

ZNF740 facilitates the malignant progression of hepatocellular carcinoma via the METTL3/HIF-1A signaling axis

HAO ZHANG^{1,2}, BING HAN³, SHE TIAN², YONGJUN GONG² and LI LIU⁴

¹College of Clinical Medicine, Guizhou Medical University, Guiyang, Guizhou 561113, P.R. China; ²Department of Hepatobiliary Surgery, The Affiliated Hospital of Guizhou Medical University, Guiyang, Guizhou 550000, P.R. China;

³Guizhou Provincial Key Laboratory of Pathogenesis and Drug Research on Common Chronic Diseases,

Guizhou Medical University, Guiyang, Guizhou 561113, P.R. China; ⁴School of Public Health,

Guizhou Medical University, Guiyang, Guizhou 561113, P.R. China

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Abstract. Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death, and efficient treatments to facilitate recovery and enhance long-term outcomes are lacking. Zinc finger proteins (ZNFs), known as the largest group of transcription factors, have gained interest for their roles in HCC by stimulating the transcription of well-known tumor-causing genes. However, the specific roles and molecular mechanisms of ZNF740 in HCC remain unknown. The present study performed bioinformatics analysis and RNA-sequencing analysis of differentially expressed genes in HCC, detected ZNF740 expression levels in HCC using reverse transcription-quantitative PCR, western blotting and immunohistochemistry, and explored the effects of ZNF740 on the progression of liver cancer *in vitro* and *in vivo* using cellular functionality assays and cell-derived xenografts. In addition, a dual-luciferase reporter assay was performed to analyze the binding of ZNF740 with the METTL3 promoter. Furthermore, cell functionality experiments were performed to analyze whether ZNF740 promotes the proliferation of liver cancer cells in a METTL3-dependent manner. Bioinformatics and immunoprecipitation assays were further used to analyze the molecular mechanism of ZNF740 in liver cancer. The present study demonstrated that ZNF740 expression was

upregulated in HCC. Mechanistically, overexpressed ZNF740 interacted with the methyltransferase-like 3 (METTL3) promoter and increased METTL3 expression, leading to the stabilization of hypoxia-inducible factor-1A (HIF1A) mRNA in an N6-methyladenosine/YTH N6-methyladenosine RNA-binding protein 1-dependent manner. Eventually, the ZNF740/METTL3/HIF1A signaling axis may facilitate the proliferation, invasion and metastasis of liver cancer via METTL3/HIF-1A signaling. The present findings revealed the important role of ZNF740 and suggested a potential therapeutic approach that might improve clinical therapies for liver cancer.

Introduction

According to estimates, hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide and has been identified as the common form of primary liver cancer (1). Notably, patients with HCC often receive a late-stage diagnosis due to the underdevelopment of early clinical diagnostic techniques, resulting in a poor prognosis and an 18% global 5-year survival rate (2,3). Currently, there is a shortage of safe and effective medications to prevent HCC recurrence and that exert long-lasting results, and surgical intervention remains the primary preference for the clinical management of HCC (4,5). Despite being approved as primary medications for the treatment of advanced HCC by the United States Food and Drug Administration, sorafenib and lenvatinib have limited efficacy due to drug resistance and tumor relapse (6,7). Therefore, the identification of early diagnostic markers and new therapeutic targets is critical and urgently needed for the development of innovative antitumor drugs.

Mutated or dysregulated transcription factors (TFs), which are widely regarded as promising therapeutic targets, are widely reported to mediate aberrant gene expression in the malignant progression of HCC, thus facilitating proliferation and metastasis, and obstructing differentiation and cell death (8-10). Zinc finger (ZNF) proteins, the largest family of TFs in the human genome, participate in driving the initiation and malignant progression of various types of cancer, including HCC (11-13). For example, ZNF143 has been

Correspondence to: Professor Li Liu, School of Public Health, Guizhou Medical University, 6 Ankang Avenue, Guian New Area, Guiyang, Guizhou 561113, P.R. China
E-mail: liuli800402@126.com

Abbreviations: HCC, hepatocellular carcinoma; ZNF, zinc finger protein; TF, transcription factor; METTL3, methyltransferase-like 3; YTHDF, YTH N6-methyladenosine RNA-binding protein; HIF1A, hypoxia-inducible factor-1 α ; NPPA, natriuretic peptide precursor A; FBS, fetal bovine serum; m6A, N6-methyladenosine; EGFR, epidermal growth factor receptor

Key words: HCC, ZNFs, ZNF740, TFs, METTL3, HIF1A

reported to be upregulated in HCC cell lines and to facilitate the expression of CDC6, ultimately enhancing HCC cell proliferation, colony formation and tumor growth *in vitro* and *in vivo* (14). Furthermore, ZNF703 has been shown to trigger epithelial-to-mesenchymal transition and confer resistance to sorafenib in HCC by directly attaching to the CLDN4 promoter and activating CLDN4 expression (15). These results outline the tumor-promoting functions of ZNFs and offer insights for investigating targets and approaches for the clinical treatment of HCC.

ZNF740 belongs to the ZNF family and has been identified as a novel TF for natriuretic peptide precursor A (NPPA). NPPA has a significant role in cardiovascular diseases and congenital malformations, making it a reliable biomarker for diagnosis and prognosis (16). Several reports have identified NPPA as an independent prognostic indicator for breast cancer (17,18). Furthermore, ZNF740 has been shown to potentially engage in the regulation of the cell cycle, cellular adhesion and tumor proliferation (19-22), demonstrating a significant overlap of >20% with TP53 target genes (23). Nevertheless, the precise roles and molecular mechanisms of ZNF740 in HCC remain unclear.

The present study examined RNA sequencing (RNA-seq) data obtained from The Cancer Genome Atlas (TCGA) database. Among them, the differential expression of ZNF740 in HCC was more significant compared with other differentially expressed genes. Therefore, the present study focused on the role of ZNF740 in liver cancer, investigating the *in vitro* and *in vivo* biological functions and molecular mechanisms of ZNF740, to deepen the understanding of the role of ZNF740 in liver cancer and to clarify whether ZNF740 can be used as a reliable indicator for its diagnosis and as a potential target for clinical therapeutic intervention.

Materials and methods

Sample collection. The study population included 45 patients with HCC who underwent surgical treatment between September 2021 and September 2023 at the Department of Hepatobiliary Surgery, Guizhou Medical University (Guiyang, China). To ensure the accuracy of the study population, the following inclusion criteria were followed: i) Preoperative imaging diagnosis of HCC; ii) proposed laparoscopic resection or open surgery; and iii) postoperative pathological diagnosis of HCC. The exclusion criteria were as follows: i) Postoperative pathological diagnosis of benign tumors or malignant tumors that were not HCC; and ii) metastatic carcinoma from other tumors that metastasized to the liver. The age range of the patients with HCC was 13-88 years (median age, 56 years) and 85.1% of the patients were male. The collection and use of clinical specimens were approved by the Ethics Committee of Guizhou Medical University (approval no. 2021220; Guiyang, China), and each patient provided written informed consent.

Cell culture. HepG2 and Hep3B liver cancer cells were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. The cell lines were genetically confirmed by short tandem repeat analysis and were routinely tested for mycoplasma contamination. HepG2 and Hep3B cells were incubated in high-glucose DMEM and MEM

(both from Gibco; Thermo Fisher Scientific, Inc.), respectively, at 37°C and 5% CO₂. In addition, 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) were added to culture medium.

Gene expression profiling interactive analysis (GEPIA) for gene expression analysis. To investigate the expression pattern of ZNF740 in liver cancer, the online tool GEPIA (<https://gepia.cancer-pku.cn/>) was employed, which facilitates comprehensive analysis of RNA-seq data from TCGA project (<https://www.cancer.gov/ccg/research/genome-sequencing/tcga>). The GEPIA platform was accessed at the official GEPIA website, and LIHC; (Liver Hepatocellular Carcinoma) was specified as the cancer type of interest. The gene of focus, ZNF740, was entered into the search query to retrieve expression data. Utilizing GEPIA, a differential expression analysis was conducted between tumor and normal samples. The platform automatically adjusts for multiple testing and provides adjusted P-values to calculate the false discovery rate (FDR). Upon completion of the analysis, the graphical output and the underlying numerical data were exported for further statistical processing and integration into the current research findings.

Lentivirus infection. The cultivation of 293T cells, procured from Pricella Biotechnology Co., Ltd. In the context of the second-generation triple plasmid packaging system, the stoichiometric ratios of the plasmids were calibrated as follows: pMD2.G/packaging support plasmid/target plasmid (pHBLV-CMV-MCS-IRES-Puro), 2:3:4 (Addgene, Inc.). Concurrently, the mass ratio of plasmid DNA (in µg) to the volume of Lipofectamine® 2000 reagent (in µl) (Invitrogen; Thermo Fisher Scientific, Inc.) was meticulously adjusted to 8:10 to optimize the transfection efficiency. Following a 48-h incubation period at 37°C in a CO₂-controlled incubator, the culture medium was harvested, with the supernatant fraction being reserved. Transfection was performed utilizing Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), in conjunction with Opti-MEM medium (Gibco; Thermo Fisher Scientific, Inc.). The transfection protocol involved the introduction of 10 µg of the designated plasmids into the 293T cells. Post-transfection, the cells were subjected to a 12-h incubation phase with fresh medium, after which they are harvested 48 h post-transfection for downstream application.

To achieve stable ZNF740 knockdown, METTL3 knockdown, ZNF740 overexpression and METTL3 overexpression, recombinant lentiviruses expressing short hairpin RNA (shRNA/sh)-ZNF740, sh-METTL3, METTL3 and ZNF740 were prepared by Guangzhou RiboBio Co., Ltd. In addition, sh-control was used as a negative control for knockdown and empty vector was used as a negative control for overexpression. For cell culture, 5×10⁴ cells/well were equally distributed into 6-well plates. The degree of cell confluence was regulated at ~30% after the cells had fully attached to the well after ~10 h. The necessary virus titer was determined using the following formula: Viral titer (TU/ml)=MOI value x number of cells/viral volume (ml). A MOI of 10 was employed to infect the cells. For subsequent culture, lentivirus-containing culture media was used in lieu of the initial culture medium. After 12 h, the medium was replaced with fresh medium and

the cells were placed in a constant temperature incubator at a 37°C. Under a fluorescence microscope fitted with the appropriate filter sets for GFP detection, the expression of the EGFP gene could be detected in HepG2 and Hep3B liver cancer cells 72 h after infection, since the lentiviruses included the EGFP gene sequence. Furthermore, because the lentiviruses included a puromycin resistance gene sequence, cells were screened using 2 µg/ml puromycin (Dalian Meilun Biology Technology Co., Ltd.) to further reduce the impact of wild-type cells on subsequent investigations. Antibiotic resistance was mostly seen in the cell population following more than three rounds of subculturing. The protein and mRNA expression levels were assessed using reverse transcription-quantitative PCR (RT-qPCR) and western blotting, respectively. The shRNA sequences are as follows: sh-control, forward 5'-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTT-3', reverse 5'-AATTCAAAAACAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTG-3'; sh-ZNF740#1, forward 5'-CCGGGCTGCTCAAGAAACAAAGGAAGTTCGAGTTCCTTTGTTCTTGAGCAGCTTTT-3', reverse 5'-AATTCAAAAAGCTGCTCAAGAAACAAAGGAAGTTCGAGTTCCTTTGTTCTTGAGCAGC-3'; sh-ZNF740#2, forward 5'-CCGGGCGATATATGTGATATGCGTTCTCGAGAACGCATATCATATATCGCTTTT-3', reverse 5'-AATTCAAAAAGCGATATATGTGATATGCGTTCTCGAGAACGCATATCATATATCGC-3'; sh-METTL3#1, forward, 5'-CCGGGCTTAACATTGCCCACTGATCTCGAGATCAGTGGGCAATGTTAAGGCTTTT-3', reverse 5'-AATTCAAAAAGCTTAACATTGCCCACTGATCTCGAGATCAGTGGGCAATGTTAAGGC-3'; sh-METTL3#2, forward 5'-CCGGGAAGTATGTTCACTATGAACTCGAGTTTCATAGTGAACATACTTGCTTTT-3', reverse 5'-AATTCAAAAAGCAAGTATGTTCACTATGAACTCGAGTTTCATAGTGAACATACTTGC-3'.

