Basic research

Gastroenterology

ENTPD1-AS1 promotes gastric cancer by activating the WNT signaling pathway through the FTO/WIF1 axis and interaction with SRSF1

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Abstract

Introduction: Gastric cancer (GC) remains a major global health burden. Emerging evidence highlights the critical regulatory roles of long noncoding RNAs (lncRNAs) in GC pathogenesis. The purpose of this study was to explore the role and potential regulatory mechanisms of a lncRNA, ENTPD1-AS1, in GC.

Material and methods: Functional assays (CCK-8, colony formation, wound healing and Transwell assays) were performed in AGS and MKN45 cells following ENTPD1-AS1/SRSF1 knockdown or WIF1 overexpression. Targets were screened via RNA-seq and bioinformatics (UALCAN and SRAMP databases). The m6A modification levels were assessed by MeRIP-qPCR, and molecular interactions were validated by RNA immunoprecipitation (RIP) and immunofluorescence (IF) assays.

Results: Knockdown of ENTPD1-AS1 & SRSF1 and overexpression of WIF1 inhibited the proliferation, migration, and invasion of GC cells. Inhibition of ENTPD1-AS1 also increased the m6A modification levels in GC cells. Mechanistically, ENTPD1-AS1 downregulated WIF1 through FTO-mediated low levels of m6A modification to promote β-catenin expression, thereby activating the WNT signaling pathway and exacerbating GC. Additionally, ENTPD1-AS1 promoted GC progression by enhancing β-catenin expression through interaction with SRSF1 to activate the WNT signaling pathway.

Conclusions: The research highlights the significance of the ENTPD1-AS1/FTO/WIF1 and ENTPD1-AS1/SRSF1 axes in collaboratively activating the WNT signaling pathway. These findings identify ENTPD1-AS1 as a potential therapeutic target for GC.

Key words: ENTPD1-AS1, m6A, WIF1, SRSF1, GC.

Introduction

Gastric cancer (GC) is a global malignant disease with high incidence and mortality rates, imposing a significant burden worldwide [1]. It is a complex disease influenced by various factors, including environmental and genetic factors, and is characterized by high invasiveness and heterogeneity [2, 3]. Treatment strategies for GC include systemic thera-

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pies such as chemotherapy, targeted therapy, and immunotherapy, as well as surgical interventions [4, 5]. Unfortunately, due to the subtle symptoms of early-stage GC, most patients are diagnosed at advanced stages, leading to significantly reduced survival rates [6]. Therefore, exploring biomarkers and therapeutic targets for GC is of great clinical significance.

In recent years, numerous studies have shown that long non-coding RNAs (lncRNAs) are widely involved in the occurrence, development, and metastasis of GC through various mechanisms, including the regulation of transcription, translation, epigenetic modifications, and signaling pathway activity. They have emerged as potential diagnostic biomarkers and therapeutic targets [7, 8]. For instance, lnc_ASNRG regulates the occurrence and development of GC through the miR-519e-5p/FGFR2 axis [9], while lncRNA SNHG4 promotes GC progression by inhibiting miR-204-5p [10]. Furthermore, LINC01268 activates the PI3K/AKT signaling pathway by targeting MARCKS, promoting epithelial-mesenchymal transition (EMT), and ultimately enhancing the invasion and metastasis of GC [11]. The role of antisense lncRNAs in GC has also been revealed. For instance, lncTM4SF1-AS1 can upregulate TM4SF1 and activate the PI3K/AKT signaling pathway, thereby promoting the proliferation, metastasis, and EMT of GC cells [12]. Additionally, GATA6-AS1 inhibits GC cell proliferation and migration by upregulating PTEN through sponging miR-543, thereby inactivating the AKT signaling pathway [13]. ENTPD1-AS1, as an antisense lncRNA, has been defined as a novel lncRNA that promotes GC [14]. However, further exploration of its specific molecular mechanisms in GC is warranted. As biomedicine continues to advance, epigenetics has gained attention as an important field for elucidating gene expression mechanisms. N6-methyladenosine (m6A) is one of the most common genetic epigenetic modifications in eukaryotic cells, dynamically regulated by "writers" (METTL3, METTL14 and WTAP) and "erasers" (FTO and ALKBH5), and identified by "readers" (YTHDF family, YTHDF family, HNRNP family and IGF2BP family). It is present in almost all RNA types and plays a key role in a variety of physiological and pathological processes, including cancer [15, 16]. This provides new perspectives for understanding the mechanisms of tumorigenesis and development.

Recently, the regulatory roles of lncRNAs and RNA-binding proteins (RBPs) in tumors have received significant attention [17]. Studies have shown that lncRNAs can interact with RBPs to regulate key signaling pathways, thereby influencing the malignant progression of tumors. For example, GAS6-AS1 activates the WNT pathway in bladder cancer by inhibiting APC expression through its interaction with PRC1 [18]. In addition, silencing SCAMP1-TV2 reduces INSM1 mRNA expression by decreasing its binding to PUM2, downregulating the inhibitory activity of INSM1 on SASH1 transcription and suppressing the activity of the PI3K/AKT signaling pathway, thereby inhibiting the malignant biological behavior of breast cancer cells [19]. However, it remains unclear whether ENTPD1-AS1 influences GC development through its interaction with RBPs. Furthermore, the WNT signaling pathway is a crucial cellular signaling pathway that regulates cellular processes to impact cell function, which contributes to the development of various cancers [20]. The classical WNT signaling pathway is β-catenin-dependent [21], making β-catenin synthesis a key activating factor.

Based on the above background, this study aims to elucidate the molecular mechanism by which ENTPD1-AS1 activates the WNT signaling pathway through β-catenin synthesis, ultimately influencing GC development.

Material and methods

Cell culture

Human GC cell lines AGS and MKN45 were obtained from the Cell Bank of the Chinese Academy of Sciences in Shanghai. These cell lines were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. They were maintained at 37°C in a humidified incubator with 5% CO_2.

Cell transfection

In this study, short hairpin RNAs (shRNAs) specifically targeting ENTPD1-AS1 (sh-ENTPD1-AS1#1/#2), WIF1 (sh-WIF1#1/#2), and SRSF1 (sh-SRSF1#1/#2), as well as overexpression vectors for FTO and SRSF1 and their corresponding control vectors, were obtained from Ribobio (Guangzhou, China). Following acquisition of the vectors, they were transfected into GC cells as per experimental requirements, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Reverse-transcription quantitative PCR (RT-qPCR)

The extraction of total RNA from AGS and MKN45 cells was performed using TRIzol reagent (Invitrogen). Following reverse transcription with PrimerScript RT Reagent (TaKaRa, Dalian, China), cDNAs were obtained and subsequently subjected to real-time PCR analysis using a SYBR Green detection system (TaKaRa) on

an Applied Biosystems Prism 7900 instrument (Life Technologies, USA).

Western blot

Total proteins were extracted using RIPA buffer (Beyotime, Shanghai, China) containing protease inhibitor. The protein concentration was determined using a BCA Kit (Beyotime). The protein samples were separated using SDS-PAGE after boiling water denaturation and then transferred to PVDF membranes (Millipore). The membranes were incubated with specific primary antibodies (anti-β-catenin, anti- SRSF1, and anti-GAPDH) at 4°C overnight after 1 h of containment using 5% non-fat milk. The next day, the primary antibodies were removed and washed three times with TBST, and the corresponding secondary antibodies were added and incubated at room temperature for 1 h. The protein blots were visualized by applying an ECL system (Beyotime).

Cell proliferation and colony formation assays

GC cells were seeded into 96-well plates at a density of 3,000 cells per well in triplicate. Following incubation for 24, 48, or 72 h, a 10 µl aliquot of the CCK-8 reagent (Dojindo, Kumamoto, Japan) was introduced to each well and allowed to incubate for an additional 2 h. Subsequently, the absorbance was assessed at 450 nm using a Multiskan Go microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

For the colony formation assay, 1000 cells were seeded into 6-well plates containing complete, allowing for incubation over a period of 14 days. Then cells were fixed with 4% paraformaldehyde, followed by a 1-hour staining process with 1% crystal violet. Finally, the resultant colonies were documented photographically and subsequently quantified using the ImageJ software application.

Migration and invasion assay

The wound healing assay was performed to assess the migratory capacity of GC cells. GC cells transfected with various vectors were seeded into 6-well plates and cultured until reaching 90% confluence. Subsequently, scratches were generated on the cell monolayer. The cells were then incubated at 37°C in a 5% CO_2 environment, and images were captured at 0 and 24 h to measure the width of the scratches.

