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TRIM47 drives gastric cancer cell proliferation and invasion by regulating CYLD protein stability

Jianguo Wang[1,2,3†], Jing Ye[1,2†], Rongqiang Liu[1,2†], Chen Chen[1,2*] and Weixing Wang[1,2*]

Abstract

The expression of TRIM47, a member of the TRIM protein and E3 ubiquitin ligase families, is elevated in various cancers, such as non-small cell lung cancer and colorectal cancer, and is linked to poor prognosis. This study aimed to investigate the role of TRIM47 in gastric cancer development. Using The Cancer Genome Atlas-Stomach Adenocarcinoma (TCGA-STAD) dataset and analysis of 20 patient samples from our center, TRIM47 was found to be significantly up-regulated in gastric cancer tissues and associated with advanced N-stage and poor prognosis. We constructed stable TRIM47 knockdown and overexpressing gastric cancer cell lines. CCK8, EDU, colony formation, wound healing, and Transwell tests were used to evaluate the effects on cell proliferation, invasion, and migration. The results showed that TRIM47 knockdown inhibited the proliferation, migration and invasion of gastric cancer cells, while TRIM47 overexpression promoted these behaviors. These results were further confirmed in vivo. In the mechanism part, we found that TRIM47 interacts with CYLD protein. Moreover, TRIM47 promotes K48-linked ubiquitination, leading to the degradation of CYLD by the proteasome, thereby activating the NF-κB pathway and regulating the biological behavior of gastric cancer cells. Taken together, our study demonstrated that TRIM47 is involved in the proliferation and metastasis of gastric cancer through the CYLD/NF-κB pathway.

Keywords TRIM47, Gastric cancer, Ubiquitination, NF-κB, CYLD

Background

Gastric cancer (GC) is the most common type of gastrointestinal tumour in China and the fifth most prevalent malignancy worldwide, with a persistently high mortality rate [1]. According to 2020 data, more than 1 million new cases of gastric cancer occurred globally, which represented 5.6% of all cancer cases, while deaths related to gastric cancer approached 770,000, which accounting for 7.7% of all cancer-related deaths [2, 3]. The incidence of gastric cancer varies significantly across different regions, and more than half of the new cases occur in developing countries, particularly in Asian nations, such as China, Japan, and South Korea, where the incidence and mortality rates continue to steadily increase [1, 4]. Despite advances in the diagnosis and treatment of gastric cancer,

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patient prognosis remains poor. The primary treatment methods for gastric cancer include surgical tumour resection, radiotherapy, chemotherapy, and adjuvant therapy. However, the prognosis of gastric cancer patients is influenced not only by tumour cell proliferation, metastasis, genetic diversity, and resistance to chemotherapy drugs, but also by an insufficient understanding of the molecular pathways involved in the disease and the lack of sensitive early detection and monitoring methods [5, 6]. Therefore, it is imperative to further explore the complex mechanisms that drive the development and progression of gastric cancer and to identify reliable early biomarkers.

The TRIM (tripartite motif-containing) family, which is part of the E3 ubiquitin ligase family, is widely distributed in eukaryotes and is notable for its structural conservation and rapid evolution [7, 8]. More than 80 proteins belong to the TRIM family, and each features four common domains from the N-terminus to the C-terminus: a RING-finger domain (R), a B-box1 domain (B1), a B-box2 domain (B2), and a coiled-coil domain (CC), which are all collectively known as the RBCC motif [9]. The RING-finger domain, characterized by its distinctive zinc-finger structure, primarily regulates the ubiquitination process of proteins, which precisely coordinates the transfer of ubiquitin, a hallmark of many E3 ubiquitin ligases. As crucial components of the E3 ubiquitin ligase family, TRIM family members regulate various biological processes, including immune responses, cell growth, development, apoptosis, and host resistance to viral infections 10. Tripartite Motif-Containing 47 (TRIM47) is a member of the TRIM protein family located on human chromosome 17q25.1. This gene encodes a protein comprising 630 amino acids, has a molecular weight of approximately 69.5 kDa, and contains 4418 base pairs in its gene sequence [11]. Research indicates that TRIM47 is involved in the pathogenesis and progression of various diseases. For instance, TRIM47 overexpression may exacerbate cerebral ischemia-reperfusion injury by promoting apoptosis and inflammatory responses [12]. In sepsis-induced acute lung injury, TRIM47 interacts with TRAF2 and mediates K63-linked ubiquitination, which activates the NF-κB and MAPK signaling pathways and promotes inflammation and tissue damage [13]. Additionally, the role of TRIM47 in various cancers, including non-small cell lung cancer, colorectal cancer, breast cancer, renal cell carcinoma, pancreatic cancer, and prostate cancer, has been investigated [14–16]. However, to date, no clear consensus has been reached on the specific role and mechanisms of TRIM47 in gastric cancer. Therefore, this study focused on TRIM47 to explore its potential role in gastric cancer development and to reveal its possible molecular mechanisms. This study also aimed to provide new molecular markers for the early diagnosis and targeted therapy of gastric cancer.

In this study, we investigated the role of TRIM47 in gastric cancer. Our findings revealed that TRIM47 expression is elevated in tumour tissues and is associated with poor patient prognosis. Further in vivo and in vitro experiments confirmed the oncogenic role of TRIM47. Moreover, we discovered that TRIM47 promotes gastric cancer progression by modifying CYLD with K48-linked ubiquitin chains, which leads to its degradation via the ubiquitin–proteasome system and subsequent activation of the NF-κB pathway.

Materials and methods

Bioinformatics analysis

We utilized TIMER 2.0 (http://timer.cistrome.org/) to analyse TRIM47 expression across 33 types of cancer using data from the TCGA database. RNA-Seq data and clinical information of gastric cancer patients were downloaded from the TCGA portal (https://portal.gdc.cancer.gov/). Survival information for these gastric cancer patients was examined using the BEST website (https://rookieutopia.com/app_direct/BEST/). The pathway enrichment analysis was performed using GSEA software. Finally, we investigated proteins interacting with TRIM47 using the BioGRID database (https://thebiogrid.org/).