RNA-seq analysis. Total RNA was extracted from liver cancer cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the quality of the RNA was evaluated with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). Subsequently, cDNA libraries were created with the TruSeq Stranded mRNA LT Sample Prep Kit (VAHTS Universal V6 RNA-seq Library Prep Kit for Illumina; cat. no. NR604-01; Vazyme, Inc.), according to the manufacturer's guidelines. The sequencing and analysis of the transcriptome were conducted on the Illumina HiSeq X Ten platform (Illumina, Inc.) using the HiSeq X Reagent Kit (cat. no. FC-501-2501; Illumina, Inc.). Nucleotide reads range from 75 bp using paired end sequencing mode. The BioAnalyzer 2100 (Agilent Technologies, Inc.) and the Ion Torrent™ General Library Quantification Kit (cat. no. A26217; Thermo Fisher Scientific, Inc.) were utilized for quality control and quantitative detection of the sequence libraries; the 10 pM sequence libraries were mutated into single-stranded DNA according to Illumina instructions, captured on an Illumina flow cell, clustered in situ, and clustered in the Illumina NovaSeq 6000 sequencer for 150 cycle sequencing. The raw data were subjected to quality control processing using Trimmomatic (24), which included the removal of low-quality reads. HISAT2 (25) was utilized to align the high-quality reads to the human genome

(GRCh38.p14), and counts were obtained for each gene. The DESeq2 package (version 1.40.2) in R (26) was utilized for conducting differential expression analysis. Statistical significance was assessed using a threshold of $P < 0.05$ and $\log_2 \text{Fold change} > 2$. The clusterProfiler package (version 3.17) in R (27) was utilized to perform functional enrichment analysis on the genes that were differentially expressed. Sequencing data have been uploaded to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE268479>) under the accession number GSE268479.

RT-qPCR. Total RNA was extracted from liver cancer cells and human liver tissues using TRIzol reagent. Follow the manufacturer's operating instructions, the PrimeScript™ RT reagent kit and gDNA Eraser kit (Takara Biotechnology Co., Ltd.) were used to perform RT, and qPCR was performed using the SYBR® Premix Ex Taq™ kit on a LightCycler 480 instrument (Takara Biotechnology Co., Ltd.). Briefly, qPCR was performed as follows: Initial denaturation was 95°C for 5 min; followed by 40 cycles of denaturation (95°C, 10 sec), annealing and extension (60°C, 30 sec). All reactions were conducted in a 20-µl reaction volume in triplicate. The relative mRNA expression levels were normalized to GAPDH levels. The comparative C_q ($2^{-\Delta\Delta C_q}$) method was used to determine the relative expression levels of mRNA (28). The RT-qPCR primers used were acquired from Sangon Biotech, Co., Ltd., as follows: GAPDH, forward 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse 5'-GGCTGTTGTCATACTTCTCATGG-3'; ZNF740, forward 5'-GCAGGTGTGAGTTGGTTCC-3', reverse 5'-CCTCAGCACATCAGGGCTAC-3'; METTL3, forward 5'-TTGTCTCCAACCTTCCGTAGT-3', reverse 5'-CCAGATCAGAGAGGTGGTGTAG-3'; hypoxia-inducible factor-1α (HIF1A), forward 5'-GAACGTCGAAAAGAAAAGTCTCG-3', reverse 5'-CCTTATCAAGATGCGAACTCACA-3'.

Western blotting. Cells and tissues were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology), which prevents protein degradation and the loss of phosphorylation. The BCA Protein Assay Kit (Beyotime Institute of Biotechnology) was used to determine the concentration of total proteins in accordance with the manufacturer's instructions. Equal quantities of protein extracts (20 µg) were separated by SDS-PAGE on 15% gels and were then transferred onto a PVDF membrane (MilliporeSigma). Subsequently, the PVDF membrane was blocked with skim milk for 1 h at 4°C and was incubated with primary antibodies overnight at 4°C. After washing with TBS-0.1% Tween three times, the membranes were incubated with a secondary antibody conjugated to HRP (1:1,000; cat. no. SA00001-2; Wuhan Sanying Biotechnology) for 60 min at 4°C. The presence of immunoreactivity was observed by employing an enhanced chemiluminescence system (Shanghai Yeasen Biotechnology Co., Ltd.), with GAPDH (1:50,000; cat. no. 60004-1-Ig; Wuhan Sanying Biotechnology) serving as a loading control. The following antibodies were used: Anti-ZNF740 (1:500; cat. no. 25411-1-AP), anti-METTL3 (1:500; cat. no. 15073-1-AP), anti-YTHDF1 (1:1,000; cat. no. 17479-1-AP), anti-YTHDF2 (1:2,000; cat. no. 24744-1-AP), anti-YTHDF3 (1:5,000; cat. no. 25537-1-AP) and anti-HIF1A

(1:2,000; cat. no. 20960-1-AP) (all from Wuhan Sanying Biotechnology).

Oligonucleotides. According to the manufacturer's instructions, cells were transfected with small interfering (si)RNAs using Lipofectamine RNAiMAX transfection reagent (cat. no. 13778-075; Invitrogen; Thermo Fisher Scientific, Inc.). The cells were cultured in 6-well plates until they achieved 60% confluence. To create the transfection mix, 150 μ l Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) was diluted with 7.5 μ l Lipofectamine RNAiMAX, and 150 μ l Opti-MEM was used to dilute 25 pmol siRNA. To create the siRNA-lipid combination, the diluted siRNA was mixed 1:1 with the Lipofectamine RNAiMAX that had previously been diluted. This compound was incubated at room temperature for 15 min to form a stable siRNA-lipid combination, which was used to transfect the cells for 6 h at 37°C. Subsequently, the cells were placed in medium containing 5% FBS and incubated at 37°C in a constant temperature incubator. The cells underwent RT-qPCR and western blot analysis 48 h after transfection, according to the aforementioned protocols. The siRNAs were purchased from Guangzhou RiboBio Co., Ltd. The si-control was used as a negative control and the siRNA sequences are as follows: si-control, forward 5'-CAACAAGAUGAA GAGCACCAAU-3', reverse 5'-UUGGUGCUCUUCUUCU UUGUUG-3'; si-METTL3, forward 5'-UCUACUCAGGA UCUGUAGCU-3', reverse 5'-CUACAGAUCCUGAGUUAG AGA-3'; si-YTH N6-methyladenosine RNA binding protein (YTHDF)1, forward 5'-UUAUCUUGUCCUUUUGU CUC-3', reverse 5'-GAACAAAAGGACAAGAUAAUA-3'.

Mouse xenograft models. Hep3B cells exposed to sh-control, sh-ZNF740#1 and sh-ZNF740#2 lentiviruses for were cultured in MEM supplemented with 10% FBS and 0.1 mM non-essential amino acids (Nanjing KeyGen Biotech Co., Ltd.). When cells reached the logarithmic phase of growth, they were collected and counted to establish a xenograft mouse model. A total of 18 BALB/c nude mice were allocated into three groups: sh-control, sh-ZNF740#1 and sh-ZNF740#2 (n=6/group). Briefly, to induce tumor growth, 0.1 ml PBS (Gibco; Thermo Fisher Scientific, Inc.) containing 2x10⁶ Hep3B cells was injected under the skin on the right side of the back of each BALB/c nude mouse (age, 4-5 weeks; weight, 18-20 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and were bred under pathogen-free conditions (temperature, 18-22°C; humidity, 50-60%; 12-h light/dark cycle). The bedding, feed and water were replaced every 2 days. The formula used to measure tumor volume was volume=(width)² x length/2, with width and length representing the shortest and longest diameters of the tumor, respectively. The Guizhou Medical University Animal Care Committee approved the present study (approval no. 2100616). The mice were sacrificed after 4 weeks of observation, or when they reached humane endpoints, such as a weight loss of 20% or behaving in a depressed manner. To reduce any possible pain or discomfort during the sacrifice, the mice were profoundly anesthetized with 5% isoflurane prior to cervical dislocation, and 3% isoflurane was administered for maintenance. Following the collection of the tumors, the tumor volume curve was created.

Immunohistochemistry (IHC). The tumor tissues collected from patients with liver cancer and from a mouse xenograft model were fixed in 4% paraformaldehyde at 4°C for 24 h and then embedded in paraffin. Sections of paraffin-embedded tissue (2-3 μ m) were dried for 60 min at 60°C and then subjected to two rounds of dewaxing in xylene for 30 min each. Subsequently, a decreasing gradient of alcohol concentrations was used for hydration. For antigen retrieval, the sections were heated in citrate buffer at 100°C for 10 min. The sections were then blocked with 5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) for 1 h at room temperature, and 3% H₂O₂ was used to block endogenous peroxidase/phosphatase activity for 10 min at room temperature. Subsequently, the sections were incubated overnight at 4°C with the following primary antibodies: Anti-ZNF740 (1:1,000; cat. no. 25411-1-AP), anti-Ki67 (1:2,000; cat. no. 27309-1-AP), anti-METTL3 (1:750; cat. no. 15073-1-AP) and anti-HIF1A (1:50; cat. no. 20960-1-AP) (all from Wuhan Sanying Biotechnology). The sections were then treated with secondary antibodies labeled with biotin (1:1,000; cat. no. SA00001-2; Wuhan Sanying Biotechnology) for 1 h at room temperature and incubated with an avidin solution labeled with horseradish peroxidase (HRP) (Wuhan Sanying Biotechnology) for 10 min at room temperature. DAB was used to identify positive staining, followed by a 3-min application of hematoxylin as a counterstain. Finally, the sections were dried by progressively adding ethanol, as well as xylene, and were subsequently sealed using neutral balsam. Representative images were obtained using a fluorescence microscope (Olympus Corporation) at a magnification of x200. Cells were categorized into grades based on the intensity of staining at the membrane, nucleus or cytoplasmic location, and then the percentage of the number of cells in each grade was counted: Grade 1 (0-10% intensity) was scored as 1 point; grade 2 (11-40% intensity) was scored as 2 points; grade 3 (41-70% intensity) was scored as 3 points; and grade 4 (71-100% intensity) was scored as 4 points. Finally, all of the scores were added up to obtain the total score.