The Transwell assay was employed to assess the invasive capacity of GC cells. GC cells in serum-free medium were seeded into the upper chamber coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The lower chamber contained medium with 10% FBS. Following 24 h of incubation, cells were stained with 1% crystal violet. The number of invaded cells was quantified under a light microscope (Olympus, Tokyo, Japan).

RNA immunoprecipitation (RIP)

RIP assays were carried out using a Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Cells were lysed, and immunoprecipitated magnetic beads were prepared to construct immunoprecipitations of RNA-binding protein-RNA complexes according to the instructions. The immunoprecipitated RNA was then purified with RNA, and the recovered RNA was quantified by RT-qPCR.

RNA pulldown + mass spectrometry analysis

To identify proteins potentially binding to ENTPD1-AS1, RNA pulldown coupled with mass spectrometry (MS) analysis was performed. The Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher, USA) was used according to the manufacturer's instructions for the RNA pulldown assay. In brief, cell lysates were incubated with biotinylated RNA probes to form RNA-protein complexes. Subsequently, streptavidin magnetic beads were added to capture the complexes. The complexes were then subjected to silver staining using the Pierce Silver Stain Kit (Thermo Fisher, USA) following the operational guidelines followed by mass spectrometry identification.

Methylated RNA immunoprecipitation

Methylated RNA immunoprecipitation (MeRIP) detection was carried out using the Magna MeRIP m6A kit (Merck Millipore, Germany) following the manufacturer's instructions. Total RNA was fragmented, and anti-m6A or anti-IgG antibodies were added for overnight incubation at 4°C. The pre-treated Protein A/G magnetic beads were then incubated at 4°C for 1 h. Subsequently, the beads were collected, washed, and the RNA was extracted for RT-qPCR analysis to assess the m6A modification level of WIF1.

TOPflash assay

The TOPflash assay was used to evaluate the activity of the WNT signaling pathway. The TOPflash reporter gene vector and the Renilla luciferase vector were transfected into GC cells. After 48 h, the cells were lysed, and the Dual Luciferase Reporter Assay System (Promega) was used according to the manufacturer's instructions for measurement. The firefly luciferase signal was detected at 560 nm, and the Renilla luciferase signal

at 480 nm. Renilla luciferase activity was used as a normalization control.

CHX chase assay

To observe the degradation of β-catenin, the CHX chase assay was performed. Two milliliters of DMEM containing 100 μg/ml cycloheximide (CHX; Sigma-Aldrich, USA) was added to each of the 4 culture dishes in each group. Total protein was extracted at 0, 2, 4, and 8 h time points and analyzed by western blot.

Immunofluorescence assay

Gastric cancer cells were plated in 24-well plates at a density of 2 × 10^4 cells per well. Following a 24-hour incubation period, the cells underwent two washes with phosphate-buffered saline before fixation with 4% paraformaldehyde for 10 min. Subsequent processing included permeabilization using 0.5% Triton X-100 for 20 min and blocking with 5% bovine serum albumin for 1 h at room temperature. Then, primary antibody against SRSF1 was applied and left to incubate at 4°C overnight. After washing, cells were treated with appropriate secondary antibody for 1 h, followed by nuclear counterstaining with DAPI. Fluorescence images were acquired using a confocal microscope (Leica, Wetzlar, Germany).

Fluorescence in situ hybridization assay

Fluorescence in situ hybridization (FISH) was carried out according to the Fluorescent In Situ Hybridization Kit protocol (RiboBio). GC cells

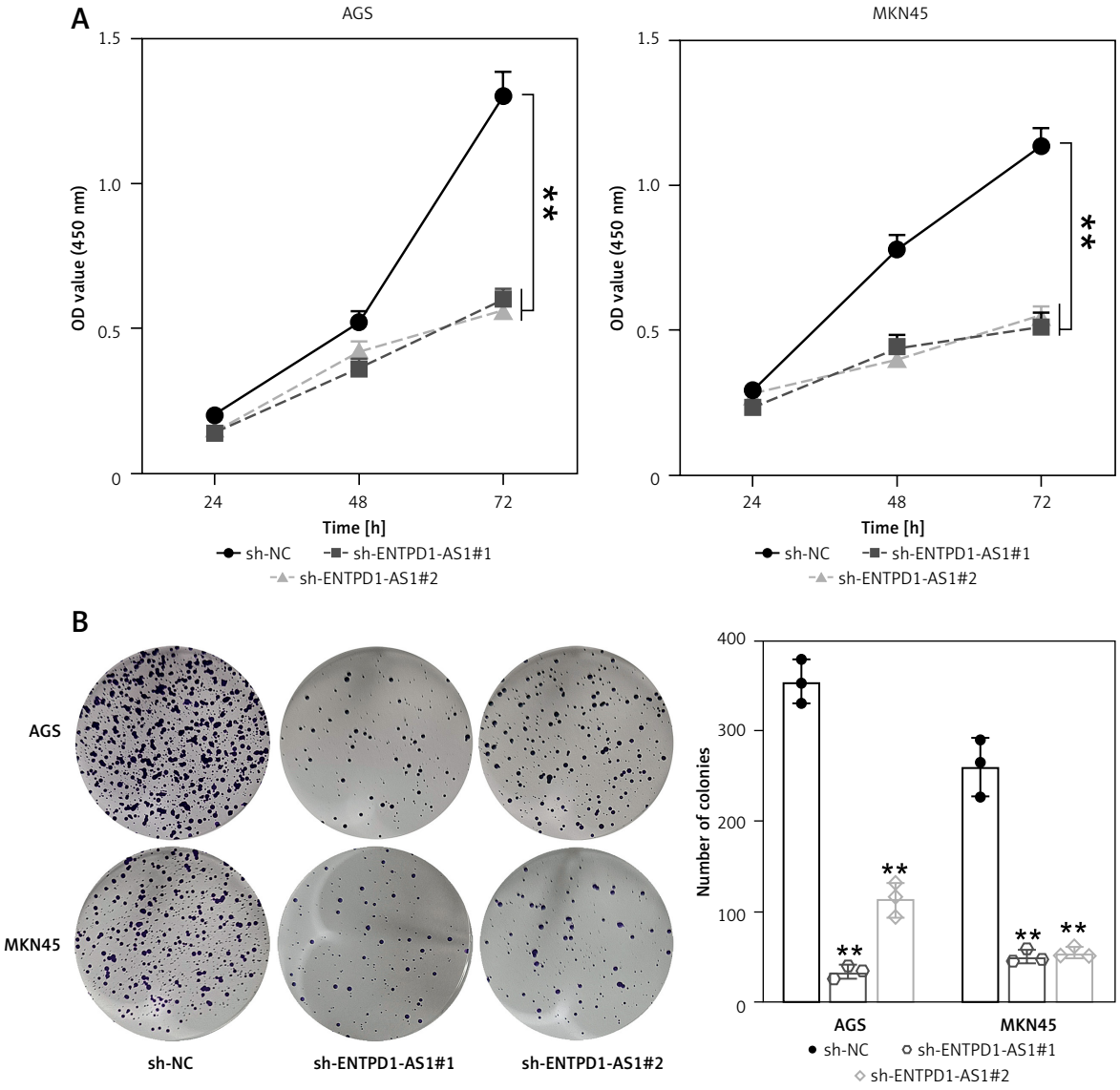


Figure 1. A, **B** – The effect of ENTPD1-AS1 knockdown on GC cell proliferation was assessed using CCK8 and colony formation assays, revealing that silencing ENTPD1-AS1 reduced GC cell proliferation. $^{**}p < 0.01$