Collection and handling of gastric cancer specimens

We collected 20 pairs of gastric cancer and adjacent tissue samples from patients who underwent surgery at the Renmin Hospital of Wuhan University (Wuhan, Hubei, China) with patient consent. All tissue experiments were approved by the Ethics Committee of the Renmin Hospital of Wuhan University. Some tissue samples were stored in liquid nitrogen or at -80 °C before protein extraction for Western blot experiments, while others were fixed and preserved in paraformaldehyde solution for subsequent immunohistochemical experiments.

Cell culture and transfection

The gastric cancer cell lines AGS and HGC-27, along with the normal gastric mucosal cell line GES-1, were obtained from the China Center for Type Culture Collection (CCTCC). GES-1 and HGC-27 cells were cultured in 1640 medium (Servicebio, Wuhan, China) supplemented with 10% Fetal Bovine Serum (Wuhan Tsingmu Biotechnology, mu001SR). AGS cells were cultured in AGS-specific medium (Procell, Wuhan). All cells were maintained in an incubator at 37 °C with 5% CO2. The TRIM47 plasmid and the packaging plasmids psPAX2 and pMD2 were cotransfected into HEK-293T cells using LIPO8000 (Beyotime) for virus packaging according to the manufacturer's instructions. Viral supernatants were collected 48 h after transfection. Fresh viral supernatant samples supplemented with 10 μg/mL polybrene (Biosharp) were

added to exponentially growing AGS and HGC-27 cells. The medium was replaced with fresh medium after 24 h. Stable cell lines in which TRIM47 was overexpressed or knocked down were selected using puromycin (10 μg/mL, Biosharp) for one week.

Immunohistochemistry

The paraffin-embedded sections were first labelled and then baked in an oven prior to deparaffinization. After the sections were washed with ultrapure water, antigen retrieval was performed for antigen unmasking. Subsequently, the sections were blocked with hydrogen peroxide, permeabilized, and incubated with primary antibodies. The next day, after the slides were warmed to room temperature and washed, the sections were incubated with MaxVision reagent for the staining reaction, which was followed by colour development. Nuclei were stained with haematoxylin dye and differentiated, followed by microscopic observation. Finally, the sections were dehydrated to remove moisture, mounted, and dried. Microscopic examination and image analysis were performed to assess the immunohistochemistry results.

Western blotting

Total protein was extracted from cells using RIPA buffer containing a phosphatase inhibitor and PMSF (RIPA: cocktail: phosphatase inhibitors: PMSF = 100:1:1:1). Lysates were heated at 98 °C for 5–10 min with SDS loading buffer. Protein abundance was analysed by SDS–PAGE and Western Blotting. The signals were visualized using Bio- Rad ChemiDoc MP. GAPDH was used as the loading control. All antibodies used in this study are listed in Table S1.

PCR

RNA was extracted from all samples using RNA-easy Isolation Reagent (Vazyme, China). The samples were digested and lysed with RNA-easy Isolation Reagent and centrifuged at 12,000 rpm, after which the total RNA was precipitated with isopropanol/ethanol. cDNA was synthesized using HiScript® III RT SuperMix for qPCR (Vazyme). RT–qPCR analysis was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme). Relative expression levels were calculated using the delta-delta Ct method with β-actin as the internal control gene. All primers used in the study are detailed in Table S2.

Cell counting Kit-8

Cell viability was measured using a Cell Counting Kit-8 (CCK8) (Biosharp, China). After synchronizing cells to the M phase to avoid cell cycle stage differences, gastric cancer cells were seeded in 96-well plates. After 24 h of culture, 10 μL of CCK8 reagent was added to each well, after which the plates were incubated at 37 °C for 2 h. The absorbance at 450 nm was measured using a microplate reader. The absorbance was measured at 24, 48, and 72 h for each group. All experiments were repeated three times, and GraphPad Prism software was used for analysis.

EdU assay

We conducted EdU experiments using the EdU Cell Proliferation Kit 555 (Beyotime, Shanghai, China) according to the manufacturer's guidelines. The cells were labelled with 10 μM EdU for 2 h at 37 °C. After labelling, the cells were washed with PBS and fixed in 4% paraformaldehyde. The cells were then stained with Azide 555 reagent for 30 min. Labelled cells were imaged with a fluorescence microscope and quantified using ImageJ software. Each EdU experiment was repeated at least three times to ensure reliability.

Colony formation assay

Gastric cancer cells were seeded in six-well plates at a density of 500 cells per well and cultured for 2 weeks. The cells were then fixed in 4% formaldehyde at room temperature for 15 min and stained with 2% crystal violet for 20 min. After washing with water and air-drying, the plates were photographed. The images were then used to count the number of colonies in each well.

Transwell assay

We performed Transwell migration and invasion assays using Corning Transwell chambers with 0.8 μm pores. For the migration assays, 5×10^5 cells were suspended in serum-free medium and added to the upper chamber, while the lower chamber contained medium supplemented with 10% FBS. For the invasion assays, 5×10^5 cells were seeded in the upper chamber precoated with Matrigel and cultured in serum-free medium, while the lower chamber contained medium supplemented with 10% FBS. After incubation for 24 h, the cells that had migrated or invaded were stained with crystal violet and observed by microscopy.

Wound healing assay

Gastric cancer cells were seeded in six-well plates and cultured until they reached 80-90% confluence. A Wound-Healing was made in the middle of each well using a 1000 μL pipette tip. The medium was then replaced with serum-free medium, and the cells were cultured for 48 h to promote migration and healing within the Wound-Healing area. The Wound-Healing distance was measured in five different areas using a microscope and image processing software (Photoshop). The percentage of Wound-Healing healing was calculated to evaluate cell migration and healing.

Apoptosis assay

The culture supernatants and wash solutions were collected, and the cells were digested with trypsin and neutralized with complete medium. The cells were then washed multiple times with PBS and were centrifuged and resuspended. Annexin V binding buffer was added once the cell suspension was adjusted to a specific concentration. A portion of the cell suspension was stained in the dark with Annexin V-APC and 7-AAD and incubated. After the Annexin V binding buffer was added, apoptosis was detected using flow cytometry. The control groups included unstained cells and cells stained with only Annexin V-APC or 7-AAD for comparative analysis.