Wound healing assay. In each well of a 6-well plate, 1x10⁷ HepG2 or Hep3B cells were cultured in DMEM or MEM supplemented with 10% FBS. When the cell confluence reached 90%, a scratch was created using a 200- μ l pipette tip. Subsequently, the cells were incubated in FBS-free medium for 24 h at room temperature. Representative images were obtained under a fluorescence microscope (Olympus Corporation) at 0 and 24 h. Mobility was assessed using the following formula: Cell migration rate (%)=(0 h scratch width-scratch width after incubation)/0 h scratch width x100.

Transwell assay. HepG2 or Hep3B cells (1x10⁵/well) were seeded and cultured in the upper chamber of a 24-well Transwell plate (pore size, 8 μ m) in FBS-free medium. In addition, 600 μ l medium supplemented with 10% FBS was added to the lower chamber of the Transwell plate. Subsequently, the dish was placed in a humidified environment with 5% CO₂ and was maintained at 37°C for 48 h. After incubation, the cells that had not migrated to the lower chamber were removed from the upper chamber, and the migrated cells were fixed with 4% formaldehyde for 10 min at room temperature and stained with 0.1% crystal violet for 10 min at room temperature. To

determine the quantity of cells that had migrated, five arbitrary fields were chosen for cell counting under a fluorescence microscope. In addition, 100 μ l Matrigel in FBS-free media was added to the upper chamber and incubated at 37°C for 4 h to conduct the Transwell invasion assay. The procedures were conducted in a similar fashion as aforementioned for the migration assay.

Colony formation assay. HepG2 or Hep3B cells transfected with siRNAs or infected with lentiviruses were seeded in 6-well plates at a density of 1×10^3 cells/well. After being cultured for 2 weeks, the cells were fixed with 4% paraformaldehyde for 15 min and then subjected to staining with 0.5% crystal violet for 10 min at room temperature. Colonies consisting of >100 cells were counted manually and included in the analysis to quantify colony formation.

Cell Counting Kit-8 (CCK-8) assay. The CCK-8 assay (Shanghai Yeasen Biotechnology Co., Ltd.) was conducted in accordance with the manufacturer's guidelines. Briefly, 96-well plates were seeded with HepG2 or Hep3B cells at a density of $\sim 4 \times 10^3$ cells/well 24 h after transfection or infection. The cells were incubated for 2 h at 37°C in the dark with 10 μ l CCK-8 reagent. The optical density was then measured using a microplate reader (Tecan Group, Ltd.) at 450 nm.

Luciferase reporter assay. METTL3 mutants were constructed, and a dual-luciferase reporter assay was performed to identify the interaction sites between METTL3 and ZNF740. The mutation sites and wild-type sequences are as follows: METTL3 binding sequences -813 to -804: 5'-CCCTCCCCAC-3'; -758 to -749: 5'-CCGCCCCCTC-3'; -296 to -287: 5'-AAACCCCCAA-3'. The corresponding mutant sequences were as follows: P1: 5'-AAAGAAAACA-3'; P2: 5'-AATAAAAAGA-3'; P3: 5'-CCCAAAAACC-3'. Lipofectamine 2000 was used to transfect HepG2 and Hep3B cells when they reached $\sim 70\%$ confluence. To conduct luciferase reporter assays, 100 ng METTL3-promoter luciferase plasmid and 2 ng *Renilla* luciferase control reporter plasmid, which was used to ensure accurate transfection efficiency, were transfected into HepG2 and Hep3B cells in 96-well plates. The day after transfection, the Dual-Luciferase Reporter Assay System (Promega Corporation) was used to measure the fluorescence intensities. Briefly, HepG2 and Hep3B cells transfected with the luciferase reporter plasmids were lysed in 20 μ l 1X lysis solution and agitated at room temperature for 20 min. Then, 10 μ l cellular lysate was mixed with 50 μ l Luciferase Assay Reagent II. Subsequently, the activity of firefly luciferase was measured for 10 sec using a luminometer, after which, 50 μ l Stop & Glo Reagent was added to the mixed solution, and the *Renilla* luciferase activity was measured for 10 sec. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Each assay was performed at least in triplicate to ensure statistical robustness.

RNA stability assay. Actinomycin D (ActD; MilliporeSigma) is a commonly used transcription inhibitor that intercalates into the DNA double helix, blocking the activity of RNA polymerase, thus inhibiting the synthesis of new mRNA. After treating cells with ActD, the half-life of mRNA can be

calculated by quantitatively measuring the decrease of mRNA over time, thereby assessing its stability. HepG2 and Hep3B cells from different groups were placed in 6-well plates and exposed to 10 μ g/ml ActD. At the onset of the experiment (i.e., the 0-h time point), cell samples were collected to serve as the experimental control group. Subsequently, cell samples were collected again at the 2, 4, 6 and 8-h time points at 37°C. The collection of samples at these various time points allowed for comparative analysis to observe and quantify the degradation rate and half-life of mRNA under the influence of ActD. By measuring the levels of mRNA at different time points, the stability of mRNA could be inferred. RT-qPCR was performed according to previously described methods (24).

Chromatin immunoprecipitation (ChIP) assay. According to the manufacturer's protocol, a ChIP assay kit (cat. no. ab500; Abcam) was used to perform the ChIP assay. After HepG2 and Hep3B cells were crosslinked, lysed and sonicated, immunoprecipitation was performed using the anti-ZNF740 antibody (1:500; cat. no. 25411-1-AP; Wuhan Sanying Biotechnology) or IgG (1:2,000; cat. no. 30000-0-AP; Wuhan Sanying Biotechnology). Subsequently, the precipitated DNA was extracted and subjected to RT-qPCR as aforementioned.

Survival analysis. The 45 patients with HCC, from whom clinical samples were collected, were followed up to assess the survival time of the patients. Survival data were plotted using Kaplan-Meier survival curves and the log-rank test was used to determine the P-value. For survival analysis, a threshold can be set to define samples with expression above the threshold as a high expression group and samples below the threshold as a low expression group. In the present study, the median value of ZNF740 expression level was set as the cutoff value for distinguishing the high (n=23) and low (n=22) expression groups.

Receiver operating characteristic (ROC) curve. In the present study, ROC curves were analyzed using ZNF740 expression levels from data collected from the 45 clinical samples. The area under the curve (AUC) reflects the magnitude of the value of the diagnostic test; the larger the area, the closer it is to 1.0, the higher the diagnostic trueness; the closer it is to 0.5, the lower the diagnostic trueness; and when it equals 0.5, there is no diagnostic value. AUC=0.5 to 0.7, lower accuracy; AUC=0.7 to 0.9, some accuracy; AUC >0.9, high accuracy.

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. Raw sequencing reads from liver cancer cells were preprocessed using FastQC (Babraham Bioinformatics) and Trimmomatic (24). Differential expression analysis was performed using the DESeq2 R package (26) with a P-value cutoff of 0.05 and FDR correction. KEGG enrichment analysis was conducted with the clusterProfiler R package (29), identifying pathways significantly enriched with differentially expressed genes. Visualization of the enrichment results was achieved using ggplot2 (27). All analyses were conducted using R version 3.6.1 (29).

Promoter region analysis. The reference sequence of the METTL3 gene was obtained from the NCBI GenBank database (Gene ID: 56339; <https://www.ncbi.nlm.nih.gov/gene/56339>).

The promoter region 2,000-3,000 base pairs upstream of the transcription start site was predicted using FGENESH software (version number 7.2.2; Softberry Inc.). Potential transcription factor binding sites within promoter regions were predicted using ALIEN software (<http://www.genome.jp/tools/alien/>) and the TRANSFAC database (BioBase GmbH). MatInspector (version number 2.2; Genomatix Software Suite) was used to further analyze these sites.

Immunoprecipitation (IP) assay. Cells were lysed in RIPA Buffer (cat. no. 89901; Cell Signaling Technology, Inc.), which contained a mixture of protease inhibitors (Roche Diagnostics) to inhibit proteolytic degradation. The cell lysate was then incubated with the primary antibodies anti-YTHDF1 (1:1,000; cat. no. 17479-1-AP), anti-YTHDF2 (1:2,000; cat. no. 24744-1-AP), anti-YTHDF3 (1:5,000; cat. no. 25537-1-AP) and IgG (1:2,000; cat. no. 30000-0-AP) (all from Wuhan Sanying Biotechnology), at a concentration of 1:250 total protein, overnight at 4°C with constant rotation. Protein A/G Plus-Agarose beads used for immunocomplex capture were purchased from Santa Cruz Biotechnology, Inc. These beads were pre-washed and pre-incubated with non-specific mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) to reduce non-specific binding. The antibody-protein complex was isolated by adding the Protein A/G agarose beads to the lysate and incubating for 2 h at 4°C with rotation. The agarose beads with bound immune complexes were pelleted by centrifugation at 1,000 x g for 5 min at 4°C and washed five times with ice-cold PBS (pH 7.4). The immune complexes were eluted by boiling in 2X Laemmli buffer (Bio-Rad Laboratories, Inc.) for 5 min at 95°C. The eluted proteins were resolved by SDS-PAGE on 15% gels using a Mini-PROTEAN® TGX gel (Bio-Rad Laboratories, Inc.) and were transferred onto a PVDF membrane. Subsequently, western blotting was performed as aforementioned.

Gene set enrichment analysis (GSEA). GSEA of the RNA-seq results comparing METTL3-overexpressing cells and control cells was performed in the present study. GSEA was performed using GSEA software version 4.3.3 (Broad Institute; <https://www.gsea-msigdb.org/gsea/downloads.jsp>) (26). For GSEA, the Molecular Signature Database v6.2 was used, which is integrated into the GSEA software suite and provides a comprehensive collection of annotated gene sets for GSEA use (26). The expression data consists of a matrix of gene expression values for different samples, which has been prepared in supported file formats such as txt. Each row in the dataset represents a gene, each column represents a sample, and the expression values indicate the level of gene expression measured under specific conditions. The GSEA software calculates an enrichment score (ES) for each gene set. The ES was then statistically evaluated to determine its significance and the FDR was calculated to correct for multiple hypothesis testing. The results of the GSEA were interpreted by examining the ES, normalized ES (NES), P-value and FDR q-value for each gene set.

