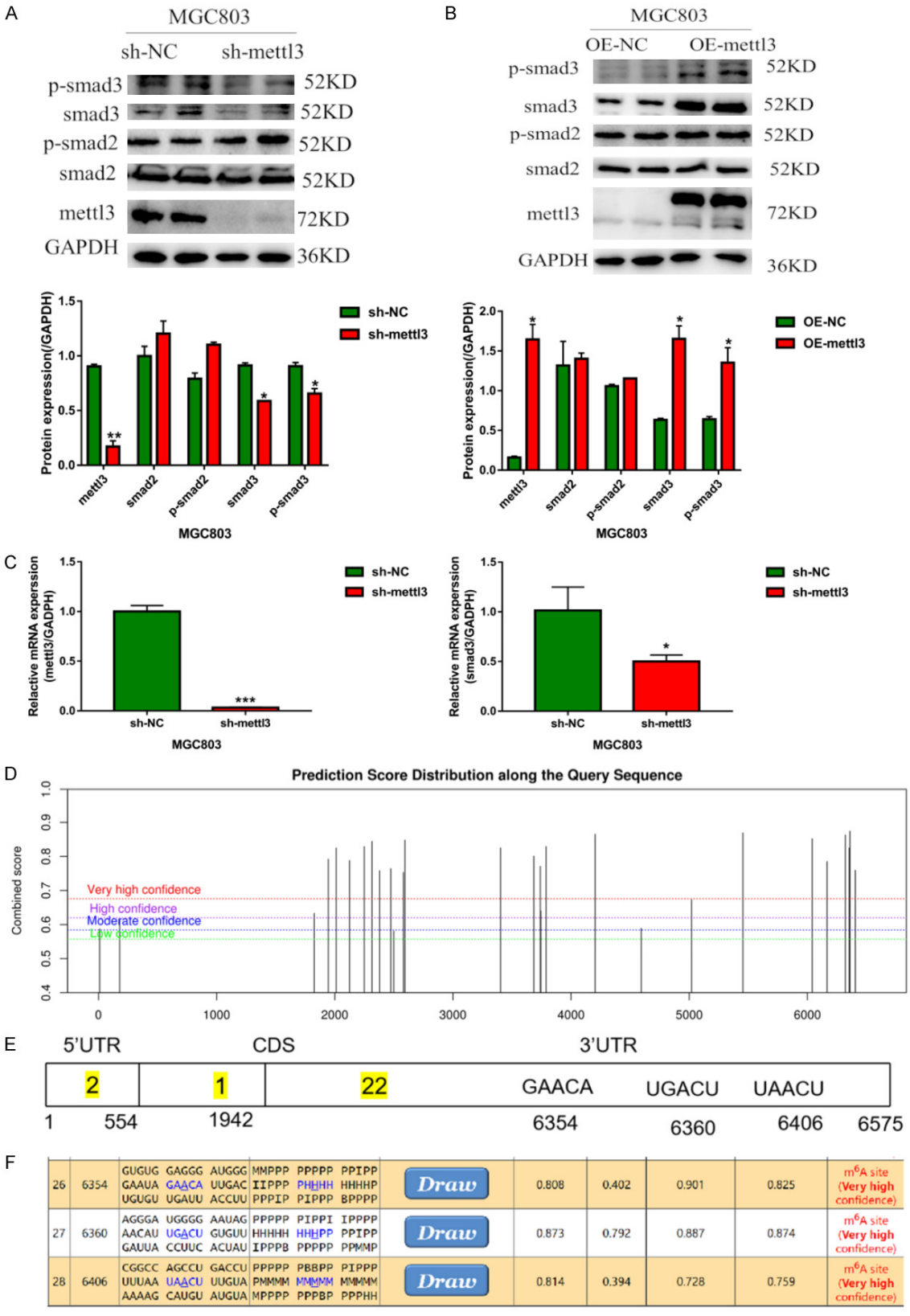

A
MGC803
sh-NC
sh-mettl3
TGF-β-
TGF-β+
100μm
MGC803
The number of cells migration
shNC+TGF-β-
shmettl3+TGF-β-
shNC+TGF-β+
shmettl3+TGF-β+
B
MGC803
sh-NC
sh-mettl3
SB431542-
SB431542+
100μm
MGC803
The number of cells migration
shNC+SB431524-
shmettl3+SB431542-
shNC+SB431542+
shmettl3+SB431542+
C
MGC803
sh-NC
sh-mettl3
TGF-β-
TGF-β+
MGC803
colony counts
shNC+TGF-β-
shmettl3+TGF-β-
shNC+TGF-β+
shmettl3+TGF-β+
D
MGC803
Sh-NC
Sh-mettl3
SB431542-
SB431542+
MGC803
colony counts
shNC+SB431524-
shmettl3+SB431542-
shNC+SB431524+
shmettl3+SB431542+

Figure 5. METTL3 knockdown reduced TGF-β-induced proliferation and metastasis of GC cells and improved the sensitivity to SB431542. A: Transwells assay of GC cells in the presence or absence of TGF-β1; B: Transwells assay of GC cells in the presence or absence of SB431542; C: The colony formation assay of GC cells in the presence or absence of TGF-β1; D: The colony formation assay of GC cells in the presence or absence of SB431542 (**P<0.05, **P<0.01, ***P<0.001*).



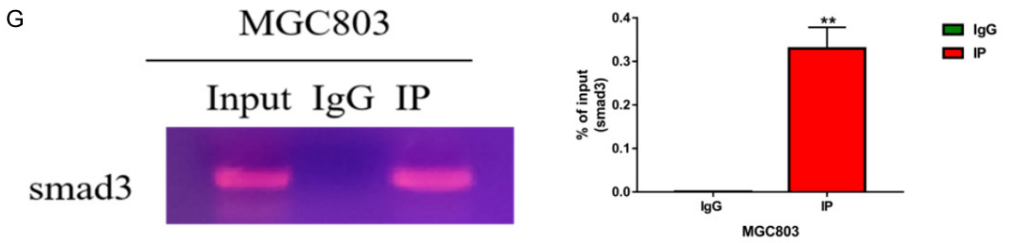


Figure 6. METTL3 positively modulated TGF-β/Smad2/3 signaling via the m6A modification of Smad3 mRNA in GC cells. A: Western blot analysis of the expression and phosphorylation of TGF-β/Smad protein after METTL3 knockdown by shMETTL3; B: Western blot analysis of the expression and phosphorylation of TGF-β/Smad protein in METTL3-expressing cells (OE); C: qPCR analysis of Smad3 mRNA level in shMETTL3 cells; D: Prediction of the m6A modification sites on