Coimmunoprecipitation (Co-IP)

Protein–protein interactions were determined using Co-IP. Gastric cancer cells or HEK-293T cells were cultured in 10 cm dishes and lysed in 1 mL of IP lysis buffer (P0013F, Beyotime) supplemented with a protease inhibitor cocktail and phosphatase inhibitors. The cells were then sonicated and centrifuged, and nonspecific binding was prevented by incubating the supernatant with Protein A/G Magnetic Beads. Subsequently, the supernatant was incubated overnight with the corresponding antibody, followed by incubation with Protein A/G magnetic beads on a rotating platform at 4 °C for 4 h. Finally, Western Blotting experiments were conducted.

Ubiquitination assay

For this assay, gastric cancer cells or HEK-293T cells were pretreated with the specified plasmids for 24 h, followed by treatment with 20 μM MG-132 for 6 h. The cells were then lysed in IP lysis buffer and fixed. The CYLD protein was immunoprecipitated using an anti-Myc antibody and Protein A/G magnetic beads and purified with a magnetic rack, after which the supernatant was discarded. Immunoprecipitates were subsequently analysed by Western Blotting.

Xenograft tumour model

Four-week-old female BALB/c nude mice were obtained from Shulaibao (Wuhan) Biotechnology Co., Ltd. Twelve mice were randomly divided into two groups, and HGC-27 cells (5×10^6 cells/100 μL) were subcutaneously injected bilaterally to establish a xenograft gastric cancer model. Tumour size was monitored weekly, and after 4 weeks, tumours were harvested. Tumour volume was calculated according to the following formula: V (cm^3) = 1/2 × length × $width^2$ 17. All animal experiments were approved by the Animal Care and Use Committee of Wuhan University.

Statistical analysis

Statistical analyses were conducted using SPSS 22.0 and GraphPad Prism 9.0 software. Significant differences between groups were assessed using t tests or one-way ANOVA. The data from three independent experiments are expressed as the means ± SDs, and p values < 0.05 indicate statistical significance.

Results

High TRIM47 expression in gastric cancer tissues is linked to a poor prognosis

Using the TIMER database, we discovered a significant increase in TRIM47 expression across various tumours, including oesophageal cancer, head and neck cancer, cholangiocarcinoma, and colon cancer. Conversely, TRIM47 expression was lower only in renal clear cell carcinoma, prostate cancer, and endometrioid carcinoma (Fig. 1A). Independent analysis of gastric cancer data from the TCGA cohort revealed a notable upregulation of TRIM47 transcription in gastric cancer tissues. Pairwise differential expression analysis further confirmed a significant increase in TRIM47 expression in gastric cancer tissues (Fig. 1B and C). Immunohistochemical staining was performed to evaluate TRIM47 expression levels in 20 pairs of gastric cancer tissues and adjacent nontumor tissues from gastric cancer patients and revealed a significant increase in TRIM47 protein in gastric cancer tissues ($P<0.05$) (Fig. 1D and E). Subsequently, western blot results demonstrated that the protein level of TRIM47 in gastric cancer was conspicuously higher than that in adjacent normal gastric tissue ($P<0.05$) (Fig. 1F and G). We analyzed the effect of TRIM47 upregulation using the BEST website. The results demonstrated that in most datasets, gastric cancer patients with high TRIM47 expression had shorter overall survival than those with low TRIM47 expression(Fig. 1H). Finally, we leveraged gastric cancer patient data from the TCGA database (comprising 379 cases) and categorized patients into TRIM47 high expression and low expression groups based on the TRIM47 expression levels. Then, the relationship between TRIM47 expression and the gender, age, tumor grade, tumor stage and TNM stage of patients was analyzed. Table 1 shows the correlation between TRIM47 expression level and N stage ($P<0.05$).

TRIM47 promotes the proliferation, invasion, and migration of gastric cancer cells

We first examined the expression of TRIM47 in both normal gastric mucosal epithelial cells (GES-1) and gastric cancer cell lines (AGS and HGC-27). TRIM47 was expressed at higher levels in AGS cells and at lower levels in HGC-27 cells (Fig. 2A). Subsequently, we established stable AGS cell lines in which TRIM47 was knocked down. Meanwhile, AGS and HGC-27 cell lines in which