Statistical analysis. Statistical analyses were conducted using SPSS 23.0 (IBM Corporation) or GraphPad Prism 7 (Dotmatics) software. Each experiment was repeated three

times. A paired or unpaired Student's t-test was used to compare the two groups. One-way ANOVA with Tukey's multiple comparisons test was used to compare more than two groups. The Kaplan-Meier method and log-rank test were used to generate and compare differences between survival curves. For statistical analysis of IHC-related data the Wilcoxon signed-rank test for paired data. Correlation analysis was performed using the Pearson correlation coefficient, -1 indicates a perfect negative correlation, 0 indicates no correlation, and 1 indicates a perfect positive correlation. The χ^2 test was used to analyze whether there was a significant association between categorical variables. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ZNF740 is upregulated in patients with HCC. To examine the involvement of ZNF740 in HCC progression, RNA-seq data acquired from TCGA database were investigated; of which 369 cases were tumor tissues and 160 cases were normal tissues. ZNF740 expression was significantly greater in HCC tissues than in adjacent healthy tissues (Fig. 1A and B). To confirm these results, RT-qPCR analysis of ZNF740 mRNA expression levels was conducted in a cohort of 45 patients with HCC to verify the increased expression of ZNF740 in HCC tissues (Fig. 1C and D). Analysis of this cohort of 45 patients revealed that ZNF740 protein expression was significantly increased in 42.1% of patients with HCC compared with the median value of ZNF740 expression in HCC samples. Another 45.6% of patients with HCC had upregulated ZNF740 expression levels in tumor tissues compared with in normal tissues, but the difference was not significant. In addition, 12.3% of patients had lower ZNF740 expression levels in tumor tissues than in normal tissues. Overall, the protein expression level of ZNF740 was upregulated in liver cancer tumor tissues (Fig. 1E). These findings were further confirmed by performing IHC and western blotting, which both revealed elevated expression of ZNF740 in HCC samples compared with in nontumor samples (Fig. 1F-H). Increased ZNF740 expression was associated with a poor prognosis in patients with HCC, as shown by the overall survival curve plotted on the basis of the collected samples (Fig. 1I). To validate the prognostic accuracy of ZNF740, a ROC/AUC analysis was performed and exhibited good performance, with an AUC value of 0.944, suggesting that ZNF740 has high accuracy as a biological marker for HCC (Fig. 1J). These findings indicated that ZNF740 may be upregulated in HCC and that elevated levels of ZNF740 could be linked to an unfavorable prognosis among patients with HCC.

ZNF740 enhances the invasive and proliferative capacity of liver cancer cells. To clarify the biological functions of ZNF740 in liver cancer, lentiviral vectors were used to achieve stable overexpression or knockdown of ZNF740 in HepG2 and Hep3B cells. The knockdown efficiency of sh-ZNF740 was detected using RT-qPCR and western blotting; sh-ZNF740 effectively knocked down the expression levels of ZNF740 in liver cancer cells (Fig. S1A). ZNF740 overexpression lentivirus effectively upregulated the expression levels of ZNF740 in liver cancer cells (Fig. S1B). A wound healing

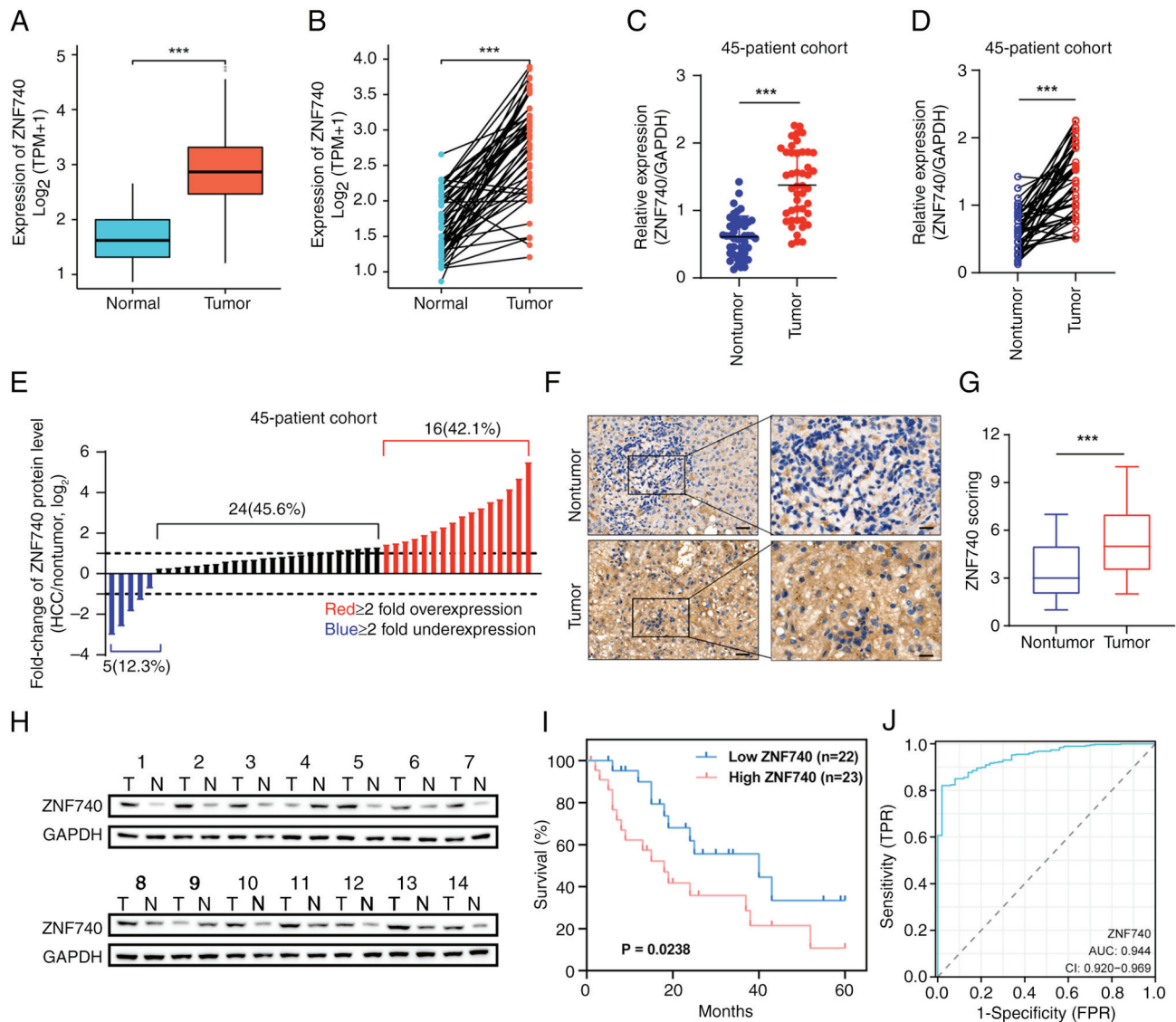


Figure 1. ZNF740 is aberrantly upregulated and associated with poor prognosis in patients with HCC. (A) Box plots of transcript levels of ZNF740 in HCC specimens and paired adjacent normal tissues. (B) Scatter matrix plot of transcript levels of ZNF740 in HCC specimens and paired adjacent normal tissues. (A and B) Data were obtained from The Cancer Genome Atlas Project database. (C) Reverse transcription-quantitative PCR analysis of ZNF740 mRNA expression in HCC specimens from 45 patients and paired adjacent normal tissues in a honeycomb plot. (D) Scatter matrix of reverse transcription-quantitative PCR analysis of ZNF740 mRNA expression in HCC specimens from 45 patients and paired adjacent normal tissues. (E) ZNF740 protein levels in HCC specimens and paired adjacent normal tissues from a 45-patient cohort, as determined by paired analysis using IHC analysis. (F) Representative images of IHC analysis of ZNF740 in HCC specimens and paired adjacent normal tissues. Scale bars: Left, 200 μ m; right, 50 μ m. (G) Semi-quantification of IHC analysis of ZNF740 in HCC specimens and paired adjacent normal tissues. (H) Western blotting of ZNF740 in HCC specimens and paired adjacent normal tissues. (I) Survival curves for patients with HCC based on ZNF740 expression levels derived from collected clinical samples (n=45). Patients were split into high and low expression groups using median mRNA expression levels from transcriptome data. (J) Receiver operating characteristic curve evaluating the performance of the predictive model based on ZNF740 expression. ***P<0.001. AUC, area under the curve; HCC, hepatocellular carcinoma; IHC, immunohistochemical; N, nontumor; T, tumor; ZNF740, zinc finger protein 740.

assay demonstrated that ZNF740 overexpression significantly improved the migratory capacity of liver cancer cells (Fig. 2A). By contrast, specific knockdown of ZNF740 decreased the migration of liver cancer cells (Fig. 2B). In addition, the migration and invasion of HepG2 and Hep3B cells were significantly enhanced by ZNF740 overexpression, but were significantly inhibited by ZNF740 knockdown (Fig. 2C and D). The impact of ZNF740 on liver cancer cell proliferation was also assessed by conducting a colony formation assay and analyzing proliferation curves. Colony formation and proliferation were significantly increased in ZNF740-overexpressing liver cancer cells, whereas colony formation and proliferation

were significantly reduced in ZNF740-knockdown cells (Fig. 2E-H). These results indicated that ZNF740 may serve a vital role in enhancing liver cancer cell invasion, migration and proliferation.

Specific knockdown of ZNF740 inhibits HCC proliferation in a mouse xenograft model. To determine the effects of ZNF740 on the proliferation of tumors *in vivo*, a mouse xenograft model of Hep3B cells was generated. In line with the *in vitro* findings, specific knockdown of ZNF740 in Hep3B cells led to the development of smaller tumor masses and decreased tumor weight in comparison to those in the control group