Smad3 mRNA by SRAMP online database; E: Statistical analysis of m6A modification site on Smad3 mRNA; F: High confidence modification site in the 3'UTR region of Smad3; G: meRIP detected Smad3 mRNA modification by m6A (**P<0.05, **P<0.01, ***P<0.001*).

selected for further meRIP study (**Figure 6F**). MeRIP results showed that Smad3 mRNA was indeed regulated by m6A modification (**Figure 6G**).

METTL3 regulated Smad3 expression via m6A reader IGF2BP2

Furthermore, the RNA binding proteins that can interact with Smad3 mRNA were analyzed by using CLIP-seq data in starBase, and the Smad3-binding m6A reader proteins were screened out, among which insulin-like growth factor 2 mRNA binding protein (IGF2BP2) showed the highest correlation (**Figure 7A**, **7B**). Importantly, we experimentally validated the regulatory role of IGF2BP2 in Smads. Our results showed that, in IGF2BP2 knockdown GC cell lines, Smad3 and p-Smad2/3 levels were significantly decreased (**Figure 7C**), while their levels were significantly increased in IGF2BP2 overexpressing cells (**Figure 7D**). These findings were supported by qPCR results in which the mRNA level of Smad3 was increased significantly by IGF2BP2 overexpression (**Figure 7E**). Moreover, RIP results confirmed that IGF2BP2 bound to and stabilized Smad3 mRNA (**Figure 7F**). We also examined the stability of Smad3 mRNA with actinomycin D (Act D) treatment and found that knockdown of IGF2BP2 shortened the half-life of Smad3 mRNA, whereas IGF2BP2 overexpression exhibited the opposite effect (**Figure 7G**).

