

YTHDF1 Facilitates the Progression of Hepatocellular Carcinoma by Promoting FZD5 mRNA Translation in an m6A-Dependent Manner

Xiangxiang Liu,^{1,2} Jian Qin,¹ Tianyi Gao,¹ Chenmeng Li,^{1,2} Bangshun He,¹ Bei Pan,¹ Xueni Xu,^{1,2} Xiaoxiang Chen,¹ Kaixuan Zeng,^{1,2} Mu Xu,¹ Chengbin Zhu,³ Yuqin Pan,¹ Huiling Sun,¹ Li Sun,⁴ Tao Xu,¹ and Shukui Wang^{1,2,5}

¹School of Medicine, Southeast University, Nanjing 210096, Jiangsu, China; ²General Clinical Research Center, Nanjing First Hospital, Nanjing Medical University, Nanjing 210006, Jiangsu, China; ³Department of Laboratory Medicine, Nanjing First Hospital, Nanjing Medical University, Nanjing 210006, Jiangsu, China; ⁴Department of Laboratory Medicine, The Second Affiliated Hospital of Nanjing Medical University, Nanjing 210011, Jiangsu, China; ⁵Jiangsu Collaborative Innovation Center on Cancer Personalized Medicine, Nanjing Medical University, Nanjing 211100, Jiangsu, China

Hepatocellular carcinoma (HCC), one of the most aggressive malignancies, ranks as the fourth leading cause of cancer-related deaths worldwide. Emerging evidence indicates that RNA N6-methyladenosine (m6A) plays a critical role in tumor progression. However, the biological function of YTHDF1 in HCC remains unclear. Here, we found that YTHDF1 expression was strikingly elevated in HCC tissues and cell lines and significantly associated with prognosis of HCC patients. Moreover, YTHDF1 expression was transcriptionally regulated by USF1 and c-MYC in HCC. Functional studies showed that YTHDF1 can promote HCC cell proliferation and metastasis both *in vitro* and *in vivo*. Multi-omics analysis revealed that YTHDF1 can accelerate the translational output of FZD5 mRNA in an m6A-dependent manner and function as an oncogene through the WNT/ β -catenin pathway. Taken together, our study revealed an essential role of YTHDF1 in the progression of HCC cells, which indicated that targeting YTHDF1 may be a potential therapeutic strategy in HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC), an aggressive malignancy, ranks as the fourth leading cause of cancer-related deaths worldwide.¹ Due to the inconspicuous symptoms, the vast majority of HCC patients are first diagnosed at advanced stages leading to only 30%–40% of patients being eligible for curative resection.² Despite advances in treatment and surgical methods, the overall survival (OS) rate of HCC patients with metastasis and disease recurrence remains unfavorable.³ Therefore, a better understanding of the molecular mechanisms of HCC and novel therapeutic strategies for HCC treatment are urgently needed.

Previously, epigenetic regulation mainly focused on the chemical modifications of DNA and histones. However, cellular RNAs also carry hundreds of distinct posttranscriptional modifications among which N6-methyladenosine (m6A) is the most abundant modification occurring in eukaryotic mRNAs.^{4,5} m6A modification of

mRNA is a dynamic and reversible process in mammalian cells that is regulated by two types of catalytic proteins (m6A “writer” and “eraser”).⁶ The m6A writer is a methyltransferase complex whose core components include METTL3, METTL14, and WTAP.^{7,8} In contrast, FTO and ALKBH5 (m6A eraser) act as the demethylases to reverse the methylation.^{9,10} Recent investigations revealed that diverse regulatory machinery can be recruited to m6A-containing mRNAs through different m6A binding proteins (m6A “readers”), and therefore impact the fate of the target mRNAs, including mRNA stability,^{11,12} nuclear export,¹³ primary microRNAs (miRNAs) processing,¹⁴ and mRNA translation.^{15,16} YTHDF1, one of the m6A readers, can enhance the translation efficiency of m6A-modified mRNA in a cap-independent manner.¹⁵ Although YTHDF1 has been reported to be associated with ovarian cancer progression,¹⁷ axon guidance,¹⁸ anticancer immunotherapy,¹⁹ and hippocampus-dependent learning and memory,²⁰ its role in HCC has not been investigated.

In this study, we demonstrated that YTHDF1 is strikingly upregulated in HCC and significantly associated with prognosis of HCC patients. We discovered that elevated YTHDF1 is mainly regulated by c-MYC and USF1 and promotes HCC cells proliferation and metastasis by increasing the translational efficiency of m6A-modified FZD5 mRNA. In addition, YTHDF1 exerts function as an oncogene through WNT/ β -catenin pathway in HCC.

RESULTS

YTHDF1 Expression Is Significantly Upregulated in HCC and Associated with Poor Prognosis

To explore the regulatory role of YTHDF1 in HCC, we first analyzed its expression. The TCGA database, GSE14520 dataset,²¹ and

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Correspondence: Shukui Wang, School of Medicine, Southeast University, Nanjing 210096, Jiangsu, China.

E-mail: sk_wang@njmu.edu.cn



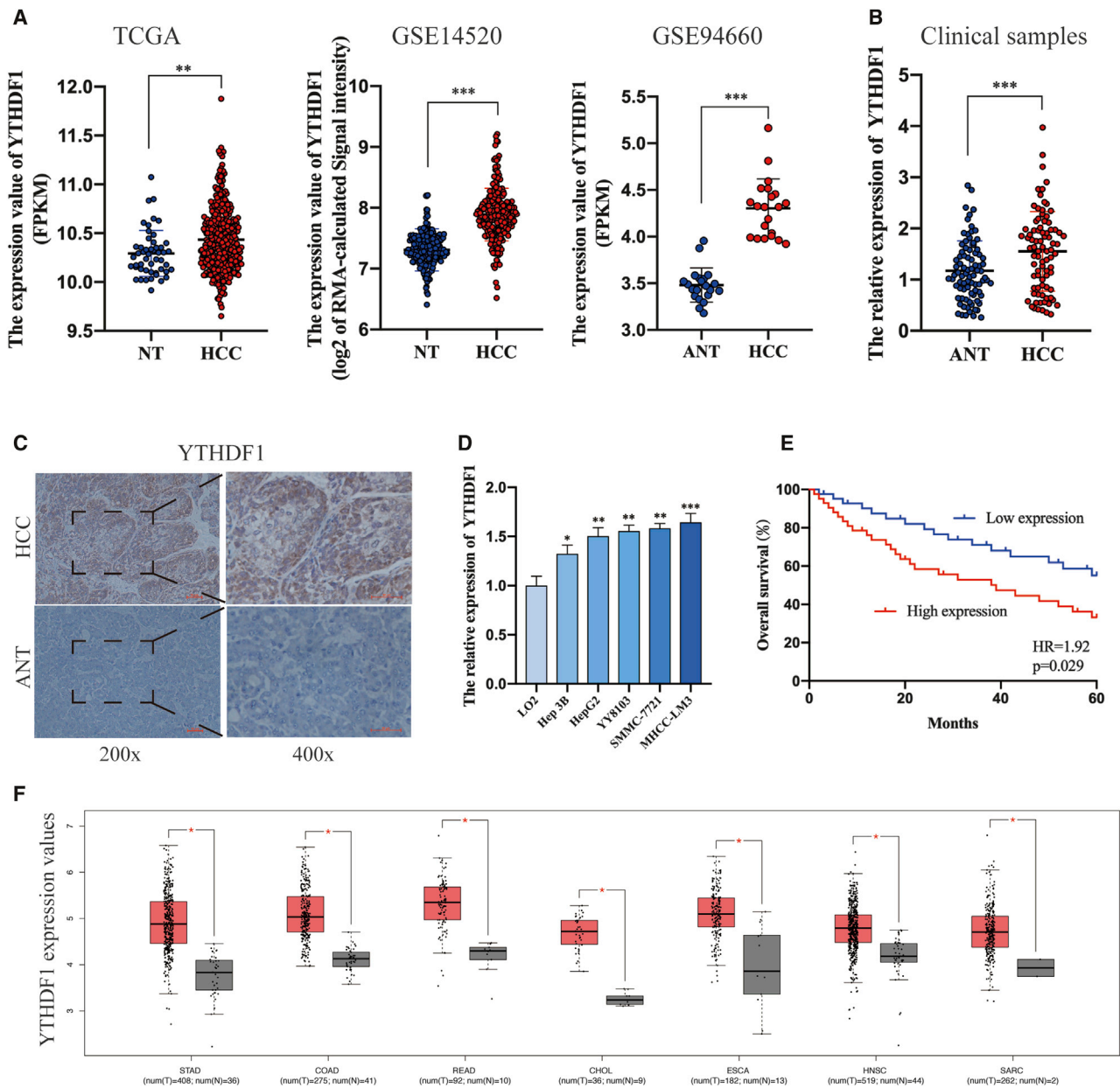
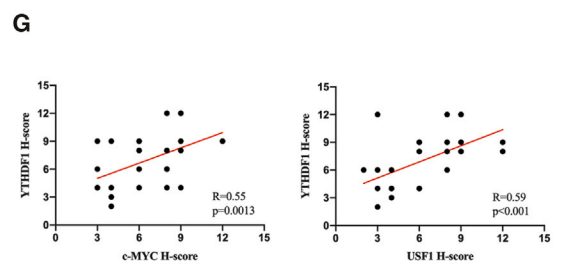
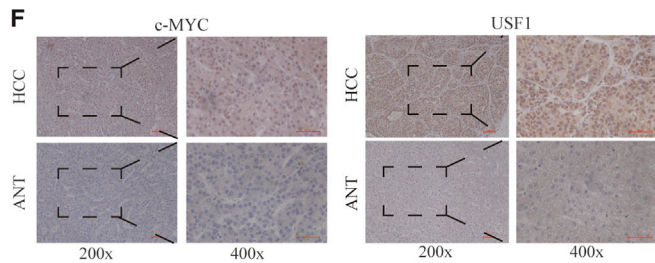
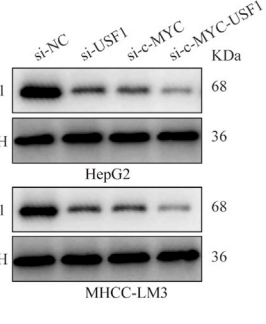
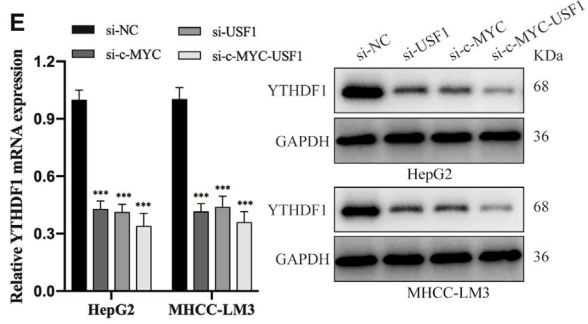
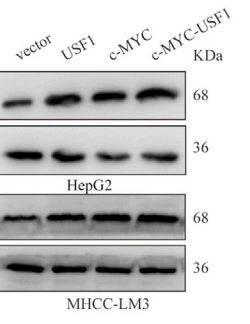
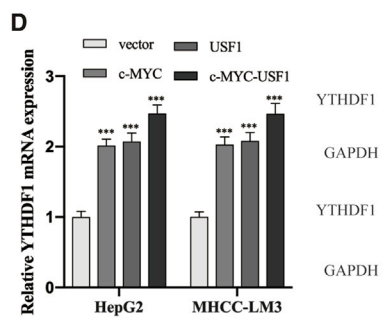
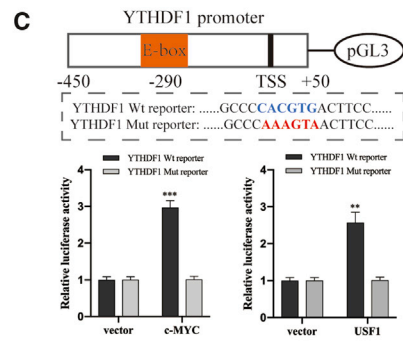
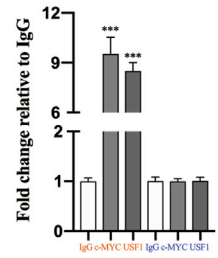
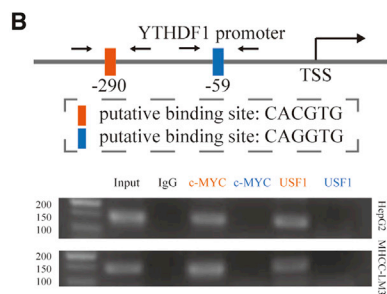
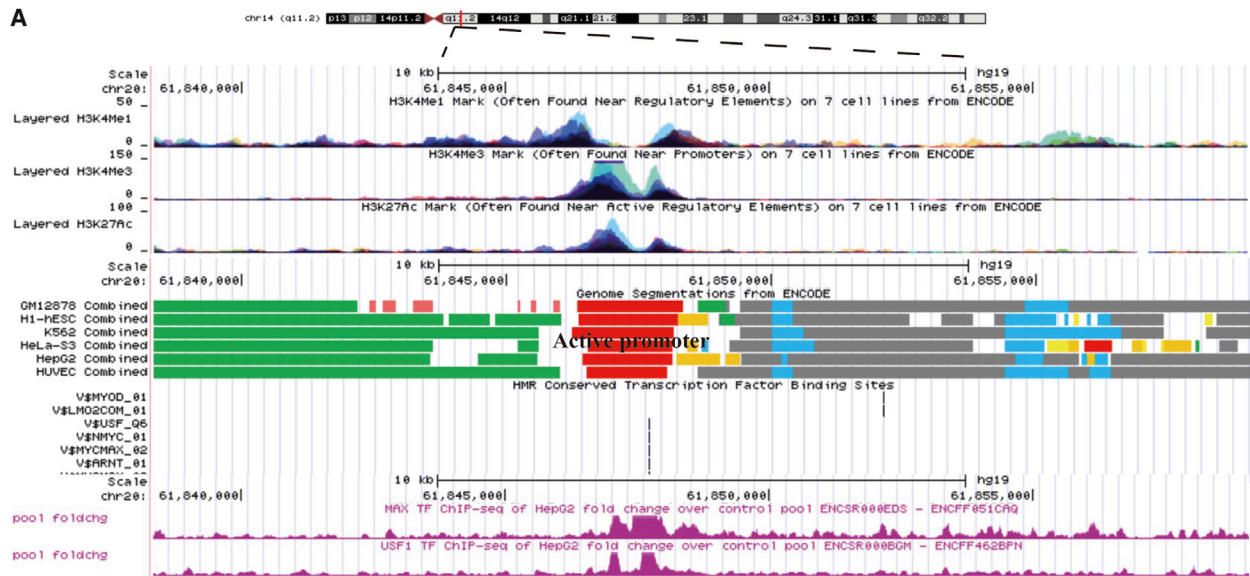