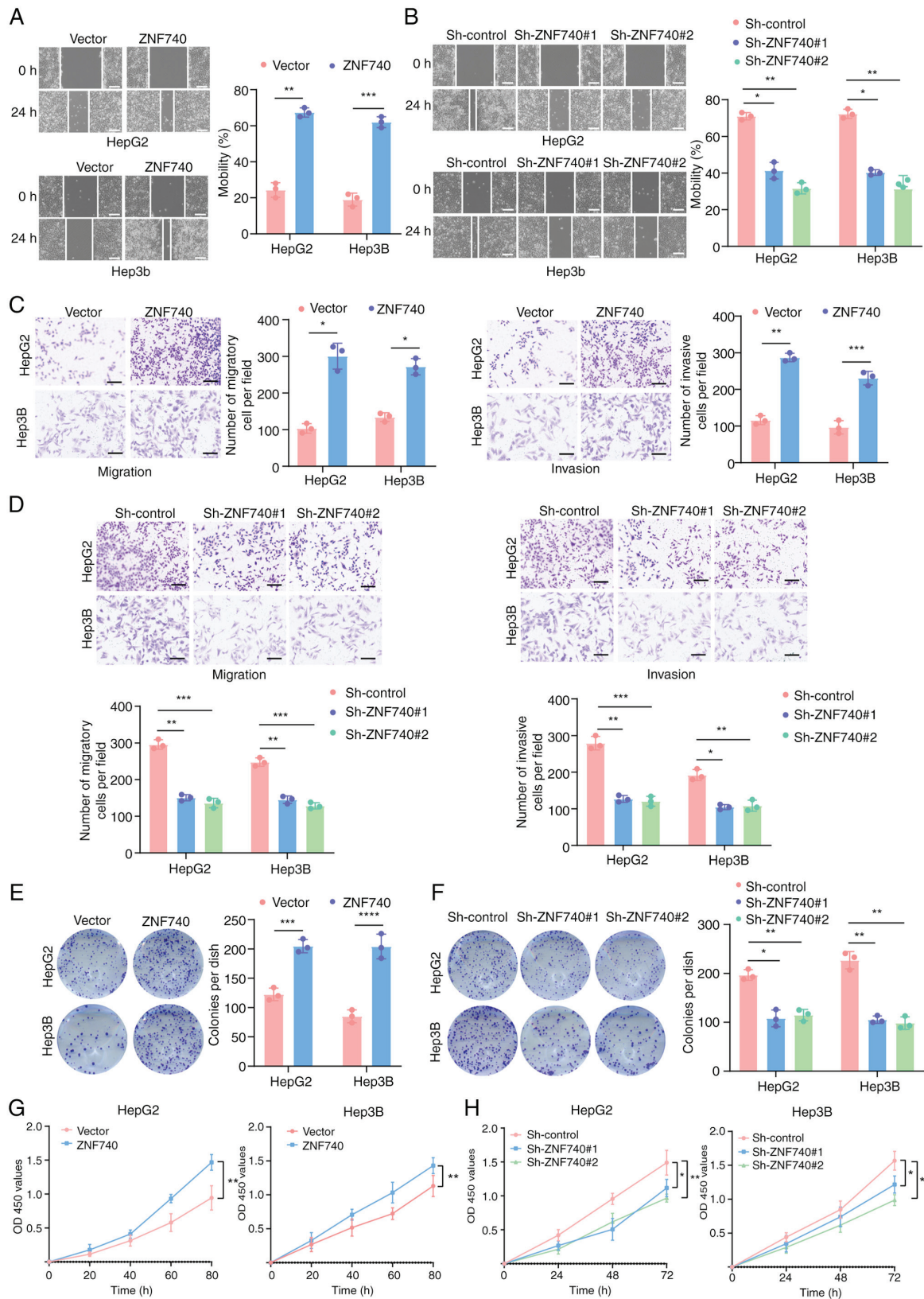


Figure 2. ZNF740 enhances the proliferative and invasive capacity of liver cancer cells *in vitro*. (A) Representative images and quantification of wound healing assays demonstrating the effect of overexpression of ZNF740 on cell migration. Scale bars, 50 μ m. (B) Representative images and quantification of wound healing assays demonstrating the effect of ZNF740 knockdown on cell migration. Scale bars, 50 μ m. (C) Representative images and quantification of Transwell assays illustrating the impact of overexpression of ZNF740 on cell invasion and migration. Scale bars, 200 μ m. (D) Representative images and quantification of Transwell assays illustrating the impact of ZNF740 knockdown on cell invasion and migration. Scale bars, 200 μ m. (E) Representative images and quantification of colony formation assays, highlighting the influence of overexpression of ZNF740 on cell colony formation. (F) Representative images and quantification of colony formation assays, highlighting the influence of ZNF740 knockdown on cell colony formation. (G) Cell proliferation curve analysis during long-term observation, depicting the role of overexpression of ZNF740 in cell proliferation. (H) Cell proliferation curve analysis during long-term observation, depicting the role of ZNF740 knockdown in cell proliferation. Data are presented as the mean $\pm$ SD from three independent experiments. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001. sh, short hairpin; ZNF740, zinc finger protein 740.

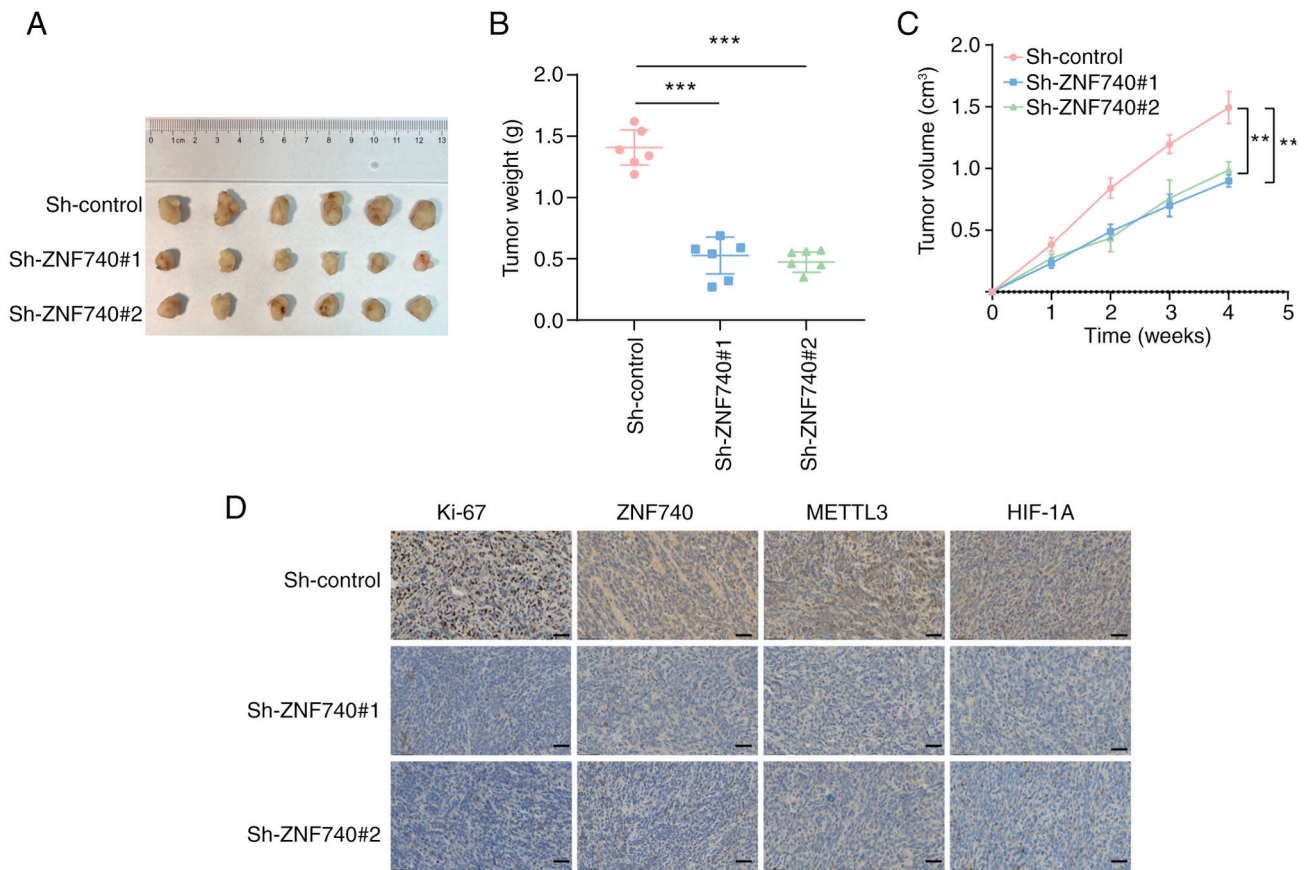


Figure 3. Specific knockdown of ZNF740 inhibits liver cancer proliferation in a mouse xenograft model. (A) Representative images of tumor nodules from the Hep3B subcutaneous tumor model in each group. (B) Tumor weights among the different groups (n=6). (C) Relative tumor volumes of different groups (n=8). Data are presented as the mean $\pm$ SD. (D) Representative images of immunohistochemical analysis of Ki-67, ZNF740, METTL3 and HIF1A expression in tumor sections from different groups. Scale bars, 50 μ m. **P<0.01, ***P<0.001. HIF1A, hypoxia-inducible factor-1 α ; METTL3, methyltransferase-like 3; sh, short hairpin; ZNF740, zinc finger protein 740.

(Fig. 3A and B). Moreover, the volume of tumor generated from Hep3B cells with targeted ZNF740 knockdown was significantly reduced after 4 weeks of xenograft transplantation (Fig. 3C). Furthermore, the results of IHC demonstrated a decrease in the ratio of Ki-67-positive cells in the ZNF740 knockdown group, along with a decrease in the expression of METTL3 and HIF1A (Fig. 3D). Collectively, these results provided convincing evidence that ZNF740 may have a vital role in enhancing the proliferation of HCC cells in a mouse xenograft model.

ZNF740 enhances the expression of the transcription factor METTL3. To examine the possible molecular mechanisms underlying the effects of ZNF740 on liver cancer, RNA-seq analysis was performed on HepG2 cells in the ZNF740-specific knockdown (sh-ZNF740#2) and control groups. The findings indicated notable differences in gene expression between the ZNF740-specific knockdown group and the control group. In the ZNF740-specific knockout group, METTL3 expression was notably reduced (Fig. 4A and B). Furthermore, KEGG enrichment analysis revealed a significant alteration in the 'RNA polymerase' (gene counts: 21; P=2.8 $\times 10^{-2}$), 'mRNA surveillance pathway' (gene counts: 18; P=6.4 $\times 10^{-2}$), 'RNA degradation' (gene counts: 20; P=1.9 $\times 10^{-3}$), 'ATP-dependent chromatin remodeling' (gene counts: 12; P=1.1 $\times 10^{-5}$), 'Spliceosome'

(gene counts: 11; P=2.3 $\times 10^{-2}$), 'Ubiquitin mediated proteolysis' (gene counts: 13; P=9.6 $\times 10^{-3}$), 'Nucleocytoplasmic transport' (gene counts: 10; P=1.7 $\times 10^{-2}$), 'Lysine degradation' (gene counts: 12; P=2.0 $\times 10^{-2}$) and 'Thermogenesis' (gene counts: 17; P=6.4 $\times 10^{-2}$) in the ZNF740-specific knockdown group (Fig. 4C). These results indicated that ZNF740 could have an essential function in controlling METTL3 expression and regulating RNA modification in liver cancer cells. METTL3 is a methyltransferase mainly involved in N6-methyladenosine (m6A) modifications of RNA. These modifications are one of the most common epigenetic modifications on eukaryotic mRNAs, and have an important impact on several processes, such as RNA stability, splicing, nucleation, degradation and translation (30). Therefore, it was hypothesized that a regulatory relationship may exist between ZNF740 and METTL3. To confirm the regulatory connection between ZNF740 and METTL3, the effect of stable overexpression or knockdown of ZNF740 on METTL3 mRNA expression was examined. The findings indicated that ZNF740 overexpression markedly elevated the mRNA expression levels of METTL3, whereas ZNF740 knockdown significantly decreased METTL3 mRNA expression levels (Fig. 4D and E). Western blotting of HepG2 and Hep3B lysates supported this conclusion (Fig. 4F). To further investigate the linkage between ZNF740 and the METTL3 promoter, the sequence of the binding motifs of