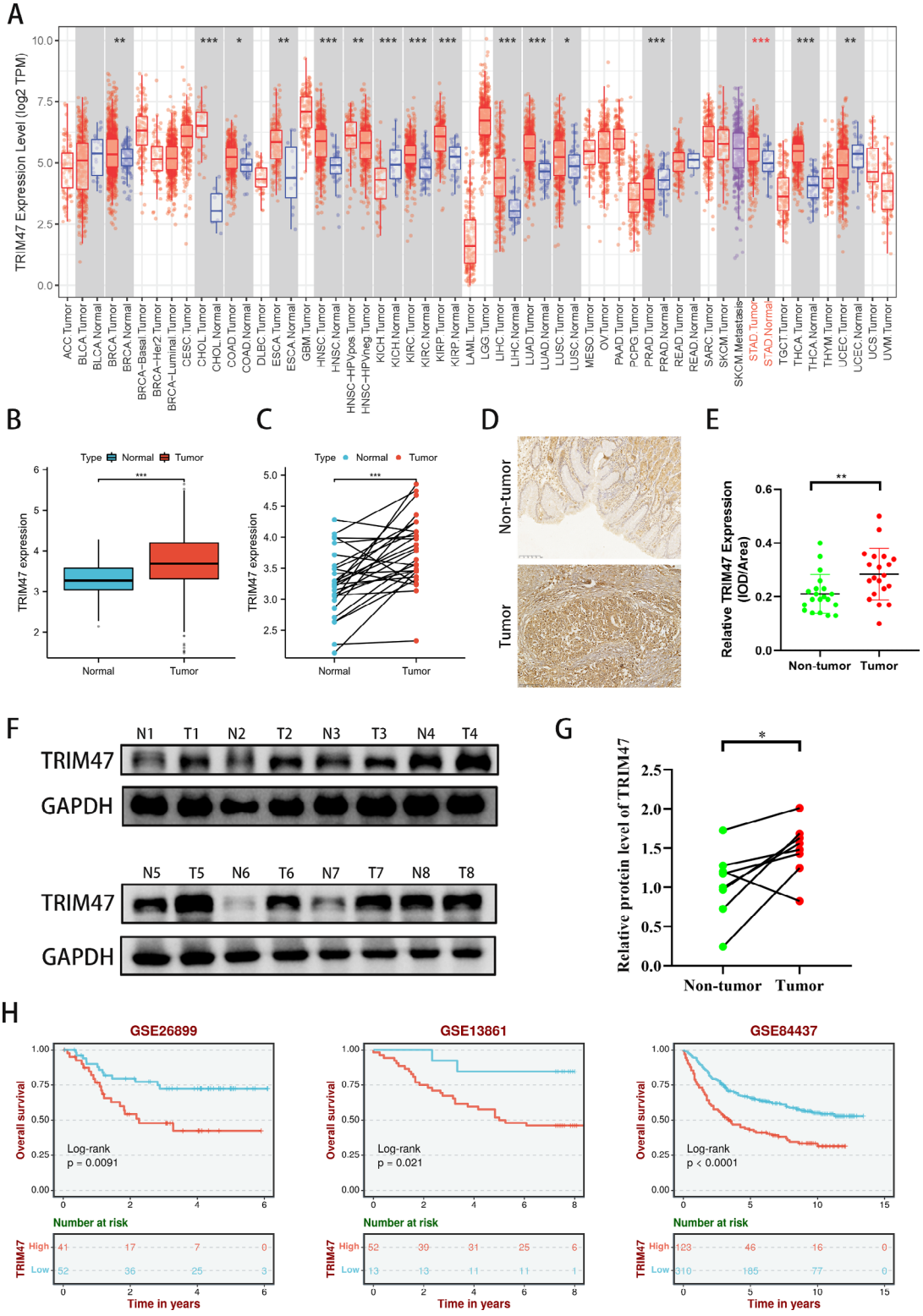

Fig. 1 Expression of TRIM47 in gastric cancer tissues. (**A**) Expression of TRIM47 across various tumor types. (**B**) TRIM47 expression in unpaired gastric cancer samples from the TCGA database. (**C**) TRIM47 expression in paired gastric cancer samples from the TCGA database. (**D**) Representative immunohistochemical staining images of TRIM47 in STAD patient tissues from our center. (**E**) Quantitative results of immunohistochemical staining images, measured by average optical density (AOD), $N = 20$. (**F**) Western Blot analysis of TRIM47 protein expression in STAD patient tissues from our center. (**G**) Quantitative results of the Western Blot analysis. (**H**) Kaplan-Meier survival curve showing overall survival in the gastric cancer dataset based on TRIM47 expression. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, t-test

Table 1 Correlations between expression of TRIM47 protein and clinicopathological factors

Characteristics	Number	TRIM47 Expression		*P* Value
		Low(*N* = 179)	High(*N* = 200)	
Age				*P* = 0.266
< 65	160	70	90	
>=65	216	107	109	
Gender				*P* = 0.861
MALE	241	115	126	
FEMALE	138	64	74	
Grade				*P* = 0.627
G1-2	149	67	82	
G3-4	223	106	117	
Stage				*P* = 0.076
Stage I-II	168	70	98	
Stage III-IV	188	96	92	
T				*P* = 0.181
T1-2	104	43	61	
T3-4	267	131	136	
M				*P* = 0.750
M0	339	159	180	
M1	23	10	13	
N				***P* = 0.019**
N0-1	216	87	129	
N2-3	146	77	69	

TRIM47 was overexpressed. We validated these alterations at the RNA and protein levels (Fig. 2B and C). The CCK8 results indicated that TRIM47 overexpression significantly enhanced cell proliferation, while TRIM47 knockdown inhibited proliferation (Fig. 2D). Consistent with these findings, the results from the EDU assays further supported this conclusion (Fig. 2E and F). Additionally, TRIM47 overexpression promoted colony formation, while TRIM47 knockdown inhibited colony formation (Fig. 2G and H). These findings suggest that TRIM47 overexpression facilitates cell proliferation, while TRIM47 knockdown suppresses proliferation, regardless of the duration.

Subsequently, the effects of TRIM47 on migration and invasiveness of gastric cancer cells were examined. Wound healing experiments showed that TRIM47 knockdown inhibited cell migration, while TRIM47 overexpression increased cell migration (Fig. 3A and B). Consistent with these findings, Transwell experiments showed that TRIM47 knockdown inhibited the cells' ability to invade and migrate. Overexpression of TRIM47 increased the invasion and migration of cells (Fig. 3C and D). Since epithelial mesenchymal transition (EMT) is a hallmark of migration and metastasis, we evaluated the expression of several EMT markers[18]. The results showed that TRIM47 knockdown decreased the expression of EMT transcription factor Snail1 and mesenchymal markers N-cadherin, while epithelial markers Occludin and E-cadherin increased expression. In contrast, overexpression of TRIM47 had the opposite effect (Fig. 3E and F). Overall, these results demonstrate that TRIM47 is a key factor that promotes the proliferation, invasion, and migration of gastric cancer cells and can induce the EMT process.

TRIM47 suppresses apoptosis in gastric cancer cells

To further investigate the effect of TRIM47 on apoptosis in gastric cancer cells, flow cytometry was performed. The results showed that the apoptosis rate of gastric cancer cells was significantly increased after TRIM47 knockdown, and overexpression of TRIM47 could reduce the apoptosis rate of gastric cancer cells (Fig. 4A-C). Subsequently, Western Blotting was used to detect the expression of apoptosis-related markers. The results showed that in cells where TRIM47 was knocked out, the expression levels of Bax and cleaved caspase-3 were increased, while the expression of Bcl-2 was decreased. Conversely, in cells overexpressed with TRIM47, Bax and cleaved caspase-3 expression were decreased, and Bcl-2 expression was increased (Fig. 4D). These observed trends at the protein level further emphasize the role of TRIM47 in promoting apoptosis when knocked down and inhibiting apoptosis when overexpressed.