Figure 1. YTHDF1 Expression Is Significantly Upregulated in HCC and Associated with Poor Prognosis

(A) The expression of YTHDF1 in HCC based analysis of TCGA database, GSE14520 dataset, and GSE94660 dataset. (B) qRT-PCR analysis of YTHDF1 expression in HCC tissues and adjacent normal tissues. (C) IHC staining of YTHDF1 in HCC tissues. (D) qRT-PCR analysis of YTHDF1 expression in HCC cell lines and LO2 cells. (E) Kaplan-Meier survival curves of OS of HCC patients based on YTHDF1 expression. (F) YTHDF1 expression is significantly upregulated in multiple cancers. ns, not significant; * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

GSE94660 dataset²² showed that YTHDF1 expression was significantly upregulated in HCC tissues (Figure 1A). Moreover, we examined YTHDF1 expression by using qRT-PCR and immunohistochemistry (IHC) analysis on clinical samples and overexpressed YTHDF1 was observed at both mRNA and protein levels in HCC tissues when compared with adjacent normal tissues (ANTs; Figures 1B and 1C; Figure S1A). Besides, qRT-PCR and immunoblotting analysis

verified elevated YTHDF1 expression in HCC cells compared to LO2 cells (Figure 1D; Figure S1B). To explore the correlation between YTHDF1 expression and clinicopathological features, we classified HCC patients into two groups according to the median value of YTHDF1 expression. As shown in Table S1, YTHDF1 expression was significantly associated with tumor size ($p = 0.028$), Tumor-Node-metastasis (TNM) stage ($p = 0.042$), and microvascular invasion



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($p = 0.016$). Survival analysis using Kaplan-Meier method showed that HCC patients with high YTHDF1 expression underwent a worse OS (Figure 1E). In addition to OS, Kaplan-Meier Plotter database analysis revealed that YTHDF1 overexpression in HCC tissues was also significantly associated with poorer relapse-free survival (RFS), progression-free survival (PFS), and disease-specific survival (DSS) of patients (Figure S1C).²³ Interestingly, YTHDF1 expression was found to be significantly upregulated in multiple solid malignancies compared to corresponding normal tissues (NTs; Figure 1F). With the data described above, we concluded that YTHDF1 expression is significantly upregulated in HCC and associated with poor prognosis of HCC patients.

YTHDF1 Expression Is Jointly Controlled by USF1 and c-MYC in HCC

The somatic mutation and copy number variations of *Ythdf1* are low in HCC.²⁴ Given the wide regulatory role of transcription factors (TFs) in gene expression, we aimed to investigate whether YTHDF1 expression was modulated by specific TFs in HCC. The UCSC Genome Browser database showed that the *ythdf1* promoter was remarkably active in multiple cell lines, including HepG2 cells (Figure 2A). We next analyzed chromatin immunoprecipitation sequencing (ChIP-seq) data of HepG2 cells downloaded from the ENCODE database.²⁵ As shown in Figure 2A, the promoter region of the *Ythdf1* gene is flanked by the deposition of histone marks typically enriched in active promoters (H3K4me3) and transcriptional regulatory elements (H4K3me1 and H3K27Ac). To identify TFs that potentially bind cis-regulatory DNA elements in the *ythdf1* promoter, we queried the HMR Conserved Transcription Factor Binding Sites deposited in the UCSC Genome Browser database and ChIP-seq data of HepG2 cells from the ENCODE database. Among others, we found the potential binding of c-MYC/MAX and USF1 in the *ythdf1* promoter (Figure 2A). Sequence analysis of the *ythdf1* promoter highlighted two consensus binding sites (E-box) for c-MYC and USF1, which are located at -290 bp (CACGTG) and -59 bp (CAGGTG) from the *Ythdf1* transcription initiation factor (TSS; Figure 2B). To verify the direct binding between c-MYC/USF1 and the *Ythdf1* promoter, we carried out ChIP experiments in HepG2 and MHCC-LM3 cells. Semiquantitative PCR and qRT-PCR analysis showed a significant enrichment of c-MYC and USF1 in the E-box located at -290 bp rather than -59 bp (Figure 2B). Besides, the luciferase activity revealed that after co-transfecting *Ythdf1* promoter reporters (wild type [WT] and mutant [Mut]) with either c-MYC or USF1 overexpression vector in HEK293T cells, only the wild-type putative binding site increased activation of the *Ythdf1* promoter (Figure 2C). To test whether up-

regulated c-MYC and USF1 accelerate YTHDF1 expression, we knocked down and increased their expression in HepG2 and MHCC-LM3 cells by using small interfering RNAs (siRNAs) and overexpression vector, respectively (Figures S2A and S2B). si-USF1#2 and si-c-MYC#1 were selected for subsequent experiments due to their higher inhibition effect (Figure S2B). qRT-PCR and immunoblotting analysis showed that c-MYC/USF1 depletion reduced but overexpression increased YTHDF1 expression at both mRNA and protein levels, even more potently when both c-MYC and USF1 were regulated (Figures 2D and 2E). IHC analysis showed that c-MYC and USF1 expression was significantly upregulated in HCC tissues compared to ANTs and exhibited positive correlations with YTHDF1 expression (Figures 2F and 2G). Moreover, c-MYC/MAX and USF1 expression was positively correlated with YTHDF1 expression in TCGA database of HCC (Figure S2C). Taken together, we concluded that YTHDF1 expression is jointly controlled by USF1 and c-MYC in HCC.