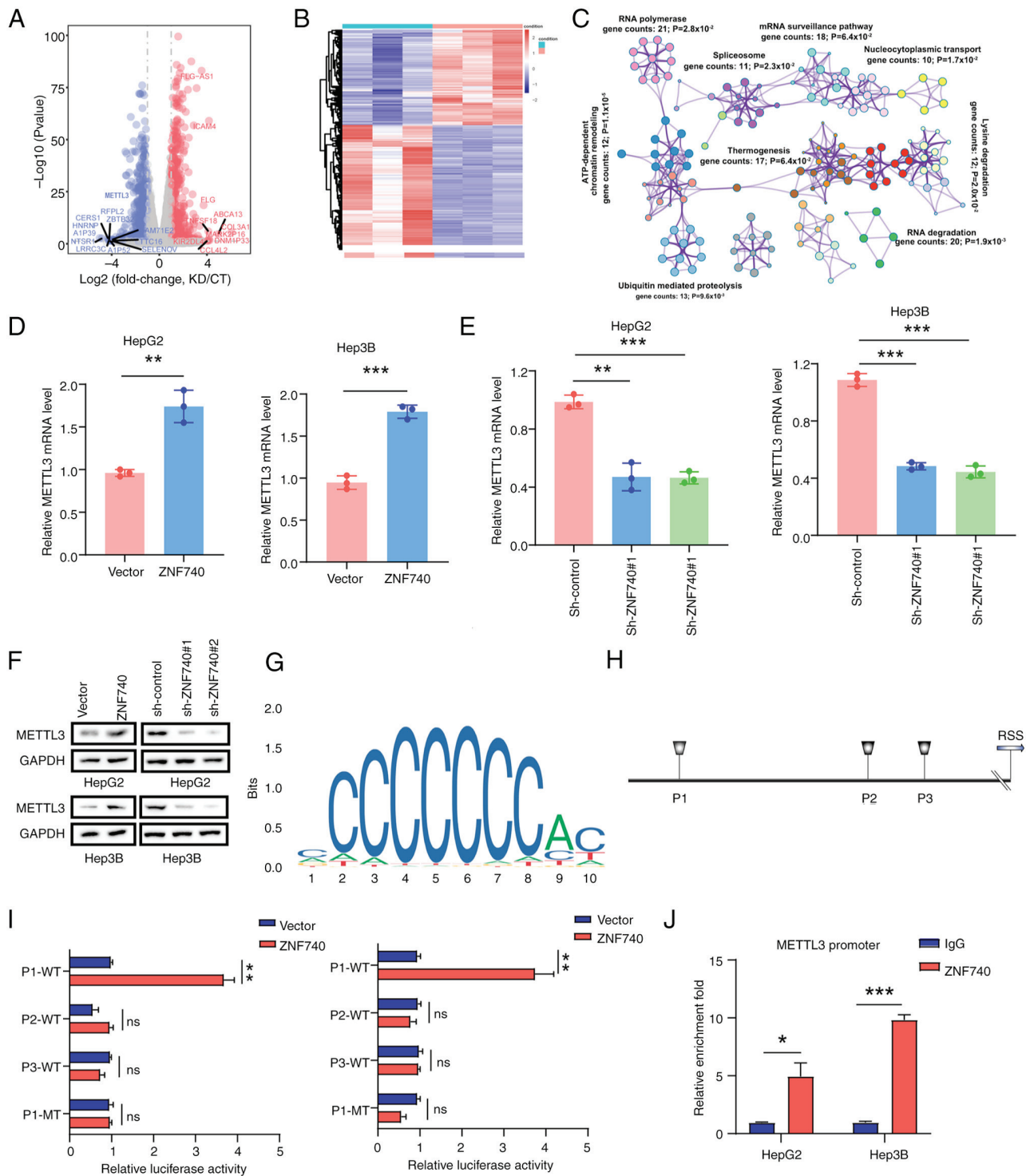


Figure 4. ZNF740 enhances the expression of the transcription factor METTL3. (A) Volcano plot depicting the differential expression of genes between ZNF740-knockdown and control HepG2 cells. (B) Heatmap illustrating the expression patterns of differentially expressed genes in HepG2 cells with ZNF740-specific knockdown and in the control group. (C) Kyoto Encyclopedia of Genes and Genomes pathway analysis demonstrating the significant alterations in RNA modification signaling pathways among differentially expressed genes. (D) Reverse transcription-quantitative PCR was performed to analyze the effect of overexpression of ZNF740 on METTL3 mRNA expression levels ($n=3$). (E) Reverse transcription-quantitative PCR analysis of the effect of knockdown of ZNF740 expression on METTL3 mRNA expression level ($n=3$). (F) Western blotting showing METTL3 protein expression levels in the different groups. (G) Binding motif sequences of ZNF740. (H) Schematic representation of the different motifs present in the METTL3 promoter region. (I) Dual-luciferase reporter assay measuring the activity of the METTL3 promoter with different motifs ($n=3$). (J) Chromatin immunoprecipitation results demonstrating the interaction of ZNF740 with the promoter region of METTL3 in HepG2 and Hep3B cells. (D, E, I and J) Data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. METTL3, methyltransferase-like 3; sh, short hairpin; ZNF740, zinc finger protein 740; KD, knockdown; CT, control.

ZNF740 was examined and a schematic representation of the different motifs (P1-P3) in the METTL3 promoter region is shown in Fig. 4G and H. Dual luciferase reporter assays were

performed to determine the activities of the different motifs of the METTL3 promoter, and the results showed that the P1 motif showed the most differential changes in METTL3

promoter activity after ZNF740 overexpression; thus, the P1 motif may be considered the binding site of ZNF740 to the METTL3 promoter (Fig. 4I). Subsequently, a ChIP assay was performed and it was demonstrated that ZNF740 could directly bind to the P1 motif on the promoter of METTL3 (Fig. 4J). Considering these findings, a regulatory connection between ZNF740 and METTL3 was revealed, and ZNF740 was identified as a novel TF of METTL3.

ZNF740 promotes liver cancer cell proliferation in a METTL3-dependent manner. To further explore the vital role of ZNF740 in enhancing the invasive and proliferative abilities of liver cancer by controlling the transcription of METTL3, ZNF740 was stably overexpressed in the HepG2 and Hep3B cell lines. Moreover, siRNAs were utilized to suppress METTL3 expression. The knockdown efficiency of si-METTL3 was detected using RT-qPCR and western blotting (Fig. S1C). After long-term observation, the migration and invasion of HepG2 and Hep3B cells were significantly increased by ZNF740 overexpression, whereas no such effects were observed in cells also transfected with si-METTL3 (Fig. 5A and B). The findings from the wound healing assay led to similar conclusions (Fig. 5C). Similarly, the proliferation of HepG2 and Hep3B cells was significantly increased by ZNF740 overexpression, whereas ZNF740 overexpression had no impact on the colony formation or proliferation of si-METTL3-transfected liver cancer cells (Fig. 5D and E). Rescue assays using si-METTL3 successfully inhibited the tumor-promoting effects of ZNF740 on the proliferation of liver cancer cells. These findings indicated that the effects of ZNF740 may rely on its ability to control METTL3 transcription.

METTL3 recruits YTHDF1 to regulate HIF1A mRNA stability. Since METTL3 is an important m6A writer (30), the present study further examined whether METTL3 exerted regulatory effects on key oncogenic signaling pathways via m6A modification in liver cancer. Infection with the METTL3 overexpression lentivirus effectively increased the expression levels of METTL3 in liver cancer cells. The comparison of gene sets between HepG2 cells overexpressing METTL3 and control cells using GSEA revealed significant activation of the HIF1A signaling pathway (Fig. 6A). The knockdown efficiency of sh-METTL3 was detected using RT-qPCR and western blotting (Fig. S1D). Subsequently, RT-qPCR and western blotting were conducted to confirm the regulatory effects of METTL3 on HIF1A. According to the RT-qPCR analysis findings, the mRNA expression levels of HIF1A were significantly greater in HepG2 and Hep3B cells with METTL3 overexpression, whereas the expression levels were significantly decreased upon METTL3 knockdown (Fig. 6B and C). Infection with the METTL3 overexpression lentivirus effectively increased the expression levels of METTL3 in liver cancer cells (Fig. S1E). Similarly, the findings from western blotting indicated a notable increase in the protein levels of HIF1A in HepG2 and Hep3B cells with METTL3 overexpression, whereas a marked decrease was observed upon METTL3 knockdown (Fig. 6D). In addition, HIF1A mRNA stability was investigated by inhibiting fresh RNA production in HepG2 and Hep3B cells using ActD. The degradation efficiency of HIF1A mRNA was detected in ActD-treated HepG2 and Hep3B cells 8 h

after METTL3 overexpression, and the results suggested that METTL3 overexpression slowed the degradation efficiency of HIF1A mRNA (Fig. 6E). IP is a laboratory technique used to isolate and enrich specific proteins from cell lysates; this method is commonly used to study protein interactions. To identify the m6A readers involved in this process, IP assays were performed with YTHDF1, YTHDF2, YTHDF3 and METTL3. The results showed a direct link between YTHDF1 and METTL3, but not between YTHDF2 or YTHDF3, thus suggesting that YTHDF1 can function in conjunction with METTL3 (Fig. 6F). The knockdown efficiency of si-YTHDF2 was detected using RT-qPCR and western blotting (Fig. S1F). Subsequently, a HIF1A mRNA stability assay was performed and demonstrated that YTHDF1 gene silencing could reverse the biological effects of METTL3 overexpression (Fig. 6G). Similarly, the RT-qPCR and western blotting results indicated a notable increase in relative mRNA and protein expression levels of HIF1A due to METTL3 overexpression; however, the expression levels were reduced in the METTL3 + si-YTHDF1 group (Fig. 6H-I). These findings indicated that METTL3 may enhance the proliferation of liver cancer cells by controlling the level of HIF1A expression in an m6A modification-dependent manner, with YTHDF1 acting as the m6A reader.

ZNF740 expression is significantly associated with the METTL3/HIF1A signaling axis. To further validate the clinical relevance of ZNF740, METTL3 and HIF1A, correlation analysis was performed on the 45-patient and TCGA cohorts. The r-values were all greater than 0. TCGA database analysis showed that the correlation analysis between ZNF740 and METTL3 had an r-value of 0.73, and the correlation analysis between ZNF740 and VEGFA had an r-value of 0.51. Due to the limited number of samples collected, the correlation of ZNF740 with METTL3, HIF1A and VEGFA detected in the clinical samples showed a positive correlation, but it was very weak ($r < 0.3$) (Fig. 7A and B); therefore, further analysis in subsequent studies is required. The results were additionally validated through IHC staining of ZNF740, METTL3 and HIF1A. Distinguishing patients with high and low expression based on median immunohistochemistry score revealed that the levels of METTL3 and HIF1A were notably decreased in samples with low ZNF740 expression (Fig. 7C). The clinical significance of ZNF740, METTL3 and HIF1A was confirmed by these findings, indicating the notable therapeutic efficacy and potential utilization of ZNF740.