METTL3 and p-Smad3 synergistically promoted the transcription of downstream target genes

Accumulating studies have shown the interactions between METTL3 and chromatin, suggesting that m6A is directly related to transcriptional regulation. Here, Pearson analysis showed that METTL3 was closely associated with p-Smad3 in a variety of tumors (**Figure 8A**). p-Smad3, as one of the most important transcription factors in TGF-β/Smad signaling pathway, directly binds to the promoters of target genes to regulate their transcription, thereby participating in the process of tumor development. Hence, we conducted protein nucleoplasmic separation experiments in MGC803 cells to show the co-localization of METTL3 and p-Smad3 in the nucleus (**Figure 8C**), which was further confirmed by immunofluorescence staining (**Figure 8B**). Furthermore, Co-IP experiments also demonstrated the interaction between METTL3 and p-Smad3 (**Figure 8D**). Next, we analyzed the CHIP seq data of METTL3 and p-Smad3, and obtained the joint regulatory downstream gene HNRNPL (**Figure 8E**) after intersection. Meanwhile, correlation analysis showed that the expression level of HNRNPL was indeed positively correlated with METTL3 and p-Smad3 (**Figure 8F**). By analyzing TCGA data, it was found that HNRNPL is highly expressed in gastric cancer and positively correlated with patient stage grading. As an oncogene, it participates in regulating the progression of gastric cancer (**Figure 8G**, **8H**). The qPCR results further confirmed that compared with the control group, knocking down METTL3 and Smad3 significantly reduced the mRNA expression levels of HNRNPL. More importantly, knocking down METTL3 and Smad3 simultaneously resulted in lower mRNA expression levels of HNRNPL (**Figure 8I**). Together, these results suggest that METTL3 interacts with p-Smad3 to promote the transcription of downstream target genes and regulate tumor progression.

A

RBP	GeneID	GeneName	GeneType	ClusterNum	ClipExpNum	ClipSiteNum	HepG2(log2FC)	K562(log2FC)	Pan-Cancer
IGF2BP2	ENSG00000166949	SMAD3	protein_coding	233	8	319	NA	NA	21
IGF2BP1	ENSG00000166949	SMAD3	protein_coding	27	7	35	0.370	NA	16
IGF2BP3	ENSG00000166949	SMAD3	protein_coding	18	4	20	NA	NA	18