YTHDF1 Promotes HCC Cell Proliferation and Metastasis *In Vitro*

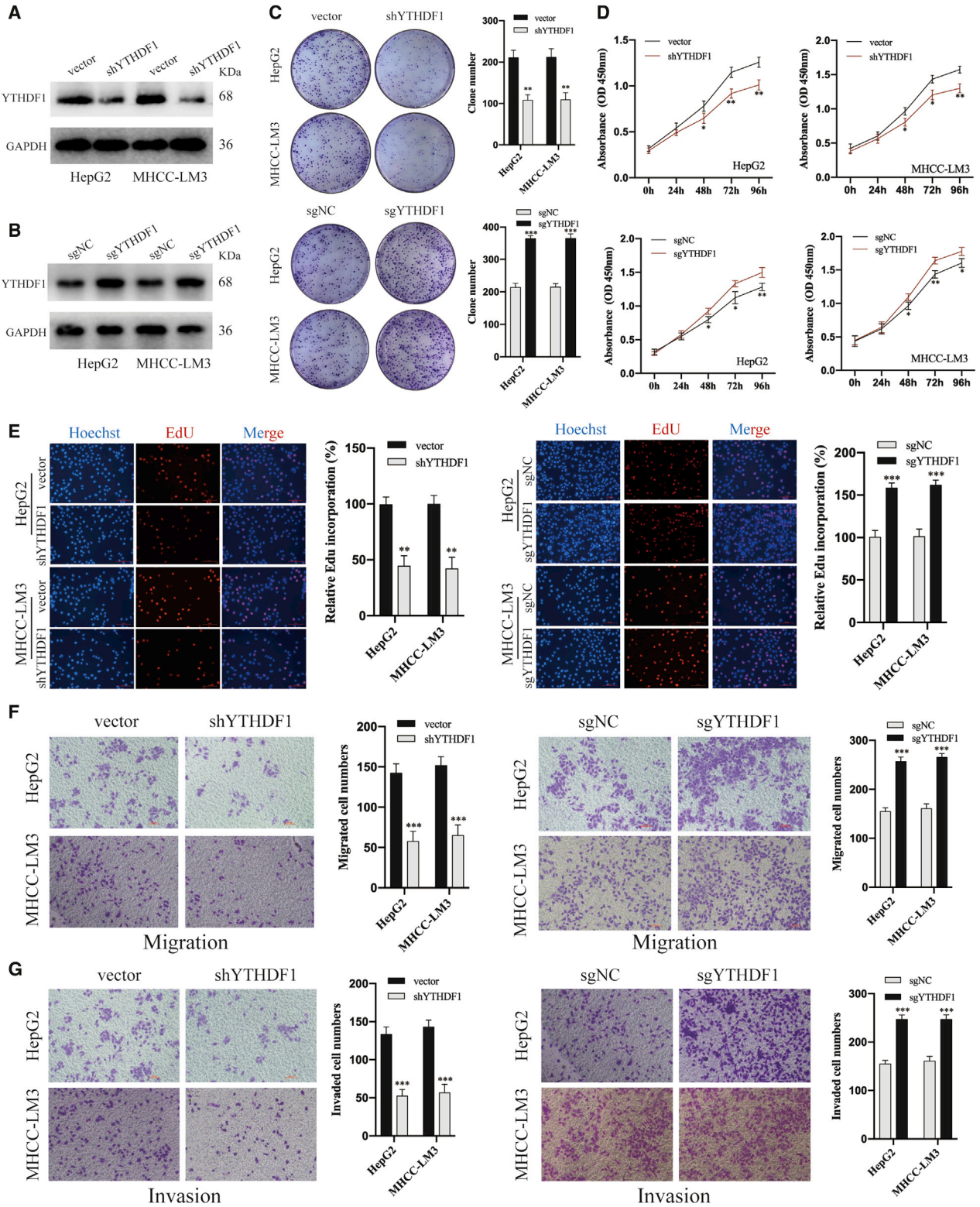
To investigate the biological function of YTHDF1 in HCC, we designed lentivirus-mediated short-hairpin RNA (shRNA; shYTHDF1) and the CRISPR-dCas9 gene activation system that has a specific single-guided RNA targeting the promoter region of the *Ythdf1* gene (sgYTHDF1) to knock down and overexpress YTHDF1 expression in HCC cells, respectively (Figures 3A and 3B). The colony formation assay showed that YTHDF1 knock-down obviously inhibited, but YTHDF1 overexpression significantly enhanced, the colony forming ability of HCC cells (Figure 3c). The Cell Counting Kit-8 (CCK-8) assay and EdU assay showed that silencing of YTHDF1 suppressed, but upregulation of YTHDF1 promoted the proliferation of HCC cells (Figures 3D and 3E). Transwell migration and Matrigel invasion assays showed that YTHDF1 downregulation and upregulation significantly inhibited and improved the migratory and invasive capabilities of HCC cells, respectively (Figures 3F and 3G). The above results demonstrated that YTHDF1 exerts oncogenic role in HCC cells.

YTHDF1 Promotes HCC Cell Proliferation and Metastasis *In Vivo*

To explore the oncogenic role of YTHDF1 in HCC *in vivo*, we performed a subcutaneous implantation experiment in nude mice by using YTHDF1-silenced MHCC-LM3 cells and YTHDF1-overexpressed HepG2 cells. The data showed that the YTHDF1 knock-down effectively reduced, but YTHDF1 overexpression significantly increased, tumor size (Figures 4A and 4B) and weight (Figures 4C and 4D). Besides, tumor tissues in different groups were verified

Figure 2. YTHDF1 Expression Is Jointly Controlled by USF1 and c-MYC in HCC

(A) Deposited histone marks, status, and c-MYC and USF1 enriched site in the promoter of *Ythdf1* in HepG2 cells based on ENCODE database and UCSC database. (B) ChIP, semiquantitative PCR, and qRT-PCR analysis of the binding sites of c-MYC and USF1 in *Ythdf1* promoter. (C) Wild-type and mutant type of *Ythdf1* promoter reporters and relative activity of reporters after being transfected with c-MYC or USF1 overexpression vector. (D) qRT-PCR and western blot analysis of the effect of c-MYC and USF1 overexpression vector on YTHDF1 expression in HepG2 and MHCC-LM3 cells. (E) qRT-PCR and western blot analysis of YTHDF1 expression after transfection of si-c-MYC and si-USF1 in HepG2 and MHCC-LM3 cells. (F) IHC analysis of c-MYC and USF1 in HCC tissues and adjacent normal tissues. (G) The correlations between c-MYC and USF1 expression and YTHDF1 expression. ** $p < 0.01$ and *** $p < 0.001$.



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through H&E-staining (Figure 4E). Next, qPCR analysis was performed to confirm YTHDF1 expression in xenografted tumor tissues. As expected, tumor tissues formed from YTHDF1-silenced MHCC-LM3 cells exhibited reduced YTHDF1 expression, whereas tumor tissues from YTHDF1-overexpressed HepG2 cells exhibited increased YTHDF1 expression (Figure 4F). To evaluate whether YTHDF1 could promote metastasis *in vivo*, we established lung metastasis models by intravenously injecting YTHDF1-silenced MHCC-LM3 cells and YTHDF1-overexpressed HepG2 cells. We observed fewer metastatic nodules on the lung surfaces in the YTHDF1 knockdown group and more metastatic nodules on the lung surfaces in the YTHDF1 overexpression group than those in the control group, respectively (Figures 4G and 4H). Collectively, our findings revealed that YTHDF1 promotes HCC cell growth and metastasis *in vivo*.

Identifying FZD5 as a Direct Target of YTHDF1 in HCC

It has been demonstrated that YTHDF1 can improve the translation efficiency of m6A-modified mRNAs.^{18,20} To identify the targets of YTHDF1, we first analyzed the public ribosome profiling data of cells with YTHDF1 knockdown (GSE63591).¹⁵ Generally, YTHDF1 depletion resulted in 1982 transcripts whose translational efficiency decreased (Figure 5A). To select targets bound with YTHDF1 directly, we downloaded the RNA immunoprecipitation sequencing (RIP-seq) and PARCLIP-seq data of YTHDF1 in GSE63591 and overlapped them with ribosome-seq data. We found that YTHDF1 depletion led to the translational efficiency of 413 genes bound with YTHDF1 decreased (Figure 5B). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that these transcripts are significantly enriched in eleven signaling pathways, such as Hippo and WNT signaling pathways (Figure 5C), among which, FZD5, FZD7, FZD9, and WNT3 were enriched in the most pathways (8/11). Interestingly, when we examined the expression abundance and translational efficiency of FZD5, FZD7, FZD9, and WNT3 in HCC by using GSE112705 dataset,²⁶ we found that the expression abundance of FZD5 was much higher than FZD7, FZD9, and WNT3 in HCC and the transcription efficiency of FZD5 was elevated in most HCC tissues when compared to ANTs (Figures 5D and 5E). Considering m6A modification is high cell-type-specific,²⁷ RNA-seq and m6A-seq data of HepG2 cells deposited in GSE90642¹² were analyzed to validate the m6A modification of FZD5 mRNA in HCC, which showed that METTL14 knockdown remarkably decreased the m6A level rather than expression level of FZD5 mRNA in HepG2 cells (Figure 5F). Above results indicated that FZD5 mRNA was m6A-modified in HCC and that m6A may influence its translation rather than stability. Hence, we selected FZD5 as the downstream target of YTHDF1 in HCC. IHC analysis showed that FZD5 expression was significantly upregulated in HCC tissues

compared to ANTs (Figure 5G) and showed a strong positive correlation with YTHDF1 expression (Figure 5H).

YTHDF1 Promotes FZD5 mRNA Translation in an m6A-Dependent Manner

To validate the direct binding between FZD5 mRNA and YTHDF1 protein, we conducted RIP experiments. Semiquantitative PCR and qRT-PCR analysis showed a significant enrichment of FZD5 mRNA binding with YTHDF1 protein when compared to immunoglobulin G (IgG; Figures 6A and 6B). In addition, knockdown of YTHDF1 resulted in a significant inhibition of FZD5 at the protein level (Figure 6C), but not at the mRNA level (Figure 6D). To exclude YTHDF1 might influence protein stability of FZD5, we treated HepG2 and MHCC-LM3 cells with cycloheximide (CHX) to block translation and discovered that the FZD5 protein expression was similarly eliminated in the vector group and the shYTHDF1 group (Figure 6E). The above results suggested that the elimination of FZD5 protein expression caused by YTHDF1 silencing was due to the decrease in mRNA translational efficiency rather than transcription or protein stability.

It is well known that YTHDF1 functions via binding m6A methylated transcripts.²⁸ Thus, we first screened m6A sites of FZD5 mRNA in HepG2 cells by using MeT-DB V2.0 database,²⁹ which showed that FZD5 mRNA m6A site were enriched in the protein-coding sequence (CDS) located at chr2:208632313–208632314 (Figure 6F). Consistently, our MeRIP-PCR assay verified a marked enrichment of m6A modification in the site (Figure 6G). Furthermore, we constructed a pGL3-FZD5 luciferase reporter by ligating the FZD5 CDS to the cloning site. The luciferase assay showed that YTHDF1-overexpression (OE) enhanced expression of FZD5-WT rather than FZD5-Mut (Figure 6H). In addition, METTL3 knockdown (Figures 6I and 6J) significantly reduced the total m6A level (Figure 6K) and FZD5 protein expression (Figure 6J) with a slight upregulation of FZD5 mRNA expression (Figure 6L) in HCC cells. Moreover, RIP assay showed that METTL3 knockdown significantly suppressed the binding between YTHDF1 and FZD5 mRNA (Figure 6M). Our results indicated that YTHDF1 selectively recognizes the m6A site in the FZD5 mRNA CDS and subsequently promotes its translation output.