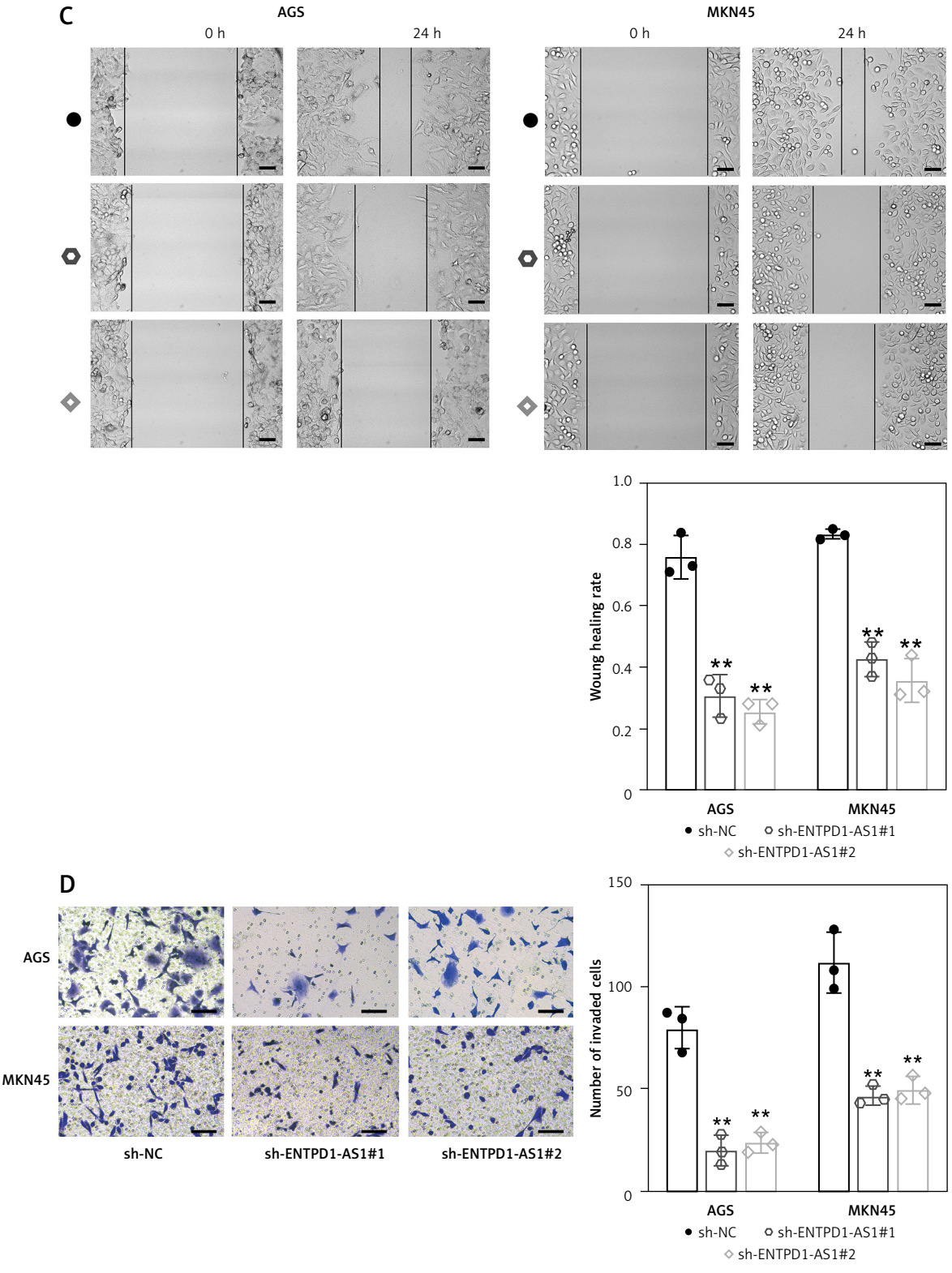


Figure 1. Cont. **C** – Wound healing assay was used to analyze the effect of ENTPD1-AS1 knockdown on GC cell migration, revealing that knockdown of ENTPD1-AS1 inhibited GC cell migration. **D** – Transwell assay was used to analyze the effect of ENTPD1-AS1 knockdown on GC cell invasion, indicating that knockdown of ENTPD1-AS1 suppressed GC cell invasion. $^{**}p < 0.01$

grown on glass coverslips at 60–70% confluence were PBS-washed, fixed with 4% formaldehyde for 20 min, and permeabilized with 0.5% Triton X-100. Overnight hybridization at 37°C was performed using ENTPD1-AS1-specific probes (RiboBio) in a humidified chamber. Following hybridization, nuclei were counterstained with DAPI and samples were imaged using a confocal microscopy.

Bioinformatics analysis

The SRAMP website was used to predict genes potentially undergoing m6A modification. The UALCAN database was used to analyze the expression of WIF1 in stomach adenocarcinoma (STAD). The GEPIA website was employed to analyze the expression of SRSF1 in various cancers.

Statistical analysis

Statistical analyses were performed using SPSS 22.0 (IBM, Armonk, NY, USA) or GraphPad Prism 7.0 (GraphPad Prism, Inc., La Jolla, CA, USA). Each experiment was repeated at least three times, with the results expressed as the mean ± SD of three independent trials. Student's *t*-test or one-way ANOVA was used to compare the means of two or more groups. A p-value < 0.05 was considered statistically significant.

Results

ENTPD1-AS1 promotes proliferation, migration, and invasion of GC cells

To investigate the role of ENTPD1-AS1 in GC, functional experiments were conducted in GC cells following knockdown of ENTPD1-AS1. Cell proliferation capacity was assessed using the CCK-8 assay and colony formation assay, revealing that knockdown of ENTPD1-AS1 led to a decrease in the proliferation ability of GC cells (Figures 1 A, B). Subsequently, changes in the migration and invasion abilities of GC cells were assessed through the wound healing assay and Transwell assay, showing that knockdown of ENTPD1-AS1 also resulted in a reduction in the migration and invasion abilities of GC cells (Figures 1 C, D). In summary, our findings indicate that ENTPD1-AS1 acts as an oncogene in GC, positively correlating with the proliferation, migration, and invasion abilities of GC cells.

ENTPD1-AS1 downregulates WIF1 through m6A modification

Next, we further explored the downstream regulatory mechanism of ENTPD1-AS1 in GC. Since m6A modification plays a crucial role in post-transcriptional gene expression regulation, we considered it while identifying the downstream target genes of ENTPD1-AS1. Using RNA-seq, we found 52 upregulated genes after knockdown of ENTPD1-AS1. Subsequently, the SRAMP database predicted 6 genes potentially undergoing m6A modification, and finally, the UALCAN database was used to screen out 4 genes (WIF1, SORBS, PHYHIPL, and CAPN13) that were downregulated in GC (Figure 2 A). It is well known that WNT inhibitory factor-1 (WIF1) is closely related to the WNT signaling pathway and exerts a negative regulatory effect on this pathway [22]. Therefore, we chose to focus on WIF1 for further investigation. Additionally, we presented the results from the UALCAN database predicting low expression of WIF1 in STAD (Figure 2 B). To confirm that ENTPD1-AS1 regulates WIF1 expression through m6A modification, we conducted meRIP-qPCR and RT-qPCR (Figures 2 C, D). The results indicated that interference with ENTPD1-AS1 upregulated the m6A modification level but increased WIF1 expression. Therefore, we can conclude that ENTPD1-AS1 downregulates WIF1 expression through m6A modification regulation.

WIF1 inhibits GC cell proliferation, migration, and invasion

To determine the role of WIF1 in GC development, we overexpressed WIF1 in GC cells and performed the following experiments. The results from the CCK-8 assay and colony formation assay indicated that overexpression of WIF1 inhibited the proliferation of GC cells (Figures 3 A, B). Additionally, the results from the wound healing assay and Transwell assay demonstrated that overexpression of WIF1 inhibited the migration and invasion abilities of GC cells (Figures 3 C, D). Therefore, we conclude that overexpression of WIF1 inhibits the proliferation, migration, and invasion of GC cells. In addition, since β-catenin is a key factor in the WNT signaling pathway, we also observed the changes in β-catenin expression under different conditions by Western blot (Figure 3 E). Based on the experimental results, we found that knockdown of ENTPD1-AS1 inhibited β-catenin expression, while knockdown of WIF1 promoted β-catenin expression. Additionally, knockdown of WIF1 reversed the inhibitory effect of ENTPD1-AS1 knockdown on β-catenin expression. This suggests that ENTPD1-AS1 promotes β-catenin expression by down-regulating WIF1.