TRIM47 regulates the proliferation, invasion, and migration of gastric cancer cells via the NF-κB pathway

To elucidate the regulatory mechanism of TRIM47 in gastric cancer progression, we first conducted gene set enrichment analysis (GSEA) on RNA-seq data, which revealed a significant overlap between genes targeted by the NF-κB signaling pathway and dysregulated TRIM47 expression (Fig. 5A). Considering the critical role of I-κBα and P65 in the NF-κB signaling pathway, we detected total protein expression and phosphorylation levels of I-κBα and P65 in TRIM47 overexpression and knockdown gastric cancer cells by Western blot assay. The results showed that the phosphorylation levels of I-κBα and P65 were significantly reduced in AGS cells with TRIM47 knockdown, while the phosphorylation levels of I-κBα and P65 were increased in AGS and HGC-27 cells with TRIM47 overexpression (Fig. 5B). It is suggested that TRIM47-mediated promotion of gastric cancer cell proliferation and invasion may depend on the activation of NF-κB pathway. To verify this possibility, we performed rescue experiments. Stably transfected AGS and HGC-27 cells were treated with the NF-κB pathway inhibitor BAY 11-7085 (10 μmol/L). The results showed that the addition of BAY 11-7085 inhibited the biology of gastric cancer cells and was able to rescue overexpression of TRIM47 on the proliferation, invasion and migration of gastric cancer cells (Fig. 5C-I). Besides, BAY 11-7085 inhibited the EMT process in gastric cancer cells and rescued the ability of TRIM47 overexpression to promote

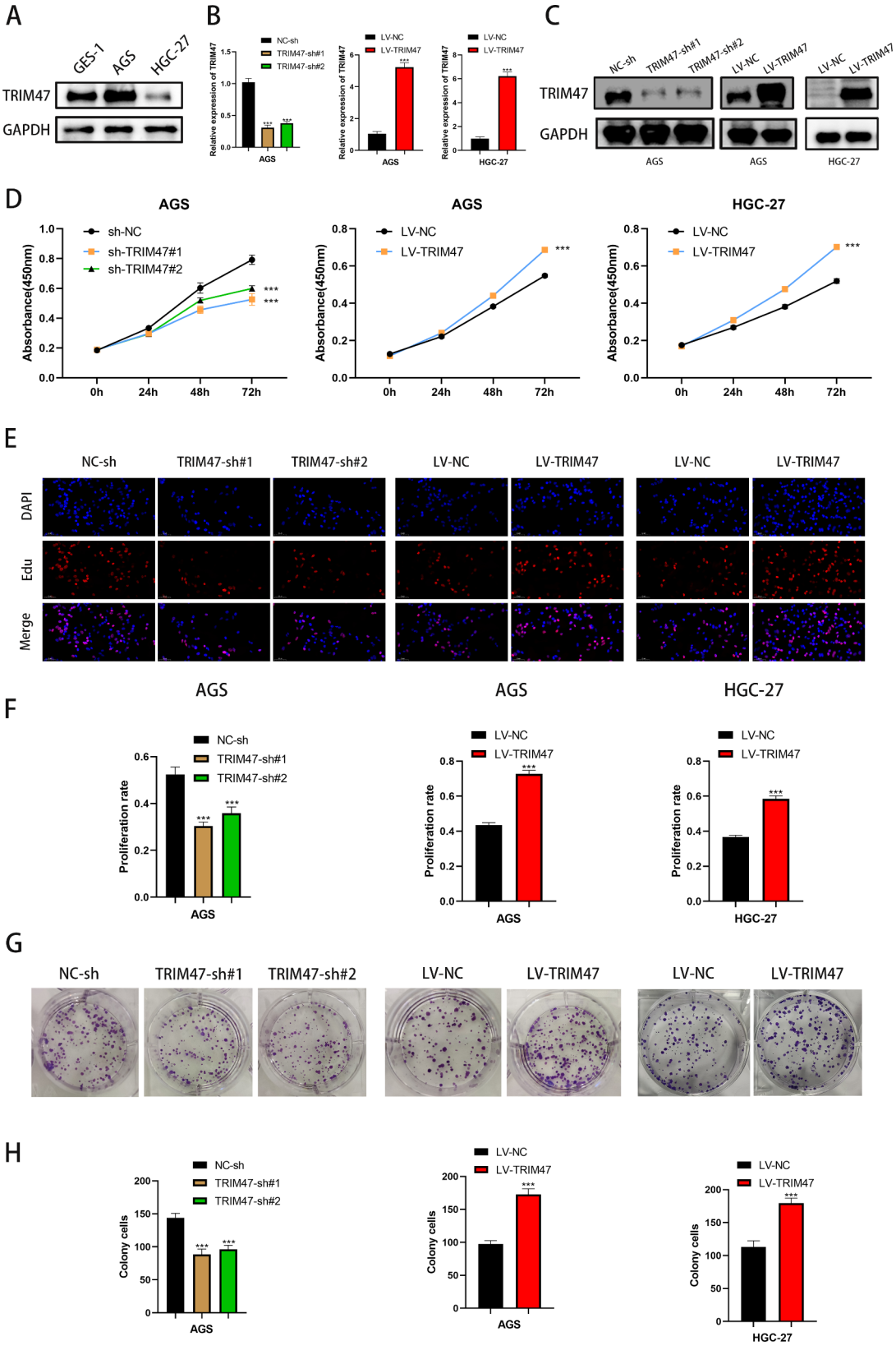

Fig. 2 Effects of TRIM47 on gastric cancer cell proliferation. (**A**) Expression of TRIM47 in gastric cancer cells and normal gastric mucosal cells. (**B**) Verification of TRIM47 shRNA and overexpression plasmid transfection efficiency by qPCR. (**C**) Validation of TRIM47 shRNA and overexpression plasmid transfection efficiency by Western Blot. (**D**) CCK8 assay to detect short-term proliferation ability of gastric cancer cells. (**E**, **F**) Edu assay to detect short-term proliferation ability of gastric cancer cells. (**G**, **H**) Colony formation assay to detect cell colony-forming ability. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, t-test