FZD5 Overexpression Effectively Reverses YTHDF1 Knockdown-Induced Inhibition of HCC Cell Progression

To explore the regulatory role of FZD5 in HCC, we overexpressed FZD5 by transfecting recombinant human FZD5 with biological activity (rFZD5; Figure 7A). The cellular phenotypes showed that FZD5 upregulation significantly promoted HCC cell proliferation, migration, and invasion (Figures 7B–7F) resembling that of YTHDF1 overexpression.

Figure 3. YTHDF1 Promotes HCC Cells Proliferation and Metastasis *In Vitro*

(A) Western blot analysis of transfection of shYTHDF1 in HepG2 and MHCC-LM3 cells. (B) Western blot analysis of transfection of sgYTHDF1 in HepG2 and MHCC-LM3 cells. (C) Clone formation assays for MHCC-LM3 and HepG2 cells with YTHDF1 knockdown or overexpression. (D) CCK-8 assays for MHCC-LM3 and HepG2 cells with YTHDF1 knockdown or overexpression. (E) EdU assays for MHCC-LM3 and HepG2 cells with YTHDF1 knockdown or overexpression. (F) Transwell migration assays for MHCC-LM3 and HepG2 cells with YTHDF1 downregulation or overexpression. (G) Transwell Matrigel invasion assays for MHCC-LM3 and HepG2 cells with YTHDF1 downregulation or overexpression. *p < 0.05; **p < 0.01; and ***p < 0.001.

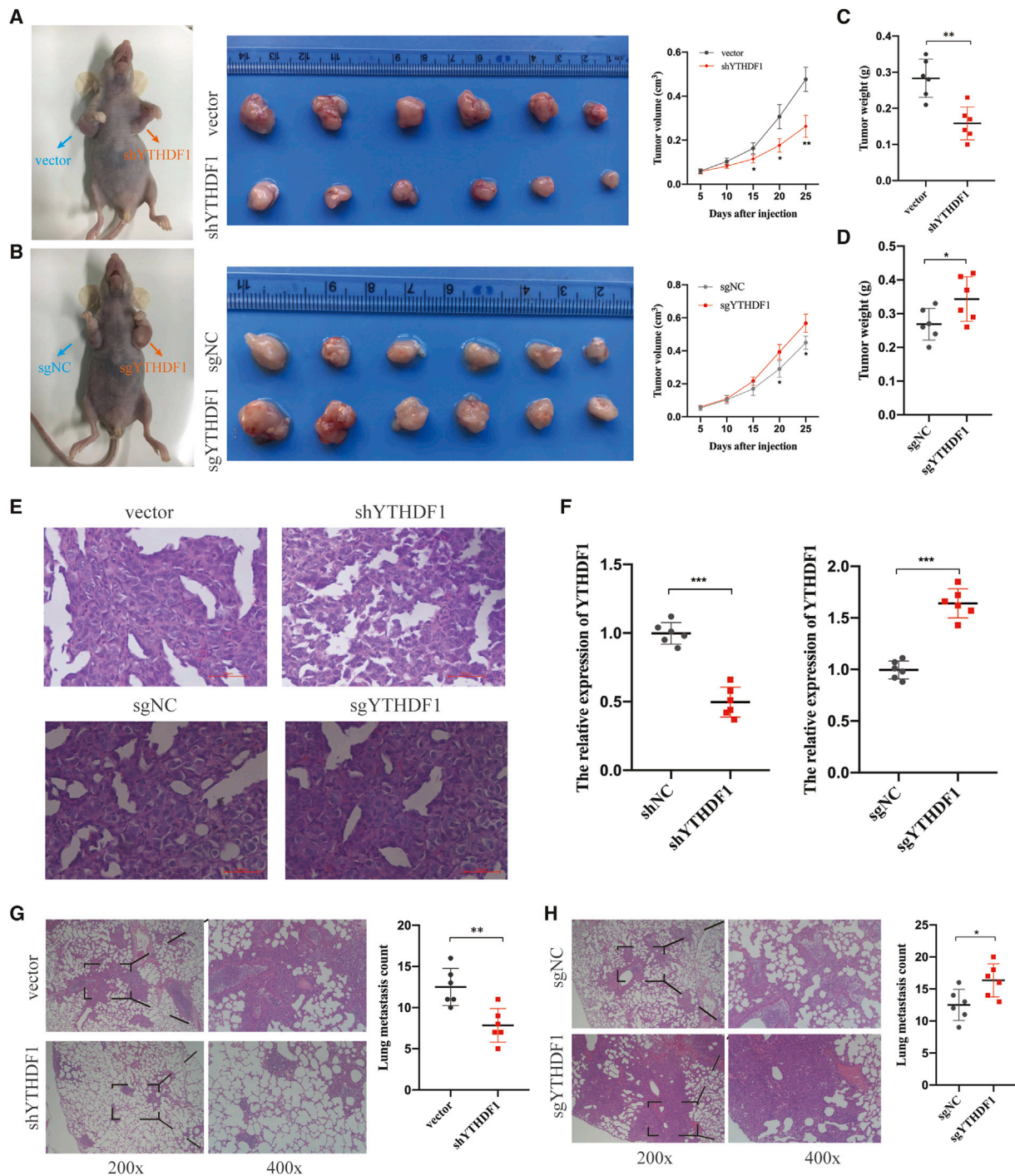


Figure 4. YTHDF1 Promotes HCC Cells Proliferation and Metastasis *In Vivo*

(A) YTHDF1 knockdown effectively reduced subcutaneous transplant tumor size. (B) YTHDF1 overexpression effectively increased subcutaneous transplant tumor size. (C) YTHDF1 knockdown effectively reduced subcutaneous transplant tumor weight. (C and D) YTHDF1 overexpression effectively increased subcutaneous transplant tumor weight. (E) H&E-staining analysis of subcutaneous transplant tumor. (F) qRT-PCR analysis of YTHDF1 expression in subcutaneous transplant tumor. (G) YTHDF1 knockdown effectively reduced the lung metastasis of HCC cells *in vivo*. (H) YTHDF1 overexpression effectively increased the lung metastasis of HCC cells *in vivo*. * $p < 0.05$; ** and $p < 0.01$.

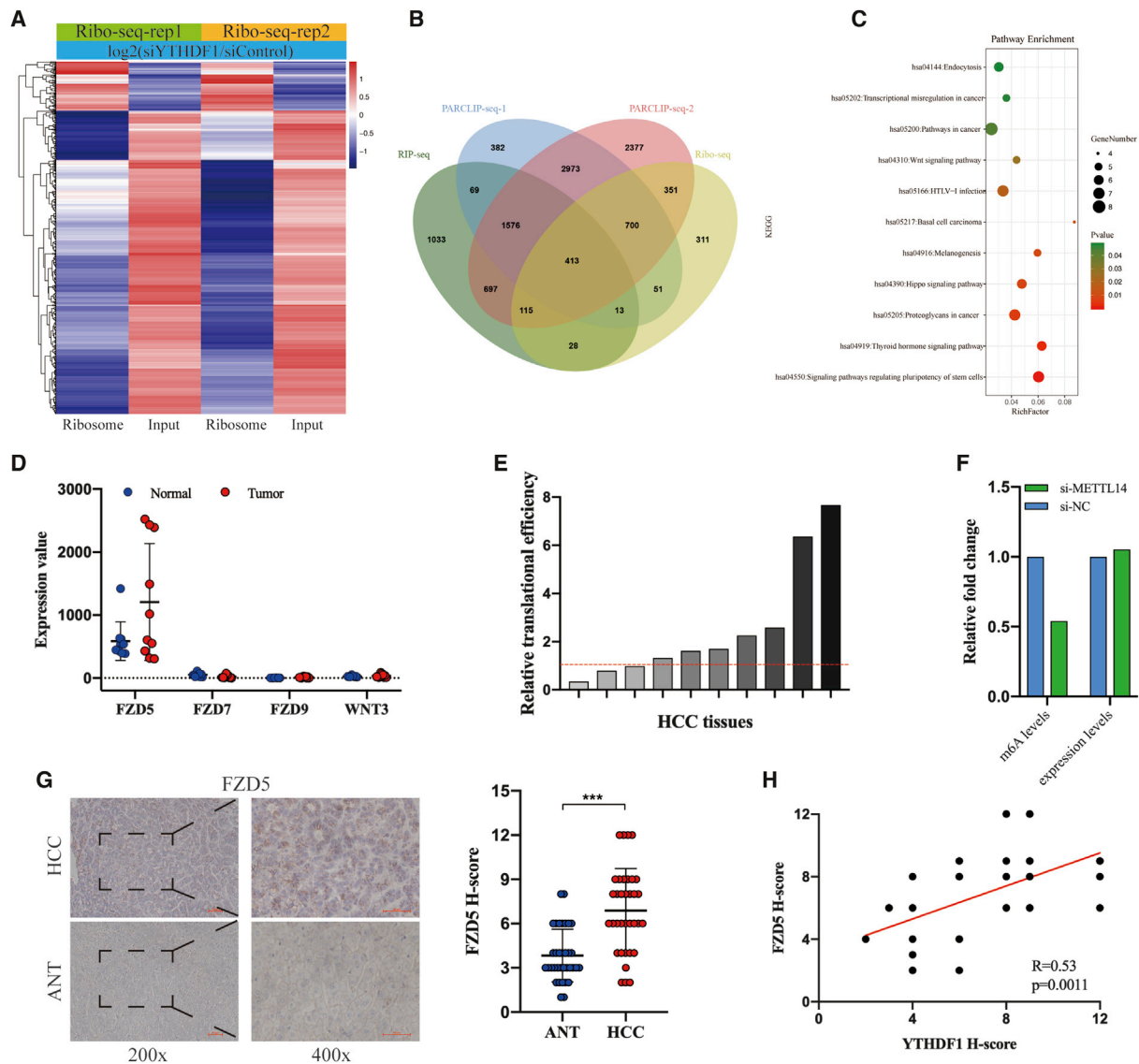


Figure 5. Identifying FZD5 as a Target of YTHDF1 in HCC

(A) Ribosome-seq of cells transfected with siYTHDF1 and control. (B) Overlapping ribosome-seq, PARCLIP-seq, and RIP-seq data. (C) KEGG analysis of putative targets of YTHDF1. (D) The expression abundance of FZD5, FZD7, FZD9, and WNT3 mRNAs in HCC tissues. (E) The transcription efficiency of most FZD5 were elevated in HCC tissues. (F) The fold change of expression levels and m6A levels of FZD5 after METTL14 knockdown in HepG2 cells. (G) IHC analysis of FZD5 in HCC tissues. (H) FZD5 protein expression was positively correlated with YTHDF1 protein expression in HCC tissues.