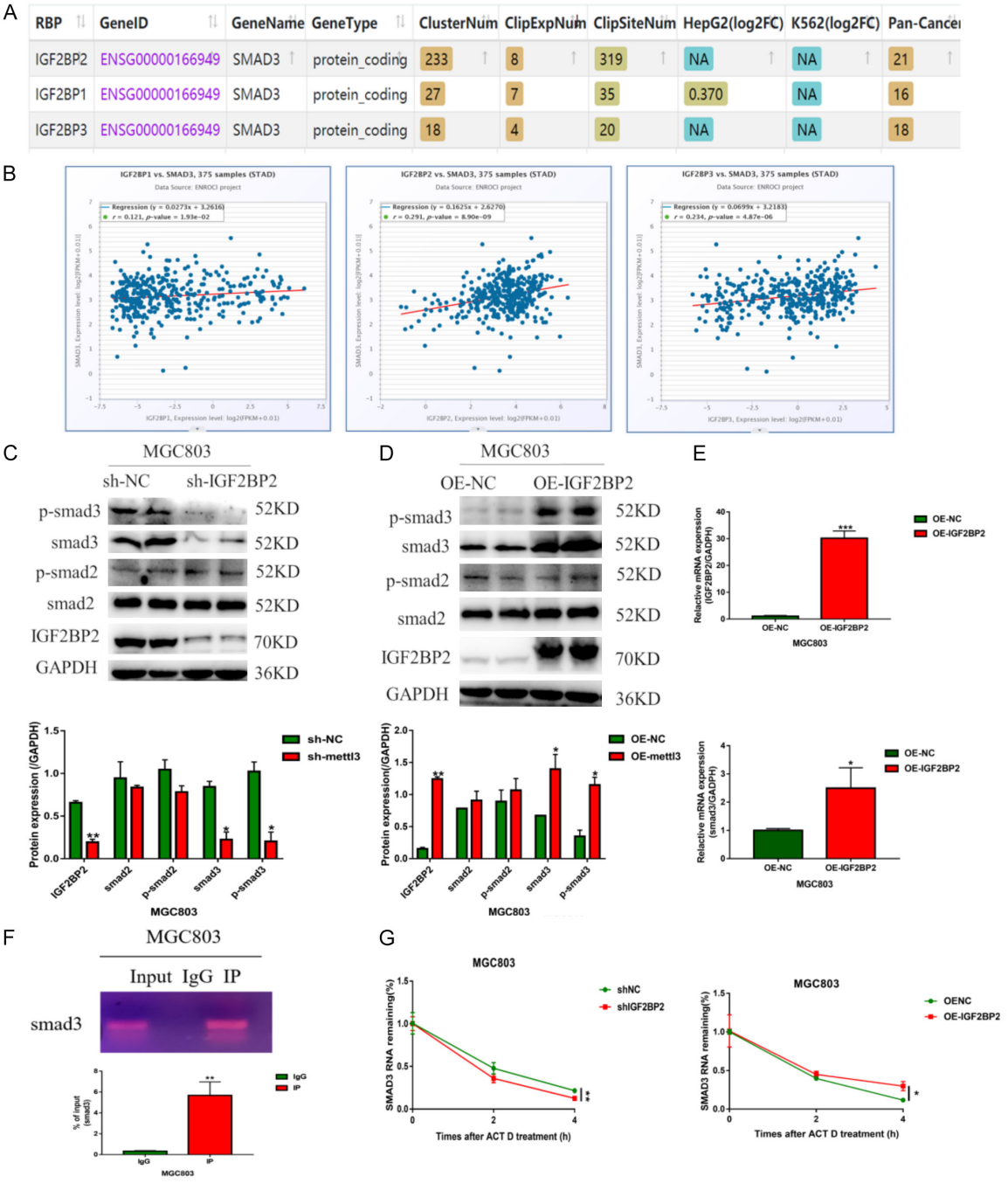


Figure 7. METTL3 regulated Smad3 expression via m6A reader IGF2BP2. A: Analysis of Smad3 mRNA binding proteins from CLIP-seq data in starBase; B: Correlation analysis between Smad3 and IGF2BP2 expression; C: Western blot analysis of the expression and phosphorylation of TGF-β/Smad protein in IGF2BP2 knockdown cells; D: Western blot analysis of the expression and phosphorylation of TGF-β/Smad protein inIGF2BP2-overexpressing cells; E: qPCR analysis of Smad3 mRNA expression in IGF2BP2-overexpressing cells; F: IGF2BP2 binding to Smad3 mRNA determined by RIP; G: Stability of Smad3 mRNA, as determined by the Act D assay (**P<0.05, **P<0.01 ***P<0.001*).

Discussion

GC, as one of the leading causes of cancer-related death globally [16], is the fifth most common cancer type and the fourth leading cause of cancer death in 2020. In addition, more than 70% of GC occurs in developing countries [17]. Treatment for advanced GC is challenging, and the complete cure rates for GC over the past 20 years is still suboptimal [18-