Discussion

Over the last few decades, there have been extensive reports indicating that TFs have significant functions in enhancing the expression of crucial oncogenes and facilitating malignant advancement in HCC (31-34). For example, the upregulation of YAP/TAZ in human HCC can enhance HCC cell proliferation by promoting the transcription of ANLN and KIF23 (34). TEA domain (TEAD) transcription factors are a class of transcription factors that serve important roles in biological development, cell proliferation, tissue regeneration and tissue homeostasis (35). The combination of sorafenib and TEAD inhibitors has been suggested as a promising future treatment for HCC (35). This suggests that studying the mechanisms of transcription factors

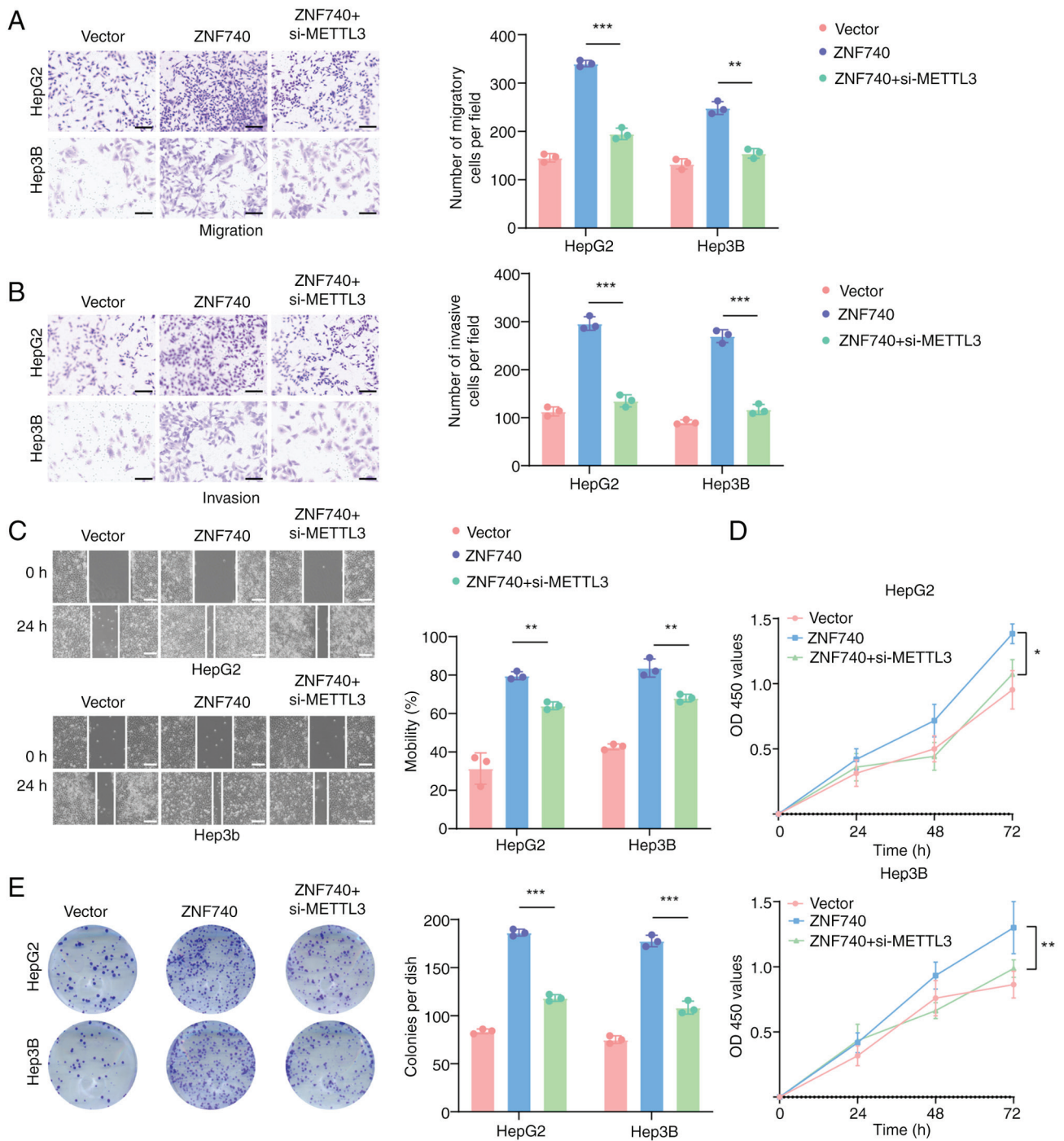


Figure 5. ZNF740 enhances liver cancer cell proliferation and migration in a METTL3-dependent manner. (A) Representative images and quantification of Transwell assays illustrating the impact of ZNF740 on cell migration via METTL3. Scale bars, 200 μ m. (B) Representative images and quantification of Transwell assays illustrating the impact of ZNF740 on cell invasion via METTL3. Scale bars, 200 μ m. (C) Representative images and quantitative analysis of wound healing assays demonstrating the effect of ZNF740 on cell migration via METTL3. Scale bars, 50 μ m. (D) Cell proliferation curve analysis during long-term observation showing the role of ZNF740 in cell proliferation via METTL3. (E) Representative images and quantitative analysis of colony formation assays, highlighting the influence of ZNF740 on cell colony formation via METTL3. Data are presented as the mean $\pm$ SD from three independent experiments. * P <0.05, ** P <0.01, *** P <0.001. METTL3, methyltransferase-like 3; si, small interfering; ZNF740, zinc finger protein 740.

in HCC may provide new opportunities for HCC treatment. Notably, ZNF proteins, which are the largest subfamily of TFs, have attracted increasing interest and have been shown to serve important roles in HCC by enhancing the transcription of well-established oncogenes in recent years (11). For example, ZNF384 has been recognized as an independent prognostic predictor for patients with HCC and as a tumor-causing gene

in HCC progression. Mechanistically, ZNF384 can directly increase Cyclin D1 expression, leading to HCC cell proliferation (36). Furthermore, zinc finger and SCAN domain containing 20 (ZSCAN20) is a transcription factor that contains zinc finger and SCAN structural domains, and it has been confirmed that ZSCAN20 has an important role in HCC cell invasion, migration and proliferation. Further examination has indicated

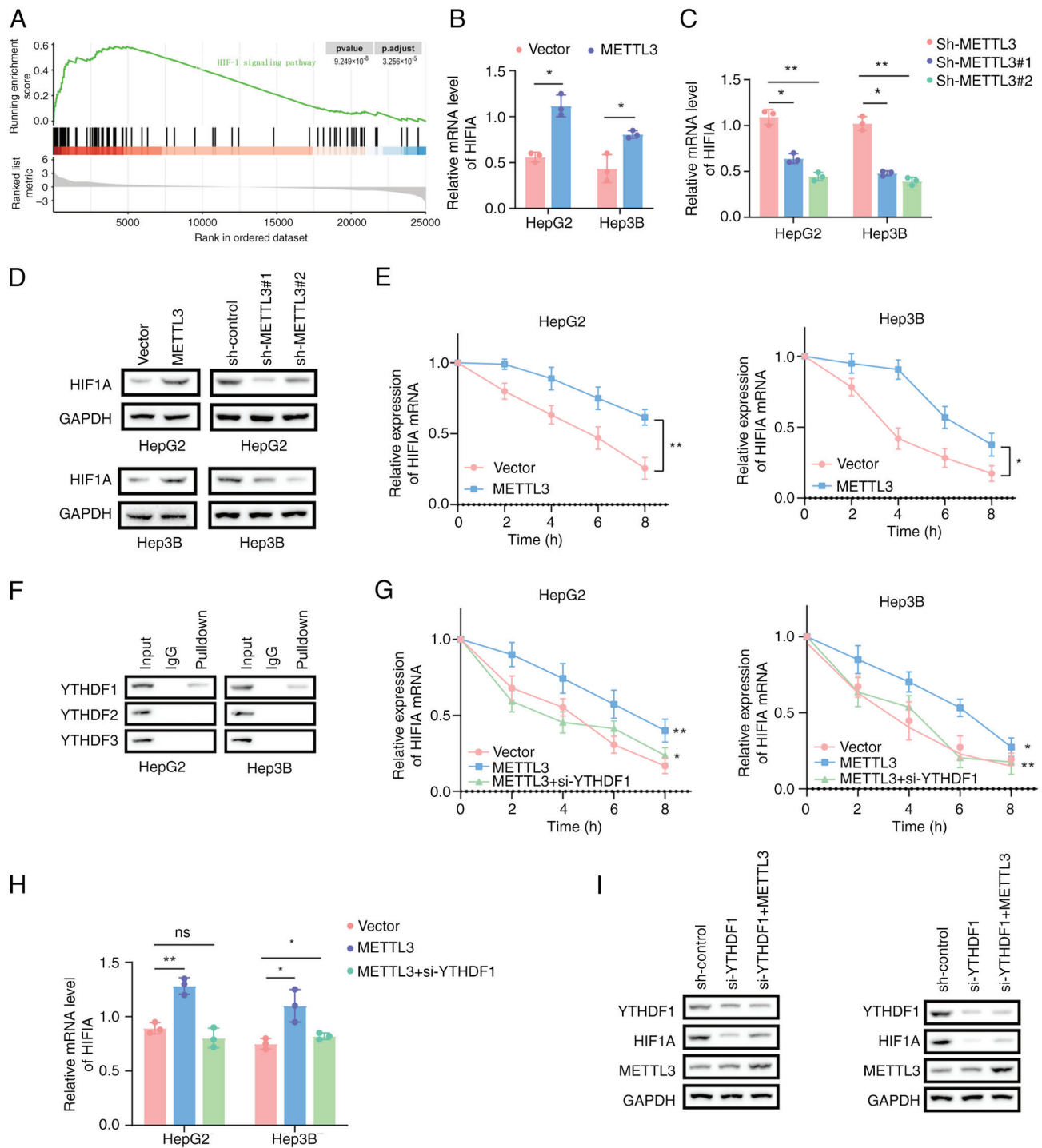


Figure 6. METTL3 recruits YTHDF1 to regulate HIF1A mRNA stability. (A) Gene set enrichment analysis of the RNA sequencing results comparing METTL3-overexpressing cells and control cells. (B) RT-qPCR analysis of the effect of overexpression of METTL3 on HIF1A mRNA expression (n=3). (C) RT-qPCR analysis of the effect of knockdown of METTL3 expression on HIF1A mRNA expression (n=3). (D) Western blotting showing HIF1A protein expression levels in the different groups. (E) RNA stability assays showed that METTL3 overexpression greatly slowed the degradation of HIF1A mRNA. (F) Immunoprecipitation assay showing the direct interaction between METTL3 and YTHDF1 (n=3). (G) RNA stability assays revealed that YTHDF1 gene silencing reversed the biological effects of METTL3 overexpression (n=3). (H) RT-qPCR analysis of HIF1A mRNA expression in different groups (n=3). (I) Western blotting showing HIF1A protein expression levels in the different groups. (B, C, E and G-I) Data are presented as the mean $\pm$ SD. *P<0.05, **P<0.01. HIF1A, hypoxia-inducible factor-1 α ; METTL3, methyltransferase-like 3; RT-qPCR, reverse transcription-quantitative PCR; sh, short hairpin; si, small interfering; YTHDF, YTH N6-methyladenosine RNA-binding protein.

a strong correlation between the expression of ZSCAN20 and genes associated with m6A modifications, as well as with the cell cycle and immune infiltration (37). Several studies have also shown that ZNF233 and ZNF689 significantly contribute to unfavorable prognosis among patients with HCC (38,39).