Next, we overexpressed FZD5 in YTHDF1-deficient HCC cells to investigate whether the oncogenic role of YTHDF1 was directly mediated by FZD5. The colony formation assay, CCK-8 assay, and EdU incorporation assay showed that YTHDF1 knockdown impaired cell colony formation and growth, whereas FZD5 upregulation reversed such effect (Figures 7B–7D). Consistently, Transwell migration and Matrigel invasion assays showed that both cell migration and invasion abilities suppressed by YTHDF1 depletion were re-established after FZD5 overexpression in HCC cells (Figures 7E and 7F).

YTHDF1 Promotes HCC Carcinogenesis through the WNT/β-Catenin Signaling Pathway

Overexpressed FZD5 was reported to activate WNT/β-catenin pathway in HCC.³⁰ Next, we investigated whether YTHDF1 functions through FZD5/WNT/β-catenin in HCC. Subcellular fractionation and immunoblotting assay showed that YTHDF1 knockdown significantly reduced the total and nuclear β-catenin expression in HCC cells, whereas FZD5 upregulation reversed such effect (Figure 8A). In addition, we found that YTHDF1 knockdown did not influence the concentration of extracellular calcium (Figure 8B) and the level of phosphorylated JNK

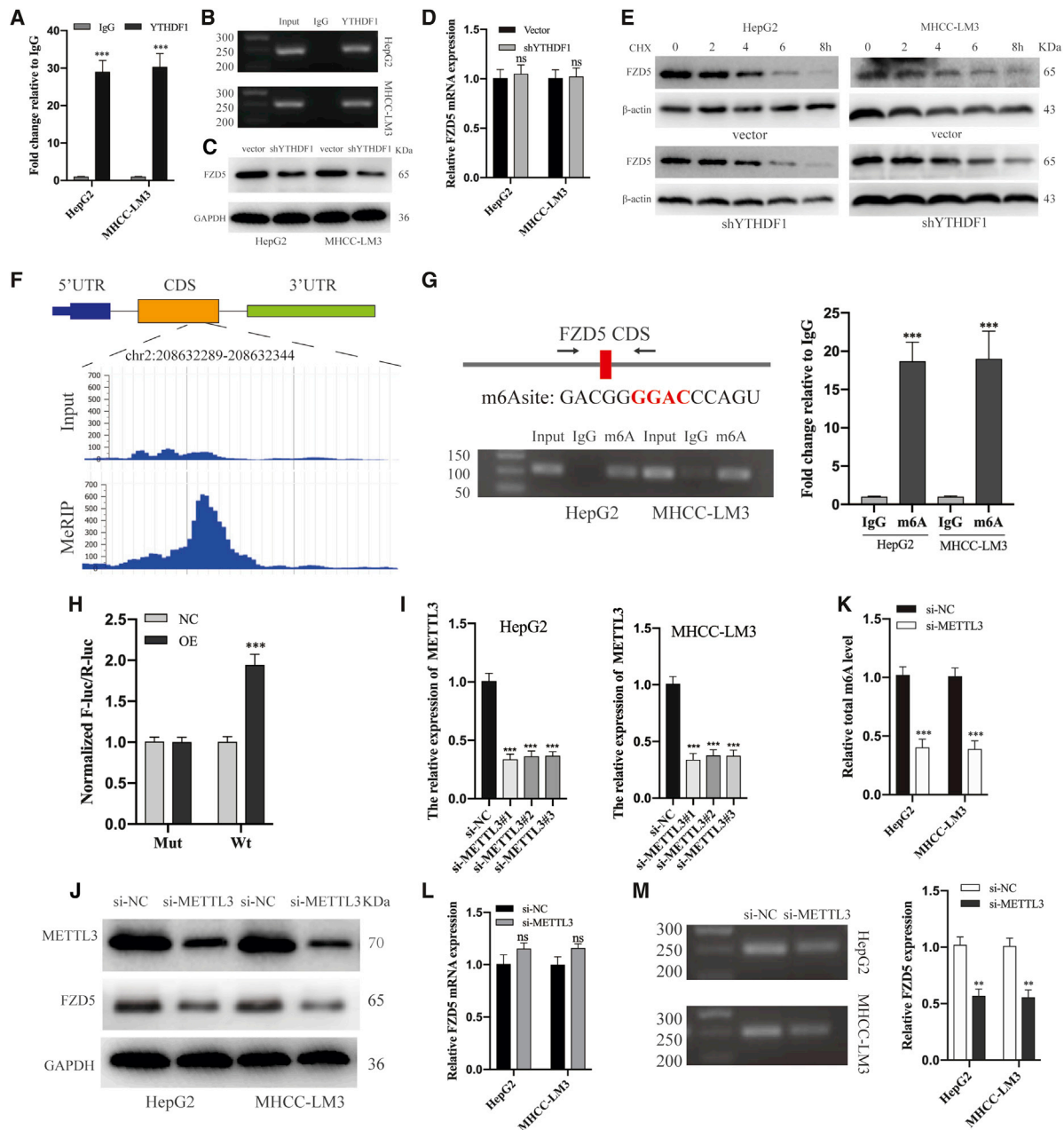
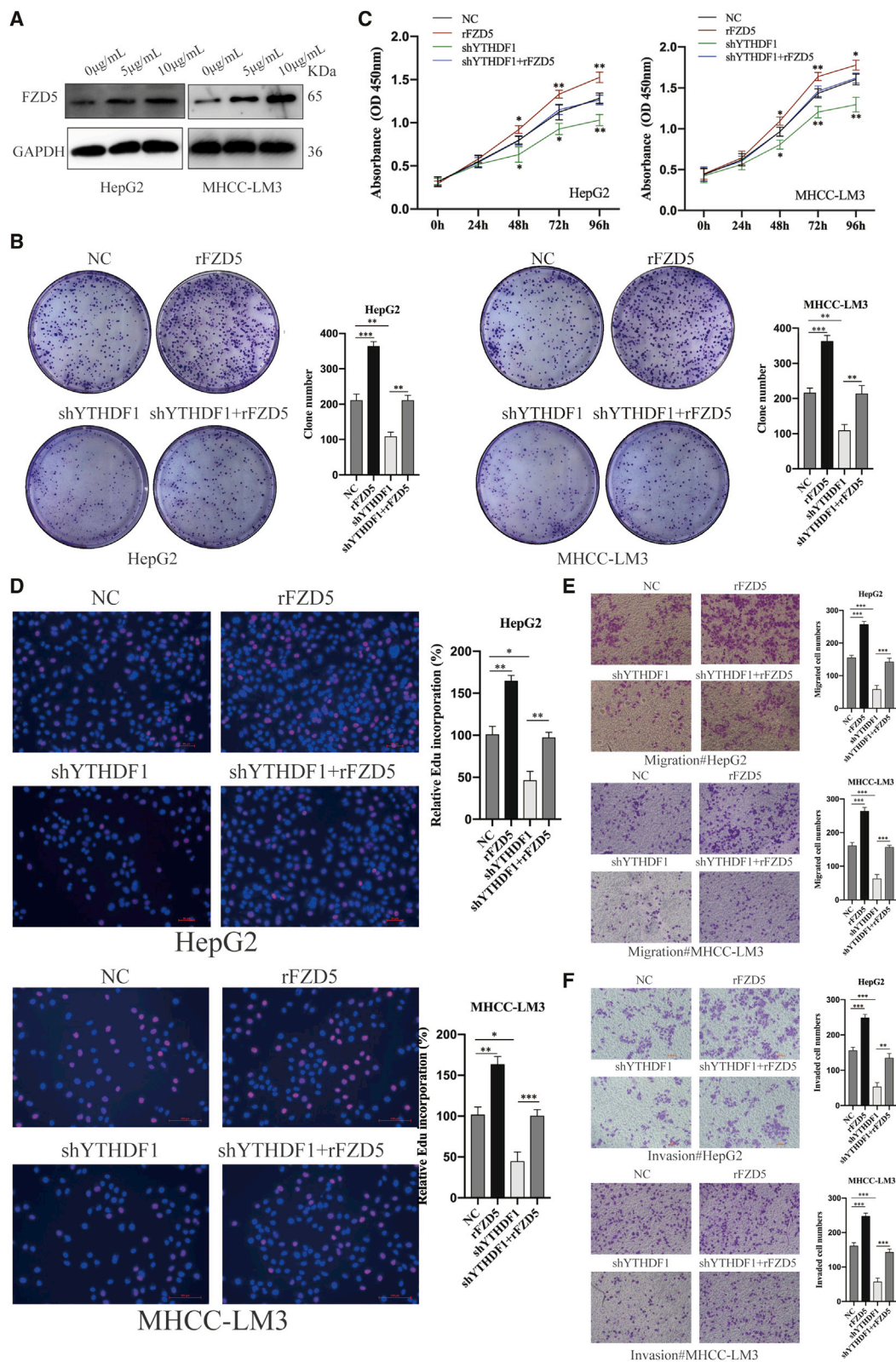


Figure 6. YTHDF1 Promotes FZD5 Translation in an m6A-Dependent Manner

(A) RIP-qRT-PCR analysis of the enrichment of FZD5 mRNA binding to YTHDF1 protein. (B) RIP-semiquantitative PCR analysis of the enrichment of FZD5 mRNA binding to YTHDF1 protein. (C) Western blot analysis of the effect of shYTHDF1 on FZD5 expression in HCC cells. (D) qRT-PCR analysis of the effect of shYTHDF1 on FZD5 expression in HCC cells. (E) Using CHX to test the stability of FZD5 protein in shYTHDF1 transfected HCC cells. (F) The location of potential m6A site in FZD5 mRNA. (G) MeRIP-qRT-PCR and semiquantitative PCR analysis of m6A site in FZD5 mRNA. (H) The luciferase assay in YTHDF1 overexpression HEK293T cells. (I) qRT-PCR analysis of the transfecting efficiency of si-METTL3. (J) Western blot analysis of the effect of si-METTL3#1 transfection on METTL3 and FZD5 expression. (K) The total m6A level of HCC cells after transfecting si-METTL3. (L) qRT-PCR analysis of the effect of transfecting si-METTL3#1 on FZD5 expression. (M) RIP-qRT-PCR and semiquantitative PCR analysis of the effect of si-METTL3 on the binding between FZD5 mRNA and YTHDF1 protein. ** $p < 0.01$ and *** $p < 0.001$.