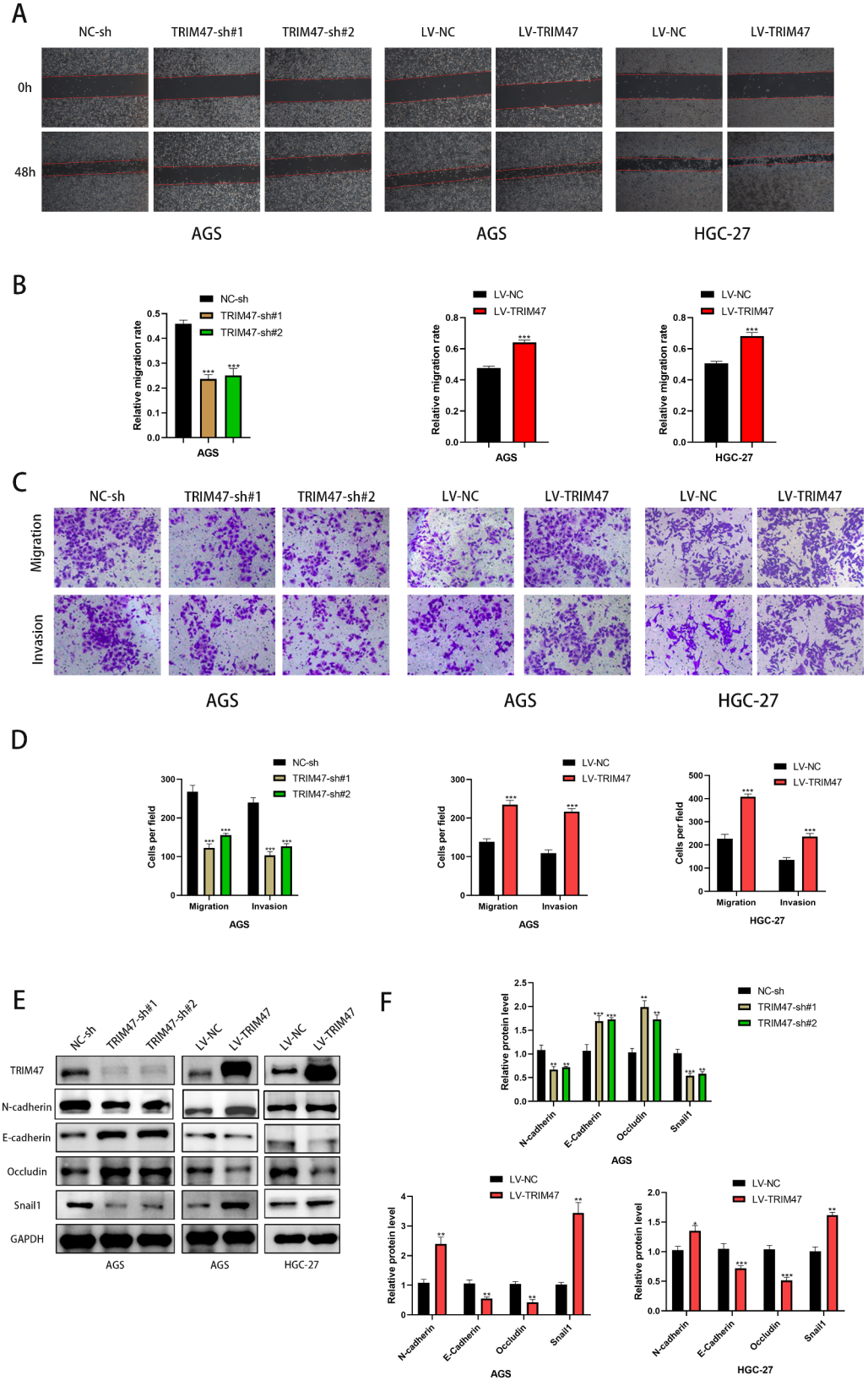

Fig. 3 Effects of TRIM47 on gastric cancer cell invasion and migration. (**A**, **B**) Wound healing assay to assess the migration capability of gastric cancer cells. (**C**, **D**) Transwell assay to evaluate the migration and invasion capability of gastric cancer cells. (**E**, **F**) Western Blot analysis of N-cadherin, E-cadherin, Occludin, and Snail1 protein expression in gastric cancer cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, t-test