ZNF740, a member of the ZNF subfamily, has been identified as a novel TF that regulates NPPA expression (16). However, the precise roles of ZNF740 in HCC and other malignancies remain largely unknown. In the present study, using a colony formation assay, Transwell assay and xenograft

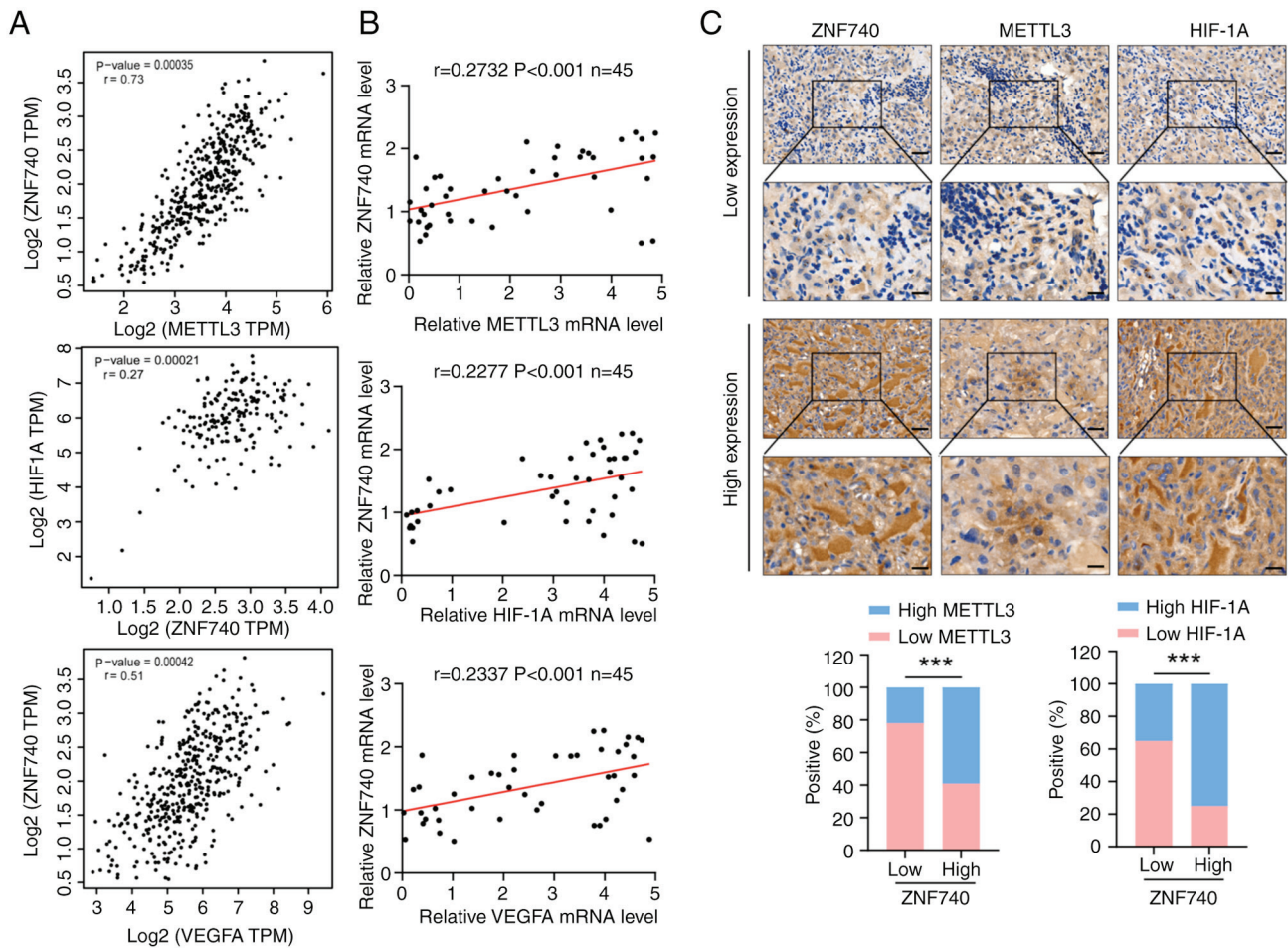


Figure 7. ZNF740 expression is significantly associated with the METTL3/HIF1A signaling axis. (A) Correlation analysis between the expression of ZNF740, METTL3, HIF1A and VEGFA in The Cancer Genome Atlas cohort. The data were first log-transformed and then statistically analyzed using Pearson correlation analysis. (B) Correlation analysis between the expression of ZNF740, METTL3, HIF1A and VEGFA in the 45-patient cohort. Data were analyzed using Pearson correlation analysis for statistical analysis. (C) Representative images and semi-quantification of immunohistochemical analysis of ZNF740 in hepatocellular carcinoma specimens. Data were analyzed using the χ^2 test. Scale bars: Upper, 200 μ m; lower, 50 μ m. ***P < 0.001. HIF1A, hypoxia-inducible factor-1 α ; METTL3, methyltransferase-like 3; ZNF740, zinc finger protein 740.

model, the results revealed that ZNF740 may facilitate the growth and invasion of liver cancer both *in vitro* and *in vivo*. To the best of our knowledge, the present findings revealed, for the first time, the elevated expression and tumor-causing function of ZNF740 in liver cancer, indicating that ZNF740 could serve as a promising target for liver cancer therapy.

METTL3 specifically acts as a methyltransferase enzyme, which is an essential component of the RNA methyltransferase complex and is responsible for the addition of m6A to RNA molecules (40). The tumor-promoting effect of METTL3 has been extensively documented in various types of cancer, such as breast cancer, colorectal cancer (CRC) and HCC (41-43). For example, METTL3 can facilitate the expression of BHLHE41 in an m6A-dependent manner, leading to the subsequent activation of CXCL1 transcription and ultimately enhancing the migration of myeloid-derived suppressor cells (42). In addition, the combination of anti-PD1 therapy and targeted suppression of METTL3 has been shown to exhibit encouraging effectiveness in combating CRC (42). Furthermore, METTL3 has been reported to enhance epidermal growth factor receptor (EGFR) expression by introducing m6A modifications to EGFR mRNA, ultimately

leading to the development of lenvatinib resistance in HCC. *In vitro* and *in vivo* experiments have revealed that METTL3 is a promising therapeutic target, and the application of a specific METTL3 inhibitor can increase the sensitivity of HCC to lenvatinib (43). The findings of the present RNA-seq analysis, RT-qPCR, ChIP and luciferase assays identified ZNF740 as a TF for METTL3. ZNF740 may bind to the P1 motif on the METTL3 promoter, leading to a significant increase in METTL3 transcription levels. These findings offer new perspectives on the dysregulation of METTL3 expression in liver cancer, despite the previous identification of several other TFs, such as p300 and GFI1, that are associated with METTL3 in various types of cancer (44-46).

Generally, the excessive proliferation of HCC cells consumes a considerable quantity of oxygen and forms a hypoxic tumor microenvironment (TME) (47). Notably, the essential regulators in the TME, known as HIFs, have been verified to facilitate various characteristics of cancer that contribute to the malignant progression of HCC and therapeutic resistance (48,49). HIFs are assembled from a functional α subunit and a constitutively expressed β subunit, which activate several major TFs and promote various cancer

hallmarks as heterodimers, including extracellular matrix remodeling, angiogenesis and aberrant glucose metabolism (5,50-52). For example, it has been shown that HIF-1A can cause the upregulation of YTHDF1, leading to the stabilization of ATG2A and ATG14 in an m6A-dependent manner, thereby controlling autophagy and metastasis in HCC (50). The HIF-1A/YTHDF1 signaling axis is a promising target for clinical cancer therapy. Furthermore, it has been discovered that HIF-1 may be linked to hypoxia-induced micropinocytosis in HCC, resulting in the absorption of extracellular fluids and promoting cell proliferation, whereas specific knockdown of HIF-1 was shown to significantly inhibit HCC progression and proliferation (51,52). Iron deficiency in HCC cells has been shown to inhibit sorafenib-induced apoptosis by increasing HIF-1A and BCL-2 to promote sorafenib resistance (53). It has also been reported that transarterial chemoembolization induces S100 calcium-binding protein A9 (S100A9) via a HIF1A-mediated pathway (54). S100A9 serves as a scaffold that recruits ubiquitin-specific peptidase 10 and phosphoglycerate mutase family member 5 (PGAM5) to form a trimer, resulting in the deubiquitination and stabilization of PGAM5, leading to mitochondrial fission and reactive oxygen species production, thereby promoting HCC growth and metastasis (54). Furthermore, tumor tissues from patients with HCC have been reported to possess upregulation of HIF-1A, and the interaction between NRF2 and HIF-1A has been confirmed (55). HIF-1A is hydroxylated under normoxic conditions by a particular HIF-prolyl hydroxylase domain protein (PHD), which makes it easier for HIF-1A to be ubiquitinated and degraded via proteases. By directly binding to the oxygen-dependent degradation domain of HIF-1A, NRF2 contributes to pseudohypoxia by impeding PHD2-mediated hydroxylation, von-Hippel-Lindau recruitment and HIF-1A ubiquitination (55). In the present study, it was demonstrated that METTL3 enhanced the stability of HIF1A mRNA through an m6A-related mechanism, thereby triggering the HIF1A signaling pathway and stimulating the proliferation of liver cancer cells. Moreover, based on the IP assay and rescue experiments, YTHDF1 was identified as the m6A reader in the METTL3/HIF1A signaling pathway. These findings enhance the understanding of m6A modifications and the hypoxic TME in liver cancer.

In conclusion, the present investigation provides a detailed understanding of TFs and m6A alterations in liver cancer. The present study revealed that ZNF740 expression was abnormally increased and associated with an unfavorable prognosis in patients with HCC. Mechanistically, overexpression of ZNF740 interacted with the P1 motif of the METTL3 promoter, leading to an increase in METTL3 and resulting in the stabilization of HIF1A mRNA in an m6A/YTHDF1-dependent manner. Thus, liver cancer progression, including the invasion and proliferation of liver cancer cells, may be ultimately facilitated by the ZNF740/METTL3/HIF1A signaling pathway. This study identified an important role of ZNF740 and suggested a potential therapeutic approach that might improve clinical therapies for liver cancer.

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Availability of data and materials

The data generated in the present study may be found in the Gene Expression Omnibus under accession number GSE268479 or at the following URL: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE268479>. All other data generated in the present study may be requested from the corresponding author.

Authors' contributions

LL contributed to the experimental design and supervision. HZ, ST and YG contributed to the experimental implementation, writing, reviewing and editing. HZ and BH contributed to the acquisition of data. LL and HZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All procedures performed were in accordance with the ethical standards of the Ethical Committee of Guizhou Medical University (approval no. 2021220; Guiyang, China), the Guizhou Medical University Animal Care Committee (approval no. 2100616), and with The Declaration of Helsinki of 1964 and later versions. All human samples were obtained with the patients' written informed consent. All institutional and national guidelines for the care and use of laboratory animals were followed.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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