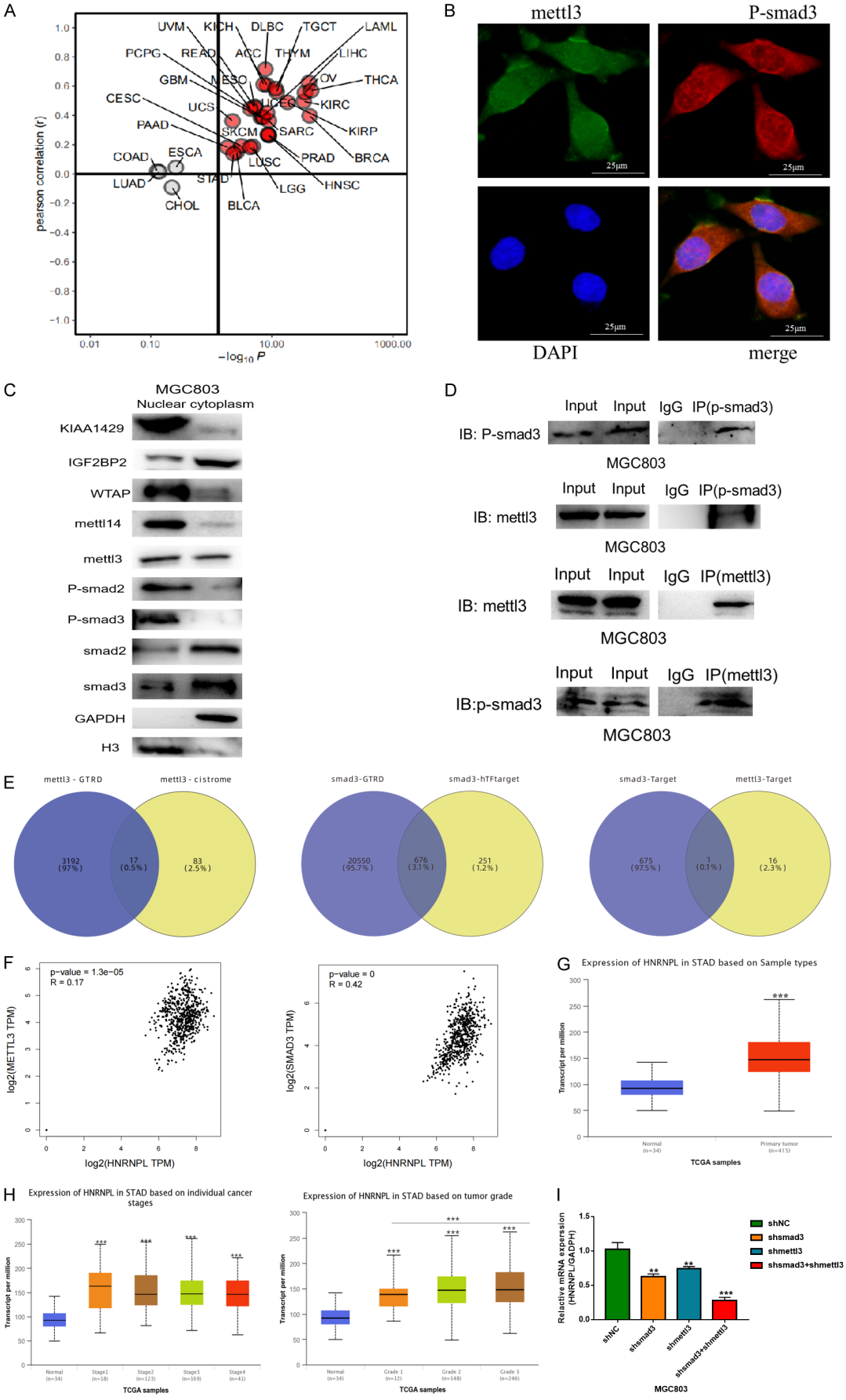

A
pearson correlation (r)
−log10 P
UVM
KICH
DLBC
TGCT
LAML
PCPG
READ
ACC
THYM
LIHC
GBM
MESO
OV
THCA
CESC
UCS
UCEC
KIRC
PAAD
SKCM
SARC
KIRP
COAD
ESCA
LUSC
PRAD
BRCA
LUAD
STAD
LGG
HNSC
CHOL
BLCA
B
mettl3
P-smad3
25μm
DAPI
merge
C
MGC803
Nuclear cytoplasm
KIAA1429
IGF2BP2
WTAP
mettl14
mettl3
P-smad2
P-smad3
smad2
smad3
GAPDH
H3
D
Input Input IgG IP(p-smad3)
IB: P-smad3
MGC803
IB: mettl3
IP(mettl3)
IB:p-smad3
E
mettl3 - GTRD
mettl3 - cistrome
3192 (97%)
17 (0.5%)
83 (2.5%)
smad3-GTRD
smad3-hTFtarget
20550 (95.7%)
676 (3.1%)
251 (1.2%)
smad3-Target
mettl3-Target
675 (97.5%)
1 (0.1%)
16 (2.3%)
F
p-value = 1.3e-05
R = 0.17
log2(METTL3 TPM)
log2(HNRNPL TPM)
p-value = 0
R = 0.42
log2(SMAD3 TPM)
G
Expression of HNRNPL in STAD based on Sample types
Transcript per million
Normal (n=34)
Primary tumor (n=415)
TCGA samples
H
Expression of HNRNPL in STAD based on individual cancer stages
Normal (n=34)
Stage1 (n=18)
Stage2 (n=123)
Stage3 (n=169)
Stage4 (n=41)
Expression of HNRNPL in STAD based on tumor grade
Grade 1 (n=12)
Grade 2 (n=148)
Grade 3 (n=246)
I
Relactive mRNA expression (HNRNPL/GADPH)
shNC
shsmad3
shmettl3
shsmad3+shmettl3
MGC803