ENTPD1-AS1 downregulates WIF1 through FTO

Given the results indicating that ENTPD1-AS1 downregulates WIF1 via m6A modification, we further explored the regulation of m6A. Based on

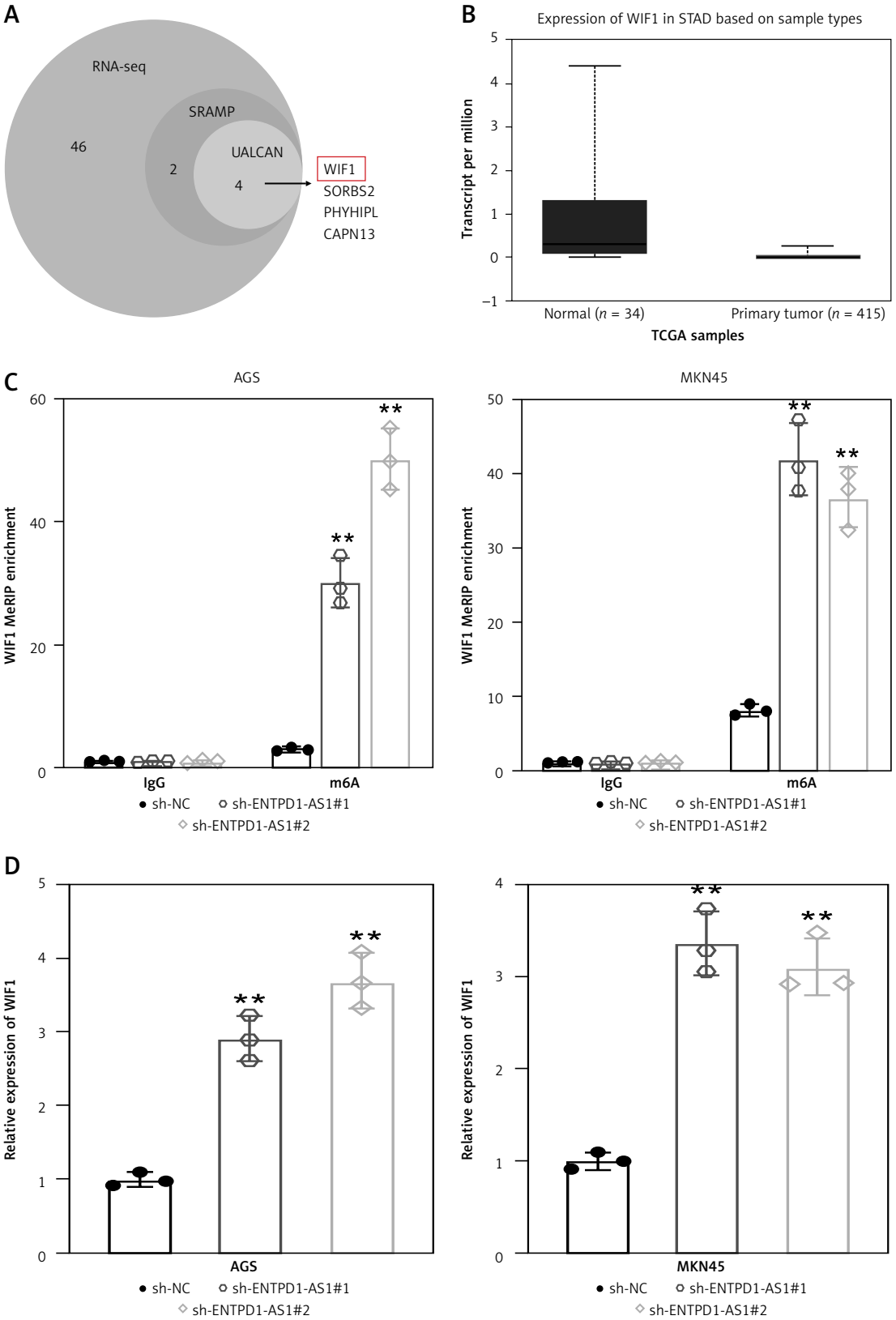


Figure 2. ENTPD1-AS1 downregulates WIF1 through m6A modification. **A** – WIF1, SORBS2, PHYHIPL, and CAPN13 were screened using RNA-seq, SRAMP, and UALCAN databases. **B** – The UALCAN database was analyzed for the expression level of WIF1 in gastric cancer tissues. **C** – MeRIP-qPCR was used to assess the effect of ENTPD1-AS1 knockdown on the m6A modification of WIF1, showing that knockdown of ENTPD1-AS1 significantly upregulated the m6A levels of WIF1. **D** – RT-qPCR demonstrated that ENTPD1-AS1 knockdown significantly increased WIF1 expression. $^{**}p < 0.01$

RT-qPCR results, we observed that knockdown of ENTPD1-AS1 significantly decreased the expression of FTO only (Figure 4 A). This suggests that the reduction in m6A modification is mediated by FTO. Simultaneously, we evaluated the interaction between FTO and WIF1 through the RIP assay (Figure 4 B). Subsequently, we simultaneously knocked down ENTPD1-AS1 and overexpressed FTO to observe the changes in m6A modification level and expression level of WIF1 (Figures 4 C, D). The results indicated that FTO overexpression reversed the promoting effect of knockdown of ENTPD1-AS1 on the m6A modification level and expression level of WIF1. In conclusion, ENTPD1-AS1 downregulates WIF1 through promoting FTO-mediated reduction in m6A modification level.

Rescue experiments confirmed that ENTPD1-AS1 promotes GC cell proliferation, migration, and invasion through WIF1

We validated the findings so far by evidence rescue experiments. After transfecting GC cells with both ENTPD1-AS1 and WIF1 knockdown vectors, cell function assays were used to observe the changes in proliferation, migration, and invasion of GC cells. CCK-8 and colony formation assays showed that knockdown of WIF1 restored the proliferative ability inhibited by knockdown of ENTPD1-AS1 (Supplementary Figures S1 A, B). The results from the wound healing assay and Transwell assay also indicated that knockdown of WIF1 restored the migration and invasion abilities inhibited by

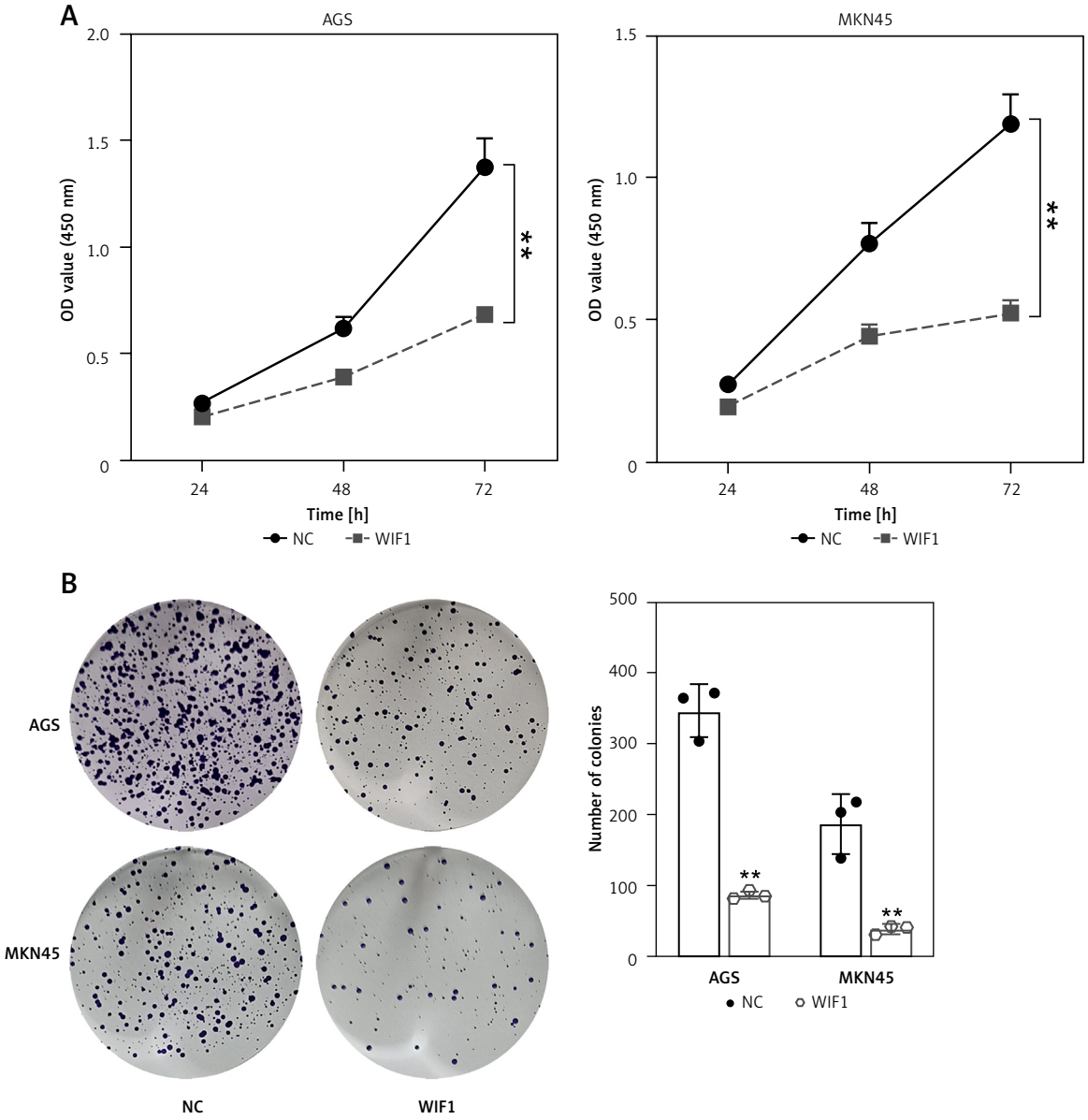


Figure 3. WIF1 inhibits GC cell proliferation, migration, and invasion. **A**, **B** – The effect of WIF1 overexpression on GC cell proliferation was evaluated using CCK8 and colony formation assays, revealing that WIF1 overexpression inhibited GC cell proliferation. *$p < 0.05$, **$p < 0.01$