(Figure 8C) in HCC cells. These results verified that the oncogenic role of YTHDF1 in HCC is mediated through the WNT/ β -catenin pathway rather than the WNT/ Ca^{2+} pathway or the planar cell polarity pathway.

The *c-Myc* gene, a well-known oncogene transcriptionally activated by WNT/ β -catenin signaling, is considerably involved in the progression of various cancers.^{31,32} Immunoblot assay showed that YTHDF1



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depletion suppressed but FZD5 overexpression re-established the expression of c-MYC in HCC cells (Figure 8A). In addition, IHC analysis showed that FZD5, β -catenin, and c-MYC expression was significantly decreased in YTHDF1-downregulated xenograft tumor samples when compared to negative controls (Figure 8D). Moreover, we observed a positive correlation between FZD5, β -catenin, c-MYC, and YTHDF1 expression in the TCGA database of HCC (Figure 8E). Together, our findings suggested that YTHDF1 promotes the carcinogenesis of HCC cells through the FZD5/WNT/ β -catenin signaling pathway (Figure 8F).

DISCUSSION

Accumulating evidence has shown that dysregulated m6A writers or erasers play a critical role in modifying the tumorigenicity of cancers, including melanoma,³³ gastric cancer,³⁴ glioblastoma,³⁵ colorectal cancer,³⁶ and HCC.³⁷ Here, we identified that YTHDF1, an actual implementer of m6A modification, is markedly upregulated in HCC. Given the somatic mutation and copy number variations of *Ythdf1* were low in HCC,²⁴ we discovered that YTHDF1 expression is mainly modulated by c-MYC and USF1, two well-known TFs that have been proven to promote gene expression. For example, Cinzia et al.³⁸ reported that c-MYC promotes transcription and productive splicing of the oncogenic splicing factor Sam68 in cancer. Our previous studies showed that USF1 could transcriptionally regulate ESRP1 and transforming growth factor β 1 (TGF- β 1) expression in breast cancer.³⁹ Although the regulatory role of c-MYC on YTHDF1 expression has been suggested preciously,⁴⁰ our study further identified the exact binding site and revealed that USF1 could cooperate with c-MYC to increase the transcription of YTHDF1.

YTHDF1 selectively recognizes and promotes ribosome loading of m6A-modified mRNAs.¹⁵ Liu et al.¹⁷ demonstrated that YTHDF1 promotes cell growth, migration, and invasion of ovarian cancer via augmenting EIF3C translation. Moreover, Shi and colleagues⁴¹ found that YTHDF1 links hypoxia adaptation and promotes NSCLC cell proliferation and xenograft tumor formation through regulating the translational efficiency of CDK2, CDK4, and cyclin D1. Here, we discovered YTHDF1 also functions as an oncogene in HCC. Differently, we identified FZD5 as the target of YTHDF1 in HCC. However, we had to admit that ribosome-seq, RIP-seq, and PARCLIP-seq data used to identify YTHDF1 downstream target were conducted in HeLa cells, which may result in the loss of other important targets of YTHDF1 in HCC. Although we verified our analysis results in HCC subsequently, conducting ribosome-seq, RIP-seq, and PARCLIP-seq of YTHDF1 by using HCC cells will be better. Mechanically, we found that m6A modification was enriched in the CDS of FZD5 mRNA and that YTHDF1 could directly promote FZD5 mRNA

translation in an m6A-dependent manner. Our findings showed that FZD5 carries out its biological function by activating the canonical WNT/ β -catenin pathway in HCC. Like all Frizzled members, FZD5 is a receptor that is bound and activated by the WNT family of lipoglycoproteins.⁴² Therefore, a coimmunoprecipitation (coIP) assay, mass spectrometry (MS) analysis, and WNT inhibitor treatment should be conducted to explore which WNT lipoglycoprotein binds to FZD5 in HCC. Beta-catenin was reported to be strikingly active in cancers and transcriptionally promoted the expression of various genes.^{43,44} In this study, we identified c-MYC as a downstream executor of β -catenin regulated by YTHDF1 in HCC. Given the transcriptional regulation of YTHDF1 expression by c-MYC, our findings found a novel feedback loop YTHDF1/FZD5/ β -catenin/c-MYC in HCC. Interestingly, CTNNB1 and c-MYC are also m6A-modified genes. m6A methylation can improve the stability and storage of CTNNB1 and c-MYC mRNA,^{12,45} which reveals a comprehensive regulatory network of m6A modification during cell biological processes.

In summary, our study illustrated that YTHDF1 can improve the translational output of FZD5 mRNA in an m6A-dependent manner and promote progression of HCC cells through the FZD5/WNT/ β -catenin signaling pathway. Our study provided a potential therapeutic strategy for HCC.

MATERIALS AND METHODS

Patients and Clinical Specimens

In total, 84 matched HCC tissues and ANTs were collected from Nanjing First Hospital affiliated to Nanjing Medical University. Patients without any antitumor treatment before surgery were included. The detailed characteristics of the patients are described in Table S1. Informed consent was obtained from each patient and the study was approved by the ethics committee of Nanjing First Hospital.

Cell Culture

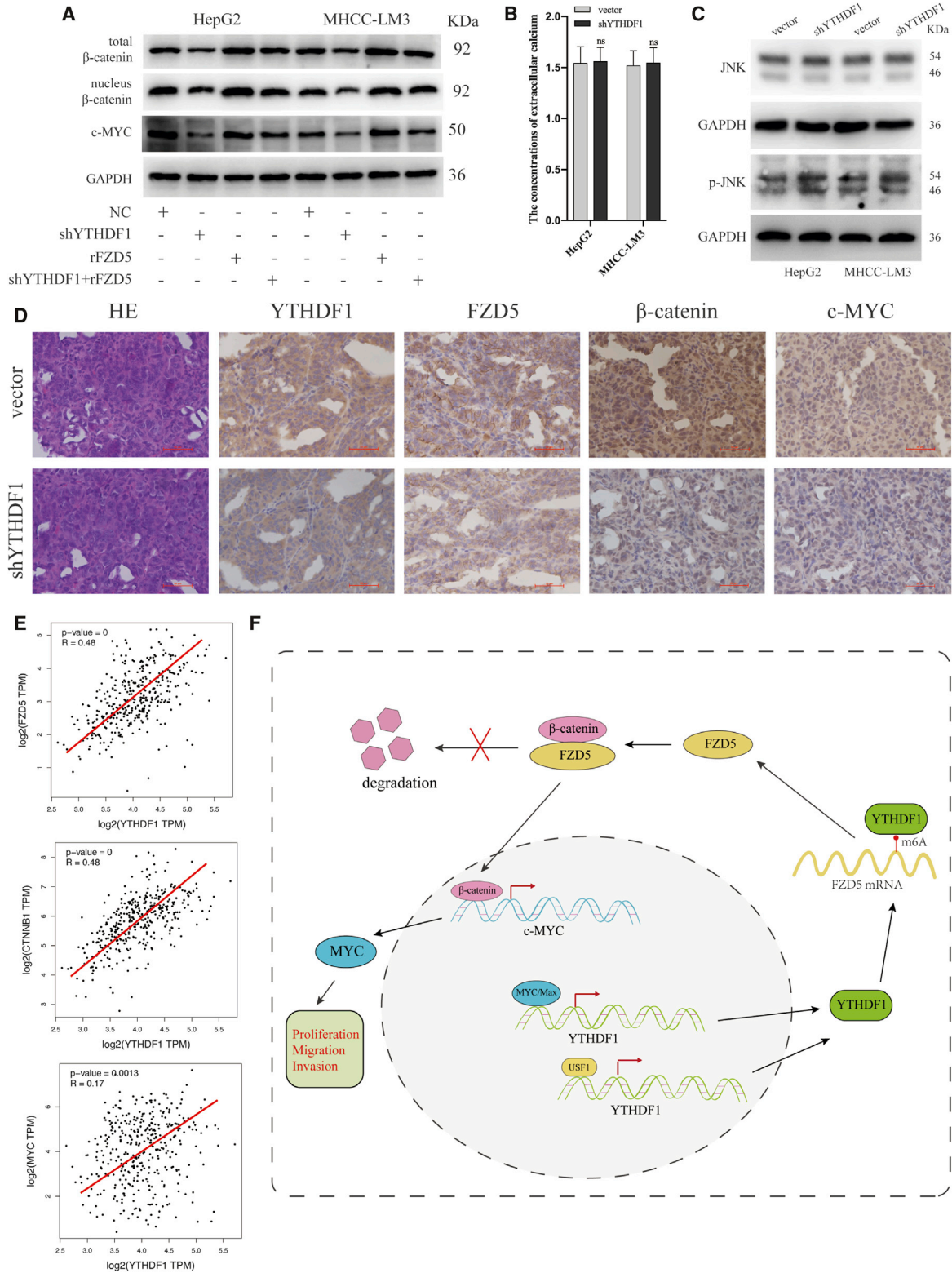
Human normal hepatocyte cells (LO2) and HCC cell lines (Hep3B, MHCC-LM3, YY8103, SMCC7721, HepG2) were purchased from Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All cells were routinely cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GIBCO, Vienna, Austria) and cultured in an incubator (Thermo Scientific, USA) with a humidified atmosphere of 5% CO₂ at 37 °C.

Cell Transfection

Stable knockdown of YTHDF1 was achieved by lentiviral-based shRNA delivery (GeneChem, China). YTHDF1 activation in HCC

Figure 7. FZD5 Overexpression Effectively Reverses YTHDF1-Knockdown-Induced Inhibition of HCC Cells Progression

(A) Western blot analysis of the transfection efficiency of rFZD5 in HCC cells. (B) Colony formation assay of HepG2 cells transfected with rFZD5 and shYTHDF1, respectively or corporately. (C) CCK-8 assay of HepG2 cells transfected with rFZD5 and shYTHDF1, respectively or corporately. (D) EdU incorporation assay of HepG2 cells transfected with rFZD5 and shYTHDF1, respectively or corporately. (E) Transwell migration assays of HepG2 cells transfected with rFZD5 and shYTHDF1, respectively or corporately. (F) Transwell Matrigel invasion assays of HepG2 cells transfected with rFZD5 and shYTHDF1, respectively or corporately. ns, not significant; *p < 0.05; **p < 0.01; and ***p < 0.001.