Figure 8. METTL3 combines with P-Smad3 to promote the transcription of downstream target genes. A: Correlation between METTL3 and p-Smad3 in a variety of tumors by Pearson analysis; B: Co-localization of METTL3 and p-Smad3 by immunofluorescence staining; C: Nucleoplasmic separation experiment; D: The interaction between proteins, as determined by Co-IP assay; E: Analyze the CHIP seq data of METTL3 and Smad3 using GTRD, Cistrome DB, and hTFarget databases; F: Correlation analysis of the correlation between HNRNPL expression and METTL3 and Smad3; G: UALCAN database analysis of the expression level of HNRNPL in gastric cancer; H: UALCAN database analysis of the relationship between HNRNPL and staging and grading of gastric cancer patients; I: qPCR analysis were used to investigate the relationship between HNRNPL and METTL3 and Smad3 expression (**P<0.05, **P<0.01, ***P<0.001*).

20]. Although the development of multimodal therapy by using surgery in combination with systemic multiline chemotherapy has improved patient survival, the overall survival of >70% GC patients is still poor [21], which highlights the need of identifying novel biomarkers for the early diagnosis and the treatment of patients with GC.

TGF-β/Smad signaling regulates a diversity of cellular processes such as cell proliferation, differentiation, migration, apoptosis, adhesion, angiogenesis, and immune surveillance. Many types of mammalian cells produce and respond to TGF-β, forming a complex network that includes epithelial cells, tumor cells, immune cells, and stromal fibroblasts. This complex network not only causes disease but also evolves over time, enabling TGF-β to have both tumor suppressive and tumor promoting effects [22]. In this study, we revealed that TGF-β/Smad2/3 pathway was upregulated in GC cells. In addition, we observed that TGF-β treatment significantly induced the EMT process, while TGF-β/Smad inhibitor attenuated the proliferation and metastasis of GC cells. Therefore, our findings indicated that the activation of TGF-β/Smads signaling pathway promotes the development of GC, and understanding its functional mechanisms can provide new insight for the clinical treatment of GC.

m6A plays an important role in almost every biological process, especially in the development of cancer; as a result, many studies have focused on identifying aberrant m6A modifications as the new mechanism of tumor development. This study used bioinformatics analysis and experimental validation to demonstrate that the mRNA and protein levels of METTL3 were significantly increased in GC tissues and were negatively correlated with the survival of patients. Xenograft tumor formation in nude mice also further verified that METTL3 knockdown significantly inhibited tumor growth, indicating that the high expression of METTL3 promotes GC cancer development. Moreover, we found that METTL3 regulated TGF-β/smad pathway-associated gene expression as a methyltransferase, and, in particular, METTL3 modified Smad3 mRNA by m6A modification. Intriguingly, IGF2BP2 was identified to stabilize Smad3 mRNA, thereby elevating Smad3 protein level.

An increasing number of interactions between METTL3 and chromatin suggest that m6A is directly related to transcriptional regulation. In this study, Pearson analysis revealed that METTL3 was closely related to p-Smad3 in a variety of tumors. p-smad3, as one of the most important transcription factors in TGF-β/Smad signaling pathway, directly bind to the promoters of downstream target genes to regulate their transcription. Here, we identified a new mechanism of p-Smad3 regulation through its interaction with METTL3.

In conclusion, TGF-β/Smad2/3 signaling activity was upregulated in GC, which promoted GC progression. In addition, our study revealed a novel mechanism of TGF-β/Smad signal transduction regulation by METTL3, which provided the rationale for targeting METTL3 as a new therapeutic strategy for the treatment of patients with GC.