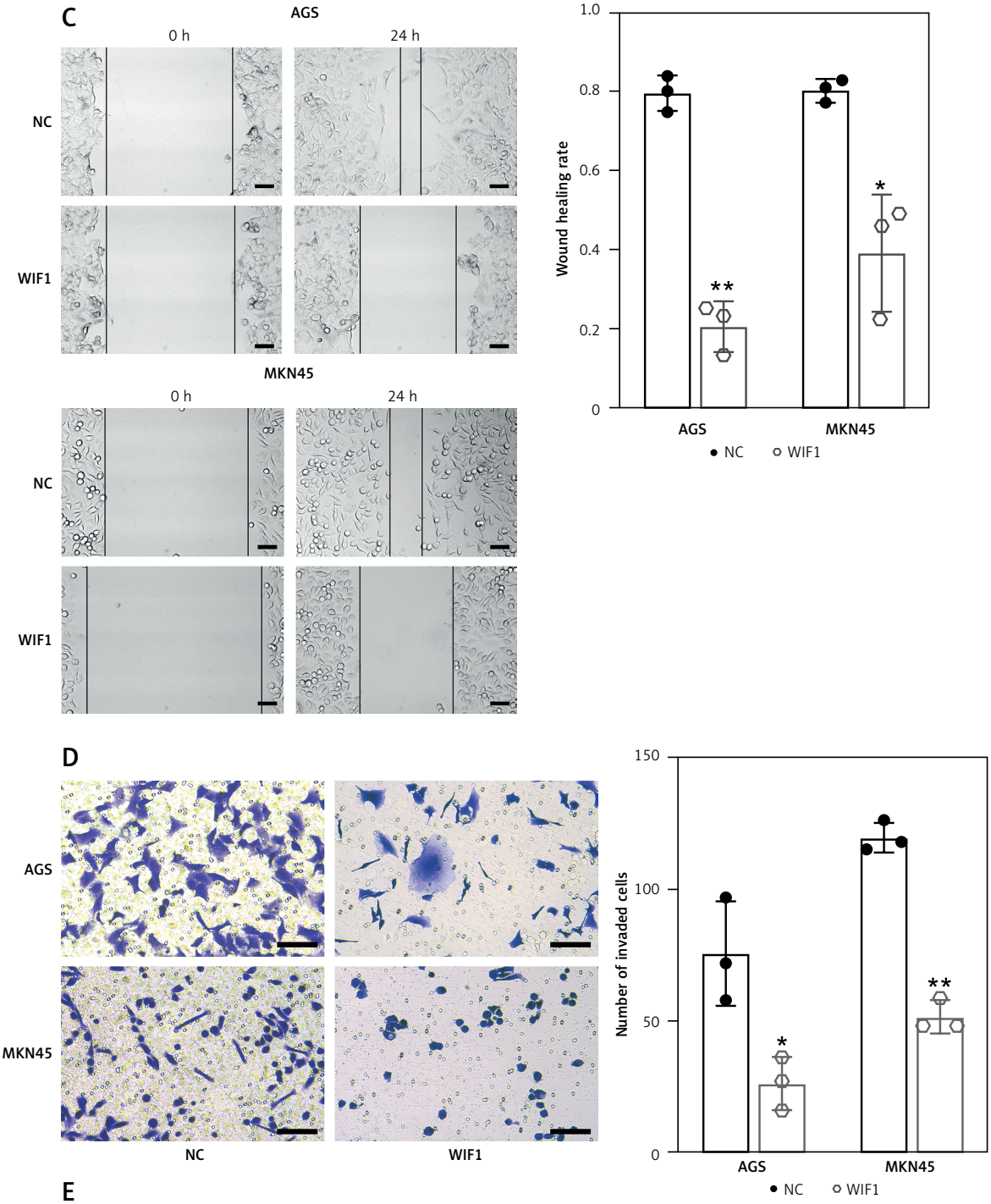


Figure 3. Cont. **C** – Wound healing assay was conducted to analyze the effect of WIF1 overexpression on GC cell migration, indicating that WIF1 overexpression reduced GC cell migration. **D** – Transwell assay was performed to evaluate the effect of WIF1 overexpression on GC cell invasion, demonstrating that WIF1 overexpression inhibited GC cell invasion. **E** – Western blot analysis of the effects of ENTPD1-AS1 and WIF1 knockdown on β-catenin expression revealed that ENTPD1-AS1 knockdown suppressed β-catenin expression, while WIF1 knockdown increased β-catenin levels, and WIF1 overexpression reversed the decrease in β-catenin expression induced by ENTPD1-AS1 knockdown. *$p < 0.05$, **$p < 0.01$

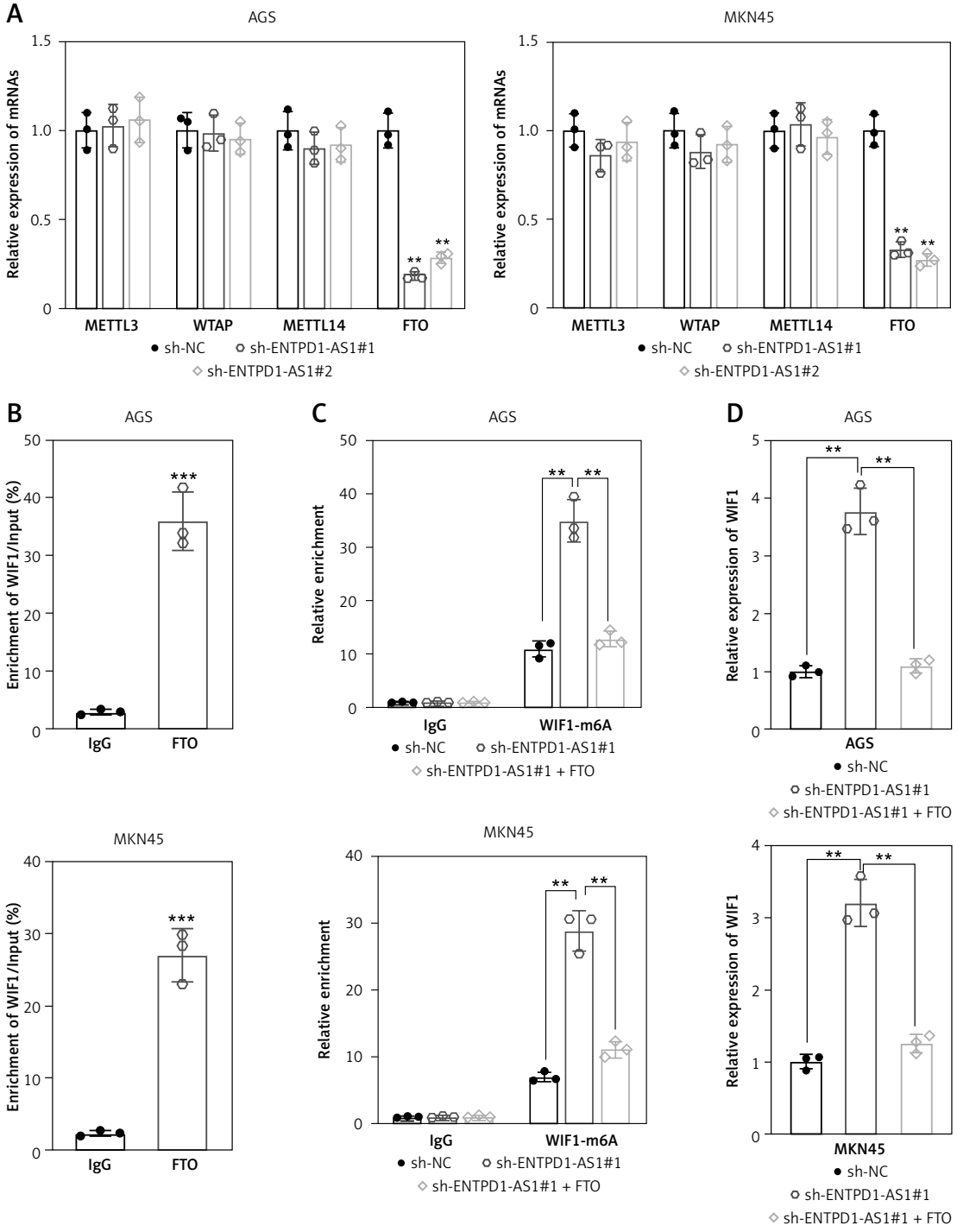
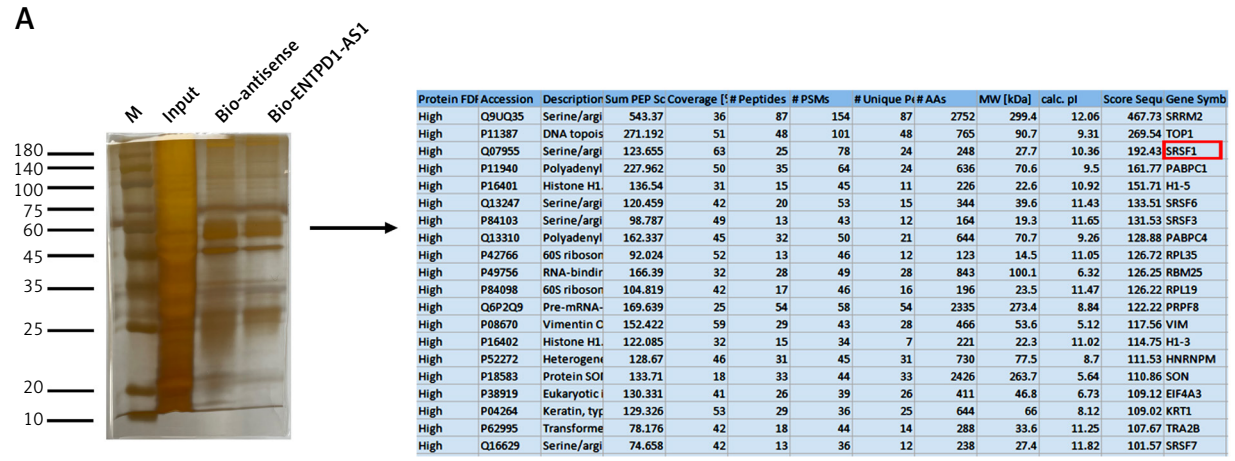