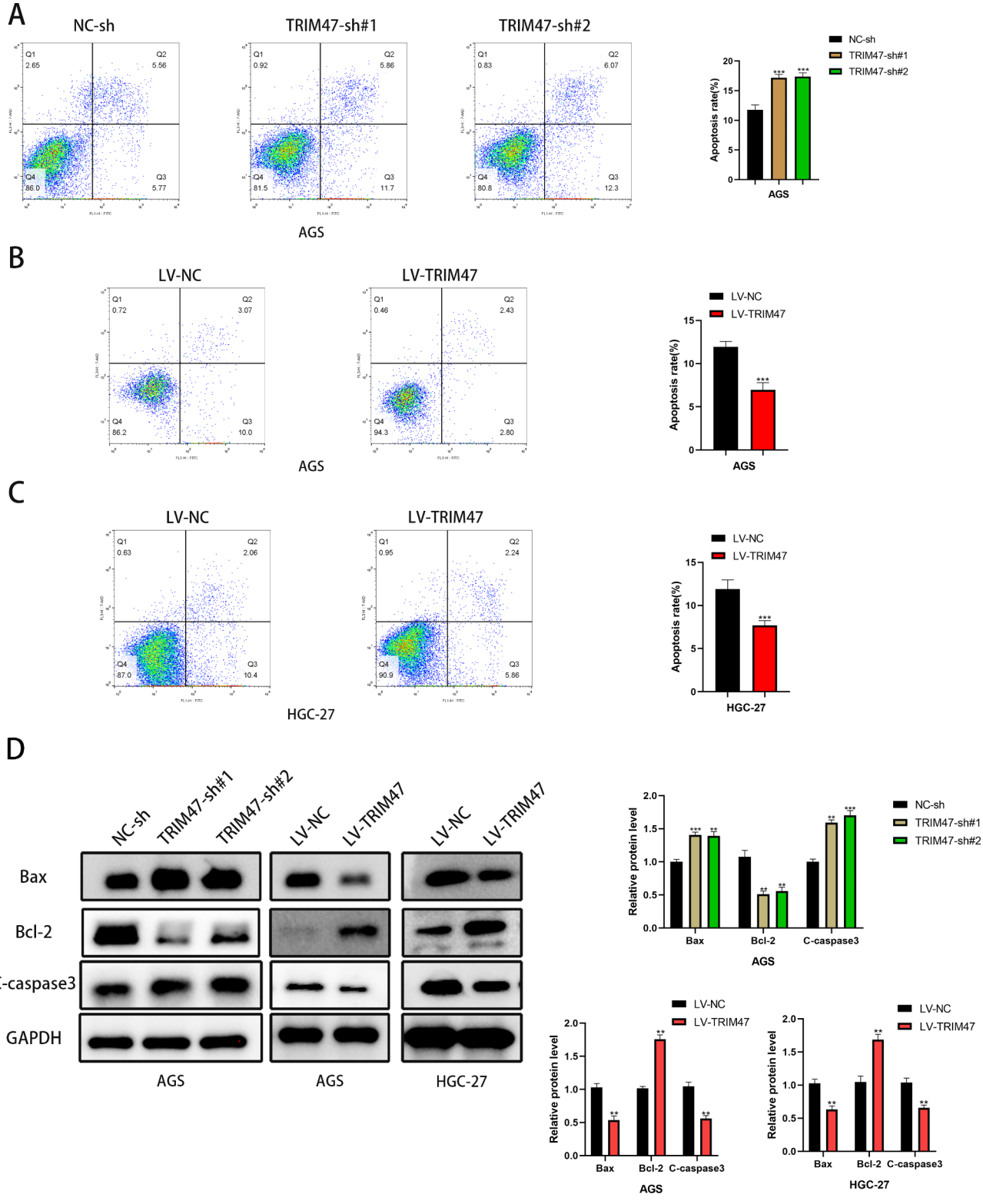

Fig. 4 Effects of TRIM47 on apoptosis of gastric cancer cells. (**A**) Left: Flow cytometry apoptosis assay in AGS cells following TRIM47 knockdown; Right: Statistical analysis. (**B**) Left: Flow cytometry apoptosis assay in AGS cells following TRIM47 overexpression; Right: Statistical analysis. (**C**) Left: Flow cytometry apoptosis assay in HGC-27 cells following TRIM47 overexpression; Right: Statistical analysis. (**D**) Western Blot analysis of apoptosis-related proteins: Bax, Bcl-2, and Cleaved-Caspase3 expression. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, t-test