Acknowledgements

Our study was supported by the “Fundamental Research Funds for the Central Universities (Grant No. 2042022kf1209)” and the “National Natural Science Foundation of China (Grant No. 81972833)” and “Basic and Clinical Medical Research Joint Fund of Zhongnan Hospital of Wuhan University (Grant No. JCZN2022008)”. We would like to thank GEO (Gene Expression Omnibus) database (http://www.ncbi.nlm.nih.gov/geo/) and TCGA (The Cancer Genome Atlas) database (https://portal.gdc.cancer.gov/) for using their data.

Disclosure of conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Address correspondence to: Lei Wei, Department of Pathology and Pathophysiology, Hubei Provincial Key Laboratory of Developmentally Originated Disease, TaiKang Medical School (School of Basic Medical Sciences), Wuhan University, Wuhan 430071, Hubei, P. R. China. Tel: +86-15327396969; E-mail: leiwei@whu.edu.cn; Yi-Hao Tian, Department of Human Anatomy, TaiKang Medical School (School of Basic Medical Sciences), Wuhan University, Wuhan 430071, Hubei, P. R. China. Tel: +86-18971158569; E-mail: yihaotian@whu.edu.cn

References

[1] Araújo D, Ribeiro E, Amorim I and Vale N. Repurposed drugs in gastric cancer. Molecules 2022; 28: 319.

[2] Hata A and Chen YG. TGF-β signaling from receptors to smads. Cold Spring Harb Perspect Biol 2016; 8: a022061.

[3] Syed V. TGF-β signaling in cancer. J Cell Biochem 2016; 117: 1279-1287.

[4] Zhang J, Thorikay M, van der Zon G, van Dinther M and Ten Dijke P. Studying TGF-β signaling and TGF-β-induced epithelial-to-mesenchymal transition in breast cancer and normal cells. J Vis Exp 2020.

[5] Stojnev S, Krstić M, Čukuranović Kokoris J, Conić I, Petković I, Ilić S, Milosević-Stevanović J and Veličković LJ. Prognostic impact of canonical TGF-β signaling in urothelial bladder cancer. Medicina (Kaunas) 2019; 55: 302.

[6] Tao S, Liu M, Shen D, Zhang W, Wang T and Bai Y. TGF-β/smads signaling affects radiation response and prolongs survival by regulating DNA repair genes in malignant glioma. DNA Cell Biol 2018; 37: 909-916.

[7] Sun T, Wu R and Ming L. The role of m6A RNA methylation in cancer. Biomed Pharmacother 2019; 112: 108613.

[8] Wang T, Kong S, Tao M and Ju S. The potential role of RNA N6-methyladenosine in cancer progression. Mol Cancer 2020; 19: 88.

[9] Deng LJ, Deng WQ, Fan SR, Chen MF, Qi M, Lyu WY, Qi Q, Tiwari AK, Chen JX, Zhang DM and Chen ZS. m6A modification: recent advances, anticancer targeted drug discovery and beyond. Mol Cancer 2022; 21: 52.

[10] Vu LP, Pickering BF, Cheng Y, Zaccara S, Nguyen D, Minuesa G, Chou T, Chow A, Saletore Y, MacKay M, Schulman J, Famulare C, Patel M, Klimek VM, Garrett-Bakelman FE, Melnick A, Carroll M, Mason CE, Jaffrey SR and Kharas MG. The N(6)-methyladenosine (m(6)A)-forming enzyme METTL3 controls myeloid differentiation of normal hematopoietic and leukemia cells. Nat Med 2017; 23: 1369-1376.

[11] Zeng C, Huang W, Li Y and Weng H. Roles of METTL3 in cancer: mechanisms and therapeutic targeting. J Hematol Oncol 2020; 13: 117.

[12] Wan W, Ao X, Chen Q, Yu Y, Ao L, Xing W, Guo W, Wu X, Pu C, Hu X, Li Z, Yao M, Luo D and Xu X. METTL3/IGF2BP3 axis inhibits tumor immune surveillance by upregulating N(6)-methyladenosine modification of PD-L1 mRNA in breast cancer. Mol Cancer 2022; 21: 60.