Figure 4. ENTPD1-AS1 downregulates WIF1 through FTO. **A** – RT-qPCR analysis of the effect of ENTPD1-AS1 knockdown on the expression of METTL3, WTAP, METTL14, and FTO, showing that ENTPD1-AS1 knockdown significantly downregulated FTO mRNA levels. **B** – RIP assay results indicated the binding of FTO to WIF1. **C** – MeRIP-qPCR analysis was performed to evaluate the effect of ENTPD1-AS1 knockdown and FTO overexpression on the m6A level of WIF1 mRNA, demonstrating that FTO overexpression reversed the increase in m6A modification levels induced by ENTPD1-AS1 knockdown. **D** – RT-qPCR was used to analyze the effect of ENTPD1-AS1 knockdown and FTO overexpression on WIF1 mRNA expression, showing that FTO overexpression reversed the increase in WIF1 mRNA levels mediated by ENTPD1-AS1 knockdown. $^{**}p < 0.01$, $^{***}p < 0.001$



Protein FDI	Accession	Description	Sum PEP Sc	Coverage [%	# Peptides	# PSMs	# Unique Pe	# AAs	MW [kDa]	calc. pI	Score Sequ	Gene Symb
High	Q9UQ35	Serine/argi	543.37	36	87	154	87	2752	299.4	12.06	467.73	SRRM2
High	P11387	DNA topois	271.192	51	48	101	48	765	90.7	9.31	269.54	TOP1
High	Q07955	Serine/argi	123.655	63	25	78	24	248	27.7	10.36	192.43	SRSF1
High	P11940	Polyadenyl	227.962	50	35	64	24	636	70.6	9.5	161.77	PABPC1
High	P16401	Histone H1.	136.54	31	15	45	11	226	22.6	10.92	151.71	H1-5
High	Q13247	Serine/argi	120.459	42	20	53	15	344	39.6	11.43	133.51	SRSF6
High	P84103	Serine/argi	98.787	49	13	43	12	164	19.3	11.65	131.53	SRSF3
High	Q13310	Polyadenyl	162.337	45	32	50	21	644	70.7	9.26	128.88	PABPC4
High	P42766	60S riboson	92.024	52	13	46	12	123	14.5	11.05	126.72	RPL35
High	P49756	RNA-bindir	166.39	32	28	49	28	843	100.1	6.32	126.25	RBM25
High	P84098	60S riboson	104.819	42	17	46	16	196	23.5	11.47	126.22	RPL19
High	Q6P2Q9	Pre-mRNA-	169.639	25	54	58	54	2335	273.4	8.84	122.22	PRPF8
High	P08670	Vimentin C	152.422	59	29	43	28	466	53.6	5.12	117.56	VIM
High	P16402	Histone H1.	122.085	32	15	34	7	221	22.3	11.02	114.75	H1-3
High	P52272	Heterogene	128.67	46	31	45	31	730	77.5	8.7	111.53	HNRNPM
High	P18583	Protein SON	133.71	18	33	44	33	2426	263.7	5.64	110.86	SON
High	P38919	Eukaryotic i	130.331	41	26	39	26	411	46.8	6.73	109.12	EIF4A3
High	P04264	Keratin, typ	129.326	53	29	36	25	644	66	8.12	109.02	KRT1
High	P62995	Transforme	78.176	42	18	44	14	288	33.6	11.25	107.67	TRA2B
High	Q16629	Serine/argi	74.658	42	13	36	12	238	27.4	11.82	101.57	SRSF7