(legend on next page)

cells was accomplished by using the lentiviral-based CRISPR-dCas9 gene activation system (GeneChem, China). MYC and USF1 siRNAs were designed and synthesized by RiboBio (Guangzhou, China) and transfected into HCC cells by using Lipofectamine 2000 (Invitrogen, USA). The shRNA, single-guided RNA (sgRNA), and siRNA sequences are listed in [Table S2](#). For c-MYC- and USF1-expressing vectors, the full open reading frame (ORF) sequences of these genes were subcloned into the pLenti-CMV-GFP vectors (ABM, Canada). Cycloheximide (CHX, HY-12320; MCE, USA) was dissolved in DMSO and treated cells at a final concentration of 100 $\mu\text{g}/\text{mL}$. Recombinant human FZD5 (rFZD5, 1617-FZC-050; R&D Systems, USA) was dissolved in PBS and added to cells at a final concentration of 10 $\mu\text{g}/\text{mL}$ according to the manufacturer's instructions.

RNA Extraction and qRT-PCR

Total RNA was isolated by using TRIzol reagent (Invitrogen, China) and subsequent cDNA synthesis was performed with PrimeScript RT Reagent (Takara, Japan). RNA expression was measured by qRT-PCR by using the SYBR Premix Ex Taq (Takara, Japan) and calculated by the $2^{-\Delta\Delta\text{Ct}}$ method with the normalization to GAPDH. The primer sequences are shown in [Table S3](#).

Immunoblot Analysis

Protein was extracted from cells by using RIPA buffer (Beyotime, China) and quantified with the Bicinchoninic Acid Protein Assay Kit (Thermo Scientific, USA). Protein extracts were separated on a 10% SDS-PAGE gel (Beyotime, China) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). Then, the membrane was incubated with the corresponding primary antibody at 4 °C overnight. Subsequently, the membrane was washed five times and incubated with secondary antibody at room temperature for 1 h. Finally, bands were visualized by using an imaging system (Bio-Rad, USA). The antibodies employed are listed in [Table S4](#).

IHC

IHC was performed on 35 matched formalin fixation and paraffin embedding (FFPE) HCC tissues and ANTs. All histologic slides were assessed by two pathologists independently. The degree of positivity was initially classified by scoring both the proportion of positively stained tumor cells and the staining intensities as previously described.⁴⁶ The score was independently assessed by two proficient pathologists. The antibodies employed are listed in [Table S4](#).

Animal Experiments

For the xenograft subcutaneous implantation model, 4-week-old male BALB/c nude mice were randomly divided into two groups. 1 week later, stably transfected MHCC-LM3 cells ($5 \times 10^6/0.2$ mL

PBS) were subcutaneously injected into the armpits of nude mice. The tumor volumes were measured every 5 days. Tumor volumes were calculated by using the following equation: volume = $1/2$ (length \times width²). 4 weeks later, the nude mice were sacrificed, and the tumor weights were recorded.

For the pulmonary metastasis model, stably transfected MHCC-LM3 cells ($1 \times 10^6/0.1$ mL PBS) were injected into each nude mouse through the tail vein. 4 weeks later, mice were euthanized, the lung tissues were collected, and the metastatic nodules were counted by using HE-staining. The animal studies were handled and treated according to protocols approved by the Animal Care Committee of Nanjing Medical College.

Total m6A Quantification Assay

The purification of polyadenylated mRNA was extracted by PolyATract mRNA Isolation Systems (Promega, USA). Then, an EpiQuik m6A RNA Methylation Quantification Kit (Colorimetric; EpiGentek, USA) was used to detect the total m6A level according to the manufacturer's protocol. Briefly, positive control (pc), negative control (nc), and 200 ng isolated mRNA were added to each well with the capture antibody. Next, the detection antibody was added. After several incubations, the m6A level was quantified colorimetrically at a wavelength of 450 nm and calculated by using the following equation: $m6A\% = \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{nc}})/S}{(\text{OD}_{\text{pc}} - \text{OD}_{\text{nc}})/P} \times 100\%$; s = 200 ng, p = 1 ng.

Methylated RNA Immunoprecipitation (MeRIP) Assay

The MeRIP assay was conducted by using the Magna MeRIP m6A Kit (17-10,499, Millipore, USA) according to the manufacturer's instructions. Briefly, 5 μg isolated mRNA was chemically fragmented into 100 nucleotides or less and immunoprecipitated with 5 μg m6A antibody or anti-mouse IgG linked to Magna IP Protein A/G Magnetic Beads. One tenth volume of fragmented RNA was saved as "10% input." After elution with 6.7 mM N6-methyladenosine 5'-monophosphate sodium salt, the expression of m6A-modified genes was determined by qRT-PCR with specific primers whose sequences are shown in [Table S3](#). Then, qRT-PCR products were further analyzed via agarose gel electrophoresis (AGE).

RIP Assay

The RIP assay was implemented by using the EZ Magna RNA Immunoprecipitation Kit (Millipore, USA) according to the manufacturer's guidelines. In short, cells were lysed by RIP lysis buffer, and magnetic beads were preincubated with antibody for 30 min at room temperature. Thereafter, the cell lysates were immunoprecipitated with beads at 4 °C overnight. Then, RNA was purified and detected by qRT-PCR. After that, qRT-PCR production was further analyzed via AGE. The primer sequences are shown in [Table S3](#).

Figure 8. YTHDF1 Promotes HCC Progression through the WNT/ β -Catenin Signaling Pathway

(A) Western blot analysis of β -catenin and c-MYC in HCC cells transfected with rFZD5 and shYTHDF1, respectively or corporately. (B) The concentrations of extracellular calcium in shYTHDF1 transfected HCC cells. (C) The phosphorylated JNK levels in shYTHDF1 transfected HCC cells. (D) H&E-staining and IHC analysis of YTHDF1, FZD5, β -catenin, and c-MYC in xenograft tumor samples. (E) The positive correlations between FZD5, β -catenin, c-MYC, and YTHDF1 expression in TCGA database of HCC. (F) The illustration summarizes the role of YTHDF1 in the regulatory network in HCC cells.

ChIP Assay

ChIP assays were carried out using a ChIP Assay Kit (Beyotime, China) according to the manufacturer's instructions with slight modifications. In brief, cells cultured in media were cross-linked with 1% formaldehyde solution for 10 min at room temperature and subsequently quenched with 125 mM glycine. DNA fragments were generated via sonication. Then, the lysates were immunoprecipitated with anti-c-MYC, anti-USF1, or normal rabbit IgG antibodies. Immunoprecipitated DNA was analyzed by qRT-PCR and AGE. Primers are listed in Table S3. Antibodies are listed in Table S4.

Luciferase Reporter Assay

For the YTHDF1 luciferase reporter assay, the core promoters of the *Ythdf1* gene (−450 to +50 containing WT or Mut E-box in −290) were synthesized and cloned into the pGL3-basic firefly luciferase reporter plasmid (GeneCreate, China). For the FZD5 CDS luciferase reporter assay, cDNAs containing the full-length CDS of FZD5 were cloned into the pGL3-basic firefly luciferase reporter (GeneCreate, China). Renilla luciferase activity was employed as a control. Luciferase activity was measured with a dual luciferase assay system (Promega, USA).

Cell Proliferation and Colony Formation Assays

For CCK-8 assays, HCC cells were seeded into 96-well plates at a density of 1×10^3 cells per well. The absorbance was measured at 450 nm after 0, 1, 2, 3, and 4 days by using a CCK-8 kit (Keygen, China). The EdU assay was carried out by using a Cell-Light EdU DNA Cell Proliferation Kit (RiboBio, China). In brief, after incubation with EdU for 2 h, cells were fixed with 4% paraformaldehyde followed by staining with Apollo Dye Solution and Hoechst 33342. Then, the cells were photographed and counted using an Olympus FSX100 microscope (Olympus, Japan). For the cell colony formation assay, after transfection for 24 h, 500 HCC cells were seeded into 6-well plates and cultured at 37°C and 5% CO₂. 2 weeks later, the cells were stained with crystal violet (0.1%) for 15 min, and the colonies were counted and photographed.

Transwell Migration and Matrigel Invasion Assays

Transwell migration and Matrigel invasion assays were conducted by using a Transwell chamber (Corning, USA) which was coated with (invasion assay) or without (migration assay) Matrigel mix (BD Biosciences, USA) according to the manufacturer's instructions. After incubation for 24 h, non-migrated and noninvaded cells were scraped off with a cotton swab, and the cells located on the bottom of the chamber were fixed with 4% paraformaldehyde for 10 min, then stained with crystal violet (0.1%) for 15 min. Thereafter, the stained cells were photographed and counted in five randomly selected fields.

Public Databases Analysis

The raw gene expression data for HCC in TCGA database were downloaded from the UCSC Xena database (<https://xenabrowser.net/>) and further analyzed by using R “limma” package. GEO: GSE14520,²¹ GSE94660,²² and GSE112705,²⁶ datasets were downloaded from GEO database (<https://www.ncbi.nlm.nih.gov/geo/>)

and further analyzed by using online tool GEO2R. The raw value of GEO: GSE63591¹⁵ and GSE90642¹² were directly downloaded from Series Matrix Files deposited in GEO database and genes with changed translation efficiency or m6A levels were analyzed by using t test and further visualized by using OmicShare tools (<http://www.omicshare.com/tools>). The prognostic value of YTHDF1 in HCC patients were analyzed by using Kaplan-Meier plotter database (<http://kmplot.com/analysis/>) with the median expression values set to be the cutoff values.²³ The DAVID website (<https://david.ncifcrf.gov>)⁴⁷ was used to carry out Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of putative targets of YTHDF1.

Statistical Analysis

All data analysis was performed by using SPSS 20.0 (SPSS, USA) and GraphPad Prism 8 (GraphPad, USA) and expressed as the mean ± SD (standard deviation) from at least three independent experiments. The differences between two groups were determined by two-tailed paired or unpaired t test. The differences between multiple groups were determined by analysis of variance (ANOVA). The chi-square test was used to analyze the different distributions of clinicopathological variables. Overall survival was estimated with the Kaplan-Meier method, and the log-rank test was employed to evaluate the difference. The correlations were analyzed by using Spearman correlation coefficients. $p < 0.05$ was considered to be statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2020.09.036>.