[13] Wang Q, Chen C, Ding Q, Zhao Y, Wang Z, Chen J, Jiang Z, Zhang Y, Xu G, Zhang J, Zhou J, Sun B, Zou X and Wang S. METTL3-mediated m(6)A modification of HDGF mRNA promotes gastric cancer progression and has prognostic significance. Gut 2020; 69: 1193-1205.

[14] Liao J, Wei Y, Liang J, Wen J, Chen X, Zhang B and Chu L. Insight into the structure, physiological function, and role in cancer of m6A readers-YTH domain-containing proteins. Cell Death Discov 2022; 8: 137.

[15] Wang J, Chen L and Qiang P. The role of IGF2BP2, an m6A reader gene, in human metabolic diseases and cancers. Cancer Cell Int 2021; 21: 99.

[16] Petryszyn P, Chapelle N and Matysiak-Budnik T. Gastric cancer: where are we heading? Dig Dis 2020; 38: 280-285.

[17] Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90.

[18] Ahn JY, Jung HY, Choi KD, Choi JY, Kim MY, Lee JH, Choi KS, Kim DH, Song HJ, Lee GH, Kim JH and Park YS. Endoscopic and oncologic outcomes after endoscopic resection for early gastric cancer: 1370 cases of absolute and extended indications. Gastrointest Endosc 2011; 74: 485-493.

[19] Aikou S, Ohmoto Y, Gunji T, Matsuhashi N, Ohtsu H, Miura H, Kubota K, Yamagata Y, Seto Y, Nakajima A, Goldenring JR, Kaminishi M and Nomura S. Tests for serum levels of trefoil factor family proteins can improve gastric cancer screening. Gastroenterology 2011; 141: 837-845, e831-837.

[20] Leja M, Park JY, Murillo R, Liepniece-Karele I, Isajevs S, Kikuste I, Rudzite D, Krike P, Parshutin S, Polaka I, Kirsners A, Santare D, Folkmanis V, Daugule I, Plummer M and Herrero R. Multicentric randomised study of helicobacter pylori eradication and pepsinogen testing for prevention of gastric cancer mortality: the GISTAR study. BMJ Open 2017; 7: e016999.

[21] Anderson LA, Tavilla A, Brenner H, Luttmann S, Navarro C, Gavin AT, Holleczek B, Johnston BT, Cook MB, Bannon F and Sant M; EUROCARE-5 Working Group. Survival for oesophageal, stomach and small intestine cancers in Europe 1999-2007: results from EUROCARE-5. Eur J Cancer 2015; 51: 2144-2157.

[22] Waddell N, Pajic M, Patch AM, Chang DK, Kassahn KS, Bailey P, Johns AL, Miller D, Nones K, Quek K, Quinn MC, Robertson AJ, Fadlullah MZ, Bruxner TJ, Christ AN, Harliwong I, Idrisoglu S, Manning S, Nourse C, Nourbakhsh E, Wani S, Wilson PJ, Markham E, Cloonan N, Anderson MJ, Fink JL, Holmes O, Kazakoff SH, Leonard C, Newell F, Poudel B, Song S, Taylor D, Waddell N, Wood S, Xu Q, Wu J, Pinese M, Cowley MJ, Lee HC, Jones MD, Nagrial AM, Humphris J, Chantrill LA, Chin V, Steinmann AM, Mawson A, Humphrey ES, Colvin EK, Chou A, Scarlett CJ, Pinho AV, Giry-Laterriere M, Rooman I, Samra JS, Kench JG, Pettitt JA, Merrett ND, Toon C, Epari K, Nguyen NQ, Barbour A, Zeps N, Jamieson NB, Graham JS, Niclou SP, Bjerkvig R, Grützmann R, Aust D, Hruban RH, Maitra A, Iacobuzio-Donahue CA, Wolfgang CL, Morgan RA, Lawlor RT, Corbo V, Bassi C, Falconi M, Zamboni G, Tortora G, Tempero MA; Australian Pancreatic Cancer Genome Initiative, Gill AJ, Eshleman JR, Pilarsky C, Scarpa A, Musgrove EA, Pearson JV, Biankin AV and Grimmond SM. Whole genomes redefine the mutational landscape of pancreatic cancer. Nature 2015; 518: 495-501.