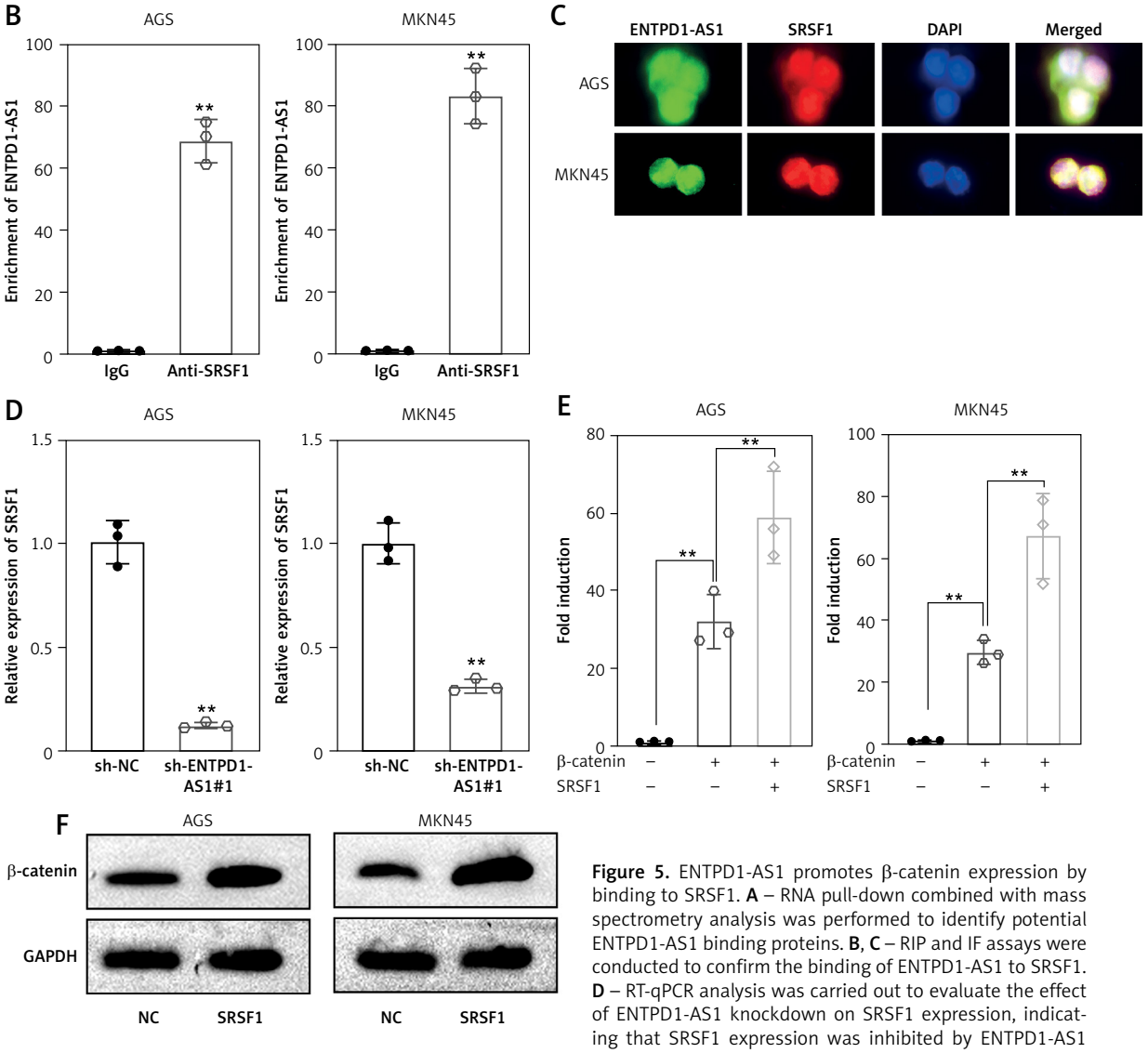


Figure 5. ENTPD1-AS1 promotes β-catenin expression by binding to SRSF1. **A** – RNA pull-down combined with mass spectrometry analysis was performed to identify potential ENTPD1-AS1 binding proteins. **B**, **C** – RIP and IF assays were conducted to confirm the binding of ENTPD1-AS1 to SRSF1. **D** – RT-qPCR analysis was carried out to evaluate the effect of ENTPD1-AS1 knockdown on SRSF1 expression, indicating that SRSF1 expression was inhibited by ENTPD1-AS1 knockdown. **E** – TOPflash assay was employed to analyze the effect of SRSF1 on the WNT signaling pathway, showing that SRSF1 promoted WNT signaling activation. **F** – Western blot analysis was performed to assess the effect of SRSF1 overexpression on β-catenin protein expression, revealing that SRSF1 overexpression increased β-catenin protein levels. $^{**}p < 0.01$

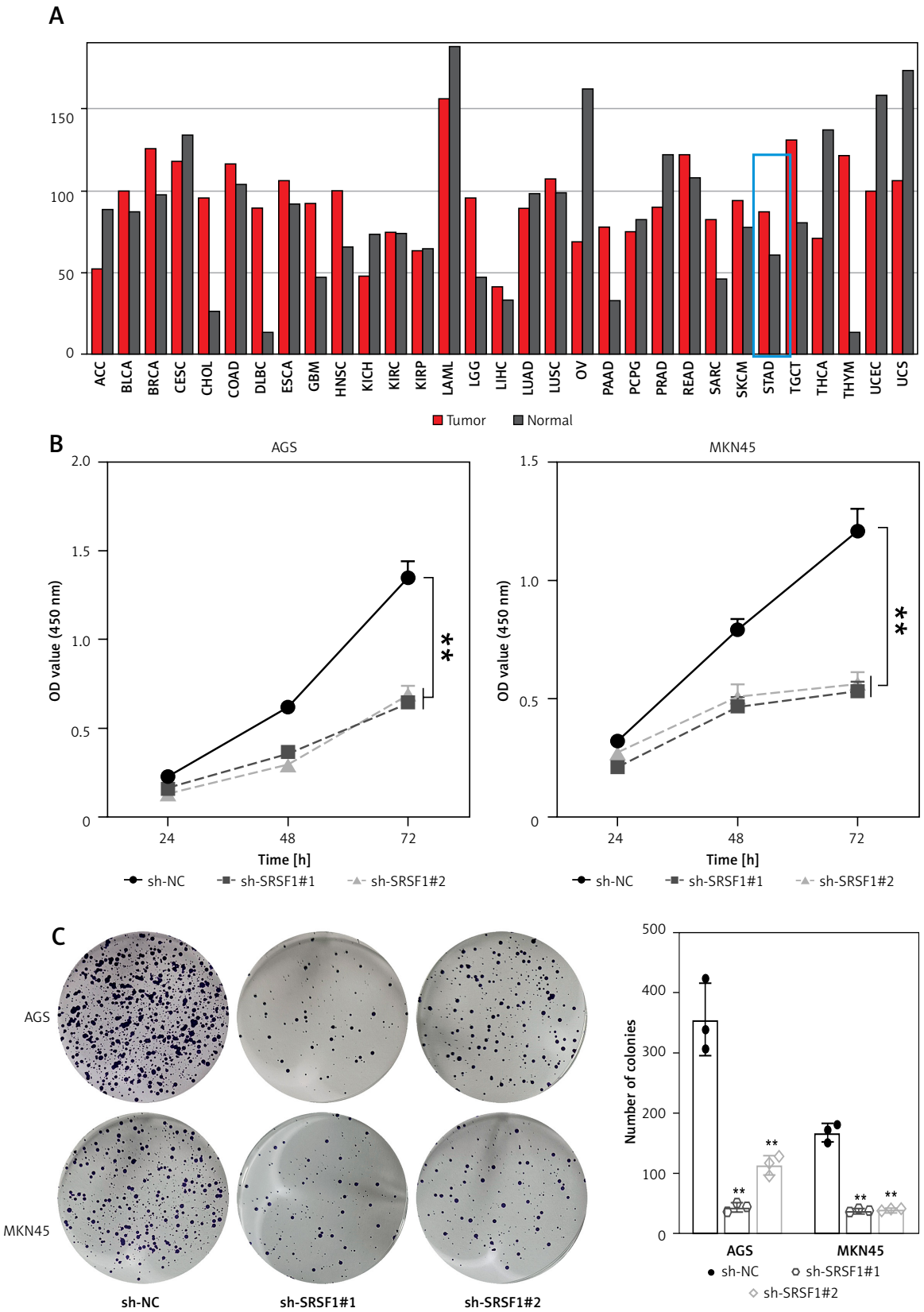


Figure 6. RSF1 is highly expressed in GC tissues and promotes proliferation, migration, and invasion of GC cells. **A** – GEPIA analysis of SRSF1 expression in pan-cancers. **B**, **C** – CCK8 and colony formation assays were used to analyze the effect of SRSF1 knockdown on the proliferation of GC cells, demonstrating that SRSF1 knockdown inhibited GC cell proliferation. *$p < 0.05$, **$p < 0.01$