AUTHOR CONTRIBUTIONS

X.L., S.W., J.Q., and B.H. discussed and designed this study, X.L., C.L., K.Z., X.C., B.P., C.Z., and X.X. performed all experiments, M.X. and T.X. analyzed TCGA and GEO databases of HCC, S.W., Y.P., L.S., and H.S. revised the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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Supplemental Information

YTHDF1 Facilitates the Progression of Hepatocellular Carcinoma by Promoting FZD5 mRNA Translation in an m6A-Dependent Manner

Xiangxiang Liu, Jian Qin, Tianyi Gao, Chenmeng Li, Bangshun He, Bei Pan, Xueni Xu, Xiaoxiang Chen, Kaixuan Zeng, Mu Xu, Chengbin Zhu, Yuqin Pan, Huiling Sun, Li Sun, Tao Xu, and Shukui Wang

Fig. S1 YTHDF1 is upregulated in HCC and associated with prognosis of patients, related to Figure 1. **a** H-score of YTHDF1 in HCC tissues and ANTs. **b** Detection of YTHDF1 protein levels in HCC cell lines and LO2 cells by western blot. **c** Kaplan-Meier survival curves of OS, RFS, PFS, and DSS of HCC patients based on YTHDF1 expression in Kaplan-Meier Plotter database.

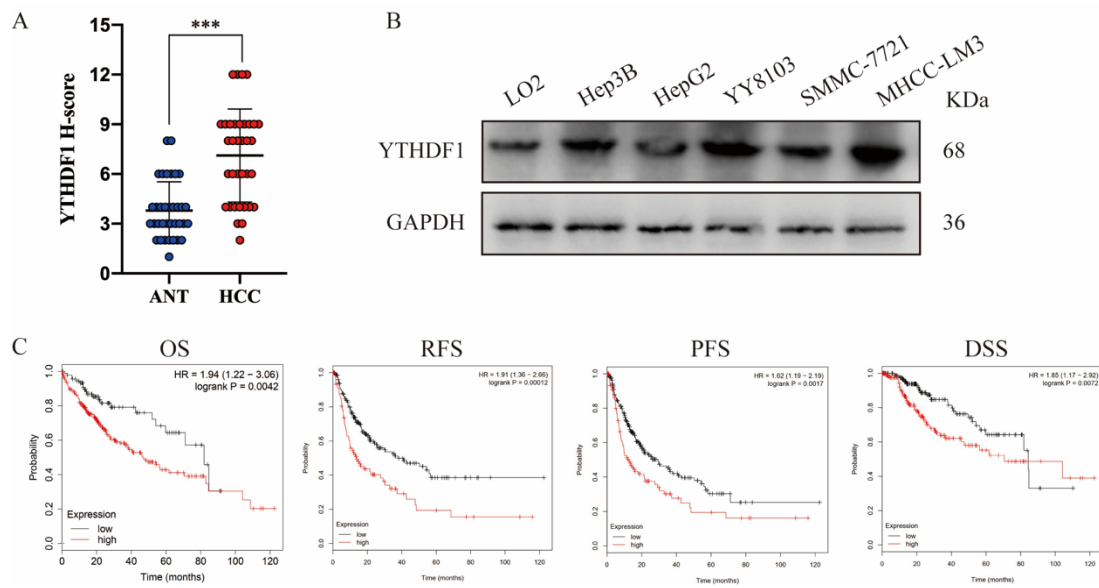


Fig. S2 c-MYC and USF1 promote YTHDF1 expression, related to Figure 2. **a** qRT-PCR analysis of the effect of transfecting c-MYC and USF1 overexpression vectors in HepG2 and MHCC-LM3 cells. **b** qRT-PCR analysis of inhibitory effect of si-c-MYC and si-USF1 in HepG2 and MHCC-LM3 cells. **c** c-MYC/MAX and USF1 expression was positively correlated with YTHDF1 expression in HCC based on TCGA database.

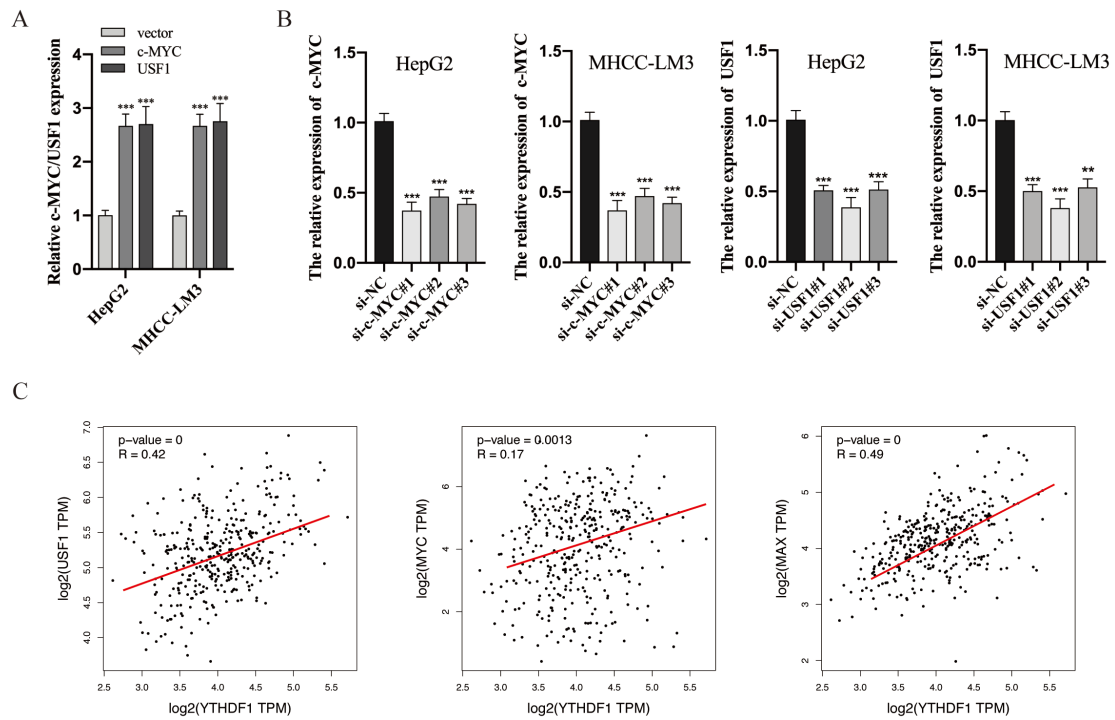


Table S1: The correlations between YTHDF1 expression and clinical characteristics of HCC patients ($n = 84$).

Clinicopathologic parameters	Number	YTHDF1 expression		P value
		High	Low	
Gender				
Male	53	28	25	
Female	31	14	17	0.65
Age (years)				
≥60	47	24	23	
<60	37	18	19	0.91
HBV surface antigen				
Negative	27	12	15	
Positive	57	30	27	0.61
Cirrhosis				
Absent	27	11	16	
Present	57	31	26	0.36
Tumor size (cm)				
≥5	45	28	17	
<5	39	14	25	0.028
Differentiation				
High + moderate	41	19	22	
Low	43	23	20	0.67
TNM stage				
I+II	65	27	36	
III+IV	19	15	6	0.042
Barcelona stage				
A+B	60	26	34	
C+D	24	16	8	0.09
Microvascular invasion				
Absent	40	14	26	
Present	44	28	16	0.016

Table S2: RNA sequences used in this study.

Name	Sequence (5' - 3')	Application
si-myc#1	GCGAGGATATCTGGAAGAA	siRNA target site
si-myc#2	CGATGTTGTTTCTGTGGAA	siRNA target site
si-myc#3	GGCGAACACACAACGUCUU	siRNA target site
si-USF1#1	GCTGGACAATGACGTGCTT	siRNA target site
si-USF1#2	GACGACTCGGGATGAGAAA	siRNA target site
si-USF1#3	CGCCGAGACAAGATCAACA	siRNA target site
si-METTTL3#1	GCACTTGGATCTACGGAAT	siRNA target site
si-METTTL3#2	CGACTACAGTAGCTGCCTT	siRNA target site
si-METTTL3#3	CAGTGGATCTGTTGTGATA	siRNA target site
sh-YTHDF1	CGAAAGAGTTTGAGTGGAA	shRNA target site
sg-YTHDF1	CACGCCCCACGTGACTTCCC	CRISPR/dcas9 target site

Table S3: Primer sequences used in this study.

Name	Sequence (5'-3')	Application
YTHDF1- forward	GACATTGGCACCTGGGATA	qRT-PCR
YTHDF1-Reverse	GCTCTGATACTGCGGTTGAG	qRT-PCR
FZD5- forward	GTCACACCCGCTCTACAACA	qRT-PCR
FZD5-Reverse	GGACGTGGAGATGAAGCACA	qRT-PCR
USF1- forward	GTGATCCAGGGTGCTTTCAC	qRT-PCR
USF1-Reverse	CCTCTGAGCCCTGGGTAGTA	qRT-PCR
c-MYC- forward	CCACGAAACTTTGCCCATAG	qRT-PCR
c-MYC-Reverse	TGCAAGGAGAGCCTTTCAGAG	qRT-PCR
METTL3- forward	CTATCTCCTGGCACTCGCAAGA	qRT-PCR
METTL3-Reverse	GCTTGAACCGTGCAACCACATC	qRT-PCR
GAPDH- forward	GAACGGGAAGCTCACTGG	qRT-PCR
GAPDH-Reverse	GCCTGCTTCACCACCTTCT	qRT-PCR
FZD5 m6A- forward	GGCGCTGAGCTCCGTGGAC	MeRIP-qRT-PCR
FZD5 m6A -Reverse	CGGCGCGTCGCTCAATCC	MeRIP-qRT-PCR
YTHDF1 promoter- forward 1	CGCGACCTGGAACACGC	ChIP-qRT-PCR
YTHDF1 promoter- Reverse 1	AGGTCCGAAGACTAAACGC	ChIP-qRT-PCR
YTHDF1 promoter- forward 2	TGTGTGGGCGGCTGTA	ChIP-qRT-PCR
YTHDF1 promoter- Reverse 2	TCCCAGTCTCGTGCGG	ChIP-qRT-PCR

Table S4: Antibodies used in this study.

Antibody	Company	Application	Concentration
YTHDF1	Proteintech (17479-1-AP)	WB	1:1000
		IHC	1:100
		RIP	2.0 μ g
FZD5	Abcam (ab75234)	WB	1:500
		IHC	4.0 μ g
c-MYC	Proteintech (10828-1-AP)	WB	1:2000
		ChIP	2.0 μ g
	Abcam (ab32072)	IHC	1:100
USF1	SANTA CRUZ (sc-390027)	IHC	1:50
		ChIP	2.0
β -catenin	Proteintech (51067-2-AP)	WB	1:2000
		IHC	1:1000
GAPDH	Proteintech (60004-1-Ig)	WB	1:5000
β -actin	Proteintech (60008-1-Ig)	WB	1:5000
HRP-conjugated Affinipure Goat Anti-Mouse IgG(H+L)	Proteintech (SA00001-1)	WB	1:10000
HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H+L)	Proteintech (SA00001-2)	WB	1:10000