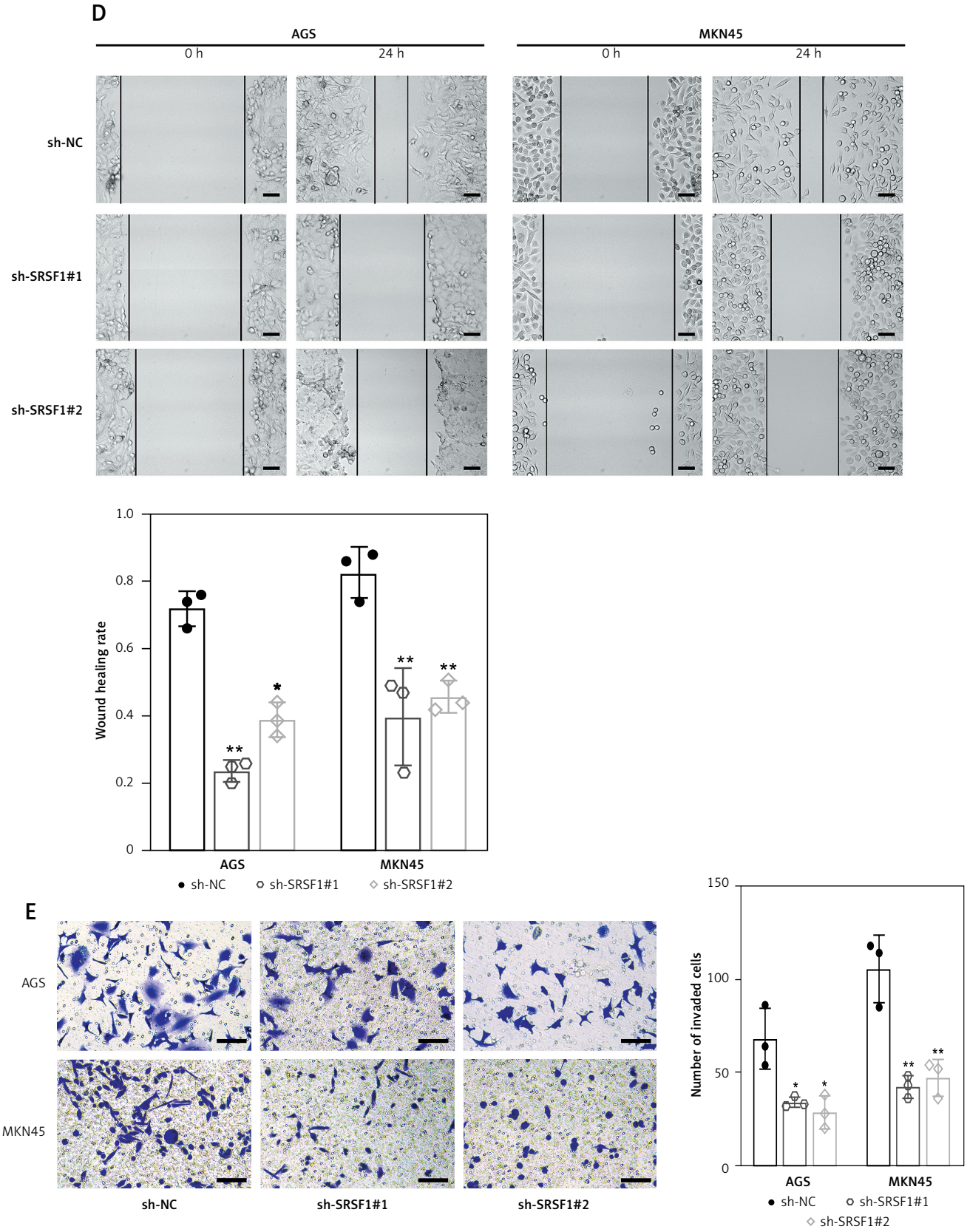


Figure 6. Cont. **D** – Wound healing assay was performed to assess the effect of SRSF1 knockdown on the migration of GC cells, indicating that SRSF1 knockdown hindered GC cell migration. **E** – Transwell assay was conducted to evaluate the effect of SRSF1 knockdown on the invasion of GC cells, showing that SRSF1 knockdown obstructed GC cell invasion. *$p < 0.05$, **$p < 0.01$

knockdown of ENTPD1-AS1 (Supplementary Figures S1 C, D). In summary, ENTPD1-AS1 exerts its pro-oncogenic effects on GC through WIF1.

ENTPD1-AS1 promotes β-catenin expression by binding to SRSF1

Since lncRNAs regulate downstream gene expression by binding to RNA-binding proteins (RBPs) [23], we hypothesized that ENTPD1-AS1 regulates β-catenin expression by interacting with an RBP. To test our hypothesis, we first conducted RNA pulldown coupled with mass spectrometry analysis to identify proteins that may interact with ENTPD1-AS1 (Figure 5 A). Among them, the RBP SRSF1 drew our attention, as it has been reported to accelerate β-catenin synthesis to activate the WNT signaling pathway, promoting tumorigenesis [24]. Subsequently, we confirmed the binding of ENTPD1-AS1 and SRSF1 through RIP and IF assays (Figures 5 B, C). Additionally, RT-qPCR results showed a positive correlation between ENTPD1-AS1 and SRSF1 expression, indicating that knockdown of ENTPD1-AS1 inhibits SRSF1 expression (Figure 5 D). Next, we investigated the impact of SRSF1 on the WNT pathway and found that SRSF1 promotes WNT signaling (Figure 5 E). Furthermore, we examined the effect of SRSF1 on β-catenin and observed that SRSF1 does not affect β-catenin degradation but enhances its synthesis through binding (Supplementary Figures S2 A, B, Figure 5 F). Therefore, based on the aforementioned experiments, it is evident that ENTPD1-AS1 promotes β-catenin expression by binding to SRSF1, thereby activating the WNT signaling pathway.

SRSF1 is highly expressed in GC tissues and promotes proliferation, migration, and invasion of GC cells

To investigate whether SRSF1 plays a pro-cancer role in GC, we conducted the following experiments. Firstly, we analyzed the expression of SRSF1 in various cancers using the GEPIA database (Figure 6 A) and found that SRSF1 is highly expressed in GC tissues. Subsequently, we knocked down SRSF1 in GC cells and observed its effects on cell proliferation, migration, and invasion. The results from the CCK-8 assay and colony formation assay showed that knockdown of SRSF1 led to inhibited proliferation of GC cells (Figures 6 B, C). The wound healing assay and Transwell assay also indicated that knockdown of SRSF1 resulted in suppressed migration and invasion of GC cells (Figures 6 D, E). Therefore, SRSF1 enhances the proliferation, migration, and invasion abilities of GC cells.

Rescue experiments confirmed that ENTPD1-AS1 promotes proliferation, migration, and invasion of GC cells through SRSF1

Finally, we performed a rescue experiment to verify whether ENTPD1-AS1 promotes GC development through SRSF1. The CCK-8 assay and colony formation assay clearly demonstrated that overexpression of SRSF1 reversed the inhibitory effect of ENTPD1-AS1 knockdown on the proliferation of GC cells (Supplementary Figures S3 A, B). The wound healing assay and Transwell assay showed a similar trend, where overexpression of SRSF1 reversed the suppression of migration and invasion caused by ENTPD1-AS1 knockdown (Supplementary Figures S3 C, D). This confirms that ENTPD1-AS1 promotes the proliferation, migration, and invasion of GC cells through SRSF1.

Discussion

Gastric cancer, as a common malignant tumor, requires urgent exploration of novel molecular markers for its diagnosis, treatment, and prognosis. LncRNAs, as a type of non-coding RNA, influence the occurrence and development of cancer through various molecular mechanisms. For example, lncRNA PTCSC3 inhibits the proliferation of oral cancer cells by inducing apoptosis and autophagy [25], while lncRNA PTTG3P regulates the development of lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) through a ceRNA mechanism [26]. Recent studies have investigated the potential molecular mechanisms of lncRNAs in GC, primarily focusing on the ceRNA mechanism. For instance, lncRNA LIFR-AS1 upregulates MTUS1 by sponging miR-4698 to inhibit GC progression [27]. Moreover, the lncRNA GIHCG promotes GC development by upregulating TLE1 expression through sponging miR-1281 [28]. Notably, the impact of ENTPD1-AS1 on GC has been reported to be mediated through the ceRNA network [14]. However, our study indicated that ENTPD1-AS1 affects GC progression through m6A modification and RBP binding.

A previous study showed that TP73-AS1 regulates the promoter methylation of WIF1 by recruiting the PRC2 complex, thereby downregulating WIF1 to activate the Wnt/β-catenin signaling pathway and ultimately promoting the development of GC [29]. However, our study revealed that ENTPD1-AS1 functions as an oncogene in GC, downregulating WIF1 through low m6A modification levels mediated by FTO, leading to enhanced β-catenin synthesis to promote the malignant characteristics of GC cells. Furthermore, the positive regulation of WIF1 expression by m6A modi-

fication has been confirmed [30], which is consistent with our research findings.

Many studies have also indicated that SRSF1 is involved in the occurrence and development of GC. For instance, CCL21 is crucial in GC progression through the MALAT1/SRSF1/mTOR axis [31], while OnclncRNA-626 accelerates GC development by affecting the p53 signaling pathway through binding to SRSF1 [32]. Additionally, SRSF1 has been found to play important roles in other diseases. For example, the lncRNA HAGLR activates the WNT signaling pathway by competitively binding to miR-93-5p to enhance SRSF1, thereby promoting the development of triple-negative breast cancer [33]. In our study, considering the interactions between lncRNAs and RBPs, and the reported impact of RBPs on cancer processes through the regulation of gene expression [34, 35], we further explored another molecular mechanism by which ENTPD1-AS1 modulates the WNT signaling pathway. Mass spectrometry revealed that SRSF1 is an RBP of ENTPD1-AS1. Further experiments demonstrated that ENTPD1-AS1 promotes β-catenin expression by recruiting SRSF1, thereby activating the WNT signaling pathway to exacerbate GC.

This study has several limitations. First, all experimental data were derived from *in vitro* cell models, lacking validation of the oncogenic role of ENTPD1-AS1 through animal experiments and clinical sample data. Future research should establish corresponding animal models and collect a substantial number of GC clinical samples to further confirm its function. Second, although the interaction between ENTPD1-AS1 and SRSF1 has been established, the specific molecular mechanisms by which SRSF1 regulates β-catenin remain to be fully elucidated, and future studies can employ additional techniques for in-depth analysis. Furthermore, the clinical translation of ENTPD1-AS1 as a therapeutic target still faces challenges that require further exploration and breakthroughs. Despite these limitations, this study provides an important theoretical basis for developing GC treatment strategies targeting ENTPD1-AS1, and the findings warrant further validation and expansion in a more comprehensive experimental framework to facilitate the clinical translation of these results.

In conclusion, this study confirms the promotive effect of ENTPD1-AS1 on GC. Mechanistically, ENTPD1-AS1 exacerbates GC through the FTO/WIF1 axis and binding with SRSF1 to activate the WNT signaling pathway. This suggests that ENTPD1-AS1 could serve as a potential biomarker and therapeutic target for GC, increasing the possibility of cure for GC patients.

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Ethical approval

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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