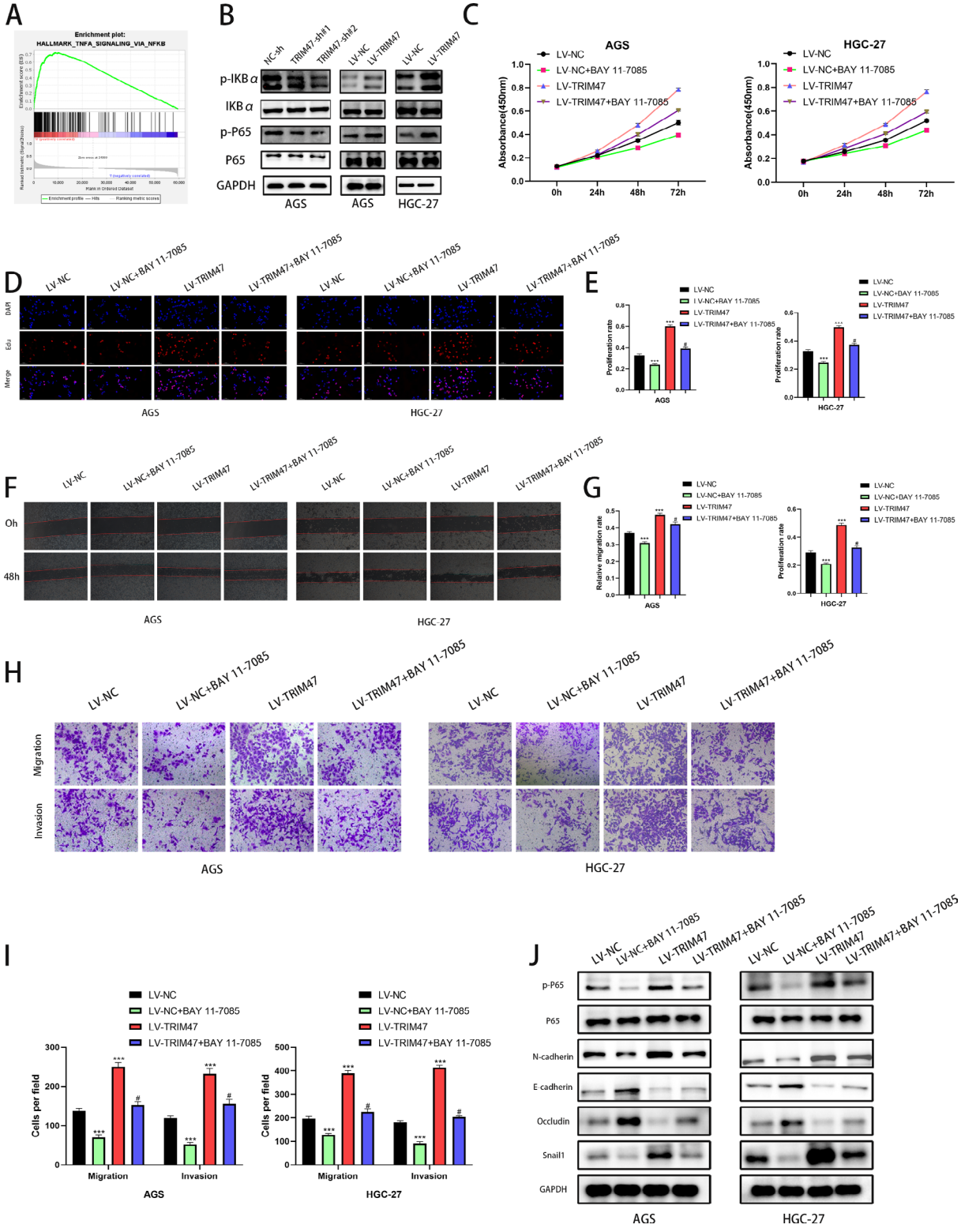

Fig. 5 TRIM47 promotes proliferation, invasion, and migration of gastric cancer cells by activating the NF-κB pathway. (**A**) Gene Set Enrichment Analysis (GSEA) results. (**B**) Western blot analysis of key molecules in the NF-κB pathway, such as p-P65, P65, p-IκBα, and IκBα. (**C**) CCK8 assay to assess proliferation of AGS and HGC-27 cells stably overexpressing TRIM47 after treatment with BAY 11-7085 (10μmol/L) for 24 h. (**D**, **E**) Edu assay to evaluate proliferation of the mentioned cells. (**F**, **G**) Wound healing assay to measure cell migration. (**H**, **I**) Transwell assay to determine cell migration and invasion capabilities. (**J**) Western blot analysis of p-P65, P65, N-cadherin, E-cadherin, Occludin, and Snail1 protein expression in cells. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, t-test

the EMT process in these cells (Fig. 5J). In summary, TRIM47 may regulate gastric cancer cell proliferation, migration, invasion, and EMT through activation of the NF-κB pathway.

TRIM47 regulates the proliferation, invasion, and migration of gastric cancer cells by modulating the Lys48-linked ubiquitination of CYLD

To further elucidate the downstream targets of TRIM47, we explored potential interacting proteins using the BioGRID website (Fig. 6A). CYLD has been implicated in the NF-κB pathway and is thus a candidate for further investigation. Immunoprecipitation experiments using HEK-293T cells revealed that TRIM47 antibody-purified cell proteins contained the CYLD protein, and vice versa, which confirms their interaction in HEK-293T cells (Fig. 6B). Additionally, we verified the interaction between TRIM47 and CYLD in the gastric cancer cell lines AGS and HGC-27 (Fig. 6C). To investigate the upstream-downstream relationship between TRIM47 and CYLD, we extracted proteins and RNA from TRIM47-overexpressing or TRIM47-knockdown AGS and HGC-27 cells and control cells. Western blot and qPCR experiments showed that TRIM47 overexpression led to a decrease in CYLD protein levels, while TRIM47 knockdown resulted in an increase, although CYLD RNA levels remained unchanged with variations in TRIM47 expression (Fig. 6D and E). Subsequently, we further validated the impact of TRIM47 on CYLD protein stability through a series of experiments. Initially, Western blot experiments revealed that as TRIM47 expression increased, CYLD protein levels gradually decreased in AGS and HGC-27 cells (Fig. 6F). To confirm the effect of TRIM47 on CYLD protein stability, we treated gastric cancer cells with the protein synthesis inhibitor cycloheximide (CHX) and monitored changes in the CYLD protein half-life. The results showed that following TRIM47 overexpression, the degradation rate of the CYLD protein in gastric cancer cells significantly increased over time compared with that in control cells (Fig. 6G and H).

Next, we treated AGS and HGC-27 gastric cancer cells with the lysosomal inhibitor chloroquine (CQ) or the proteasome inhibitor MG-132. We observed that TRIM47 overexpression significantly reduced CYLD protein levels and that the addition of MG-132 reversed the decrease in CYLD protein levels induced by TRIM47 overexpression, while CQ did not (Fig. 7A). In summary, we speculate that TRIM47 affects the protein stability of CYLD by regulating its ubiquitination level. To validate this speculation, we transfected the corresponding ubiquitin plasmids and CYLD overexpression plasmids into AGS and HGC-27 cells stably transfected with TRIM47 or empty vector. We then performed Western blot experiments to detect the ubiquitination level of the CYLD protein. The results showed that TRIM47 overexpression increased the ubiquitination level of CYLD protein in gastric cancer cells (Fig. 7B). Eight different polyubiquitin chains are common (seven lysine residues: K6, K11, K27, k29, k33, K48, k63, and one methionine residue, M1). We performed co-immunoprecipitation experiments with HEK-293T cells transfected with UB-K6R, UB-K11R, UB-K27R, UB-K33R, UB-K48R and UB-K63R plasmids, and the results showed that K48R mutation reduced the ubiquitination level of CYLD. This suggests that TRIM47 primarily stimulates Lys48-linked CYLD ubiquitination (Fig. 7C and Fig. S1). In addition, Western Blotting showed that TRIM47 overexpression only increased the ubiquitination level of CYLD in 293T cells transfected with UB-K48, while TRIM47 knockdown only decreased the ubiquitination level of CYLD in 293T cells transfected with UB-K48 (Fig. 7D and E).

To investigate the role of CYLD in TRIM47-mediated malignant transformation of gastric cancer cells, we performed corresponding rescue experiments. Our CCK-8 experiments showed that TRIM47 overexpression significantly enhanced the viability of gastric cancer cells, while CYLD overexpression restored its role (Fig. 8A). Similar results were observed in EdU experiments (Fig. 8B and Fig. S2A). In addition, Wound healing and transwell assays showed that CYLD overexpression rescued the effects of TRIM47 on migration and invasion of gastric cancer cells (Fig. 8C-D and Fig. S2B-C). Further western-blotting experiments showed that TRIM47 overexpression promoted the expression of EMT markers, whereas CYLD overexpression restored its role (Fig. 8E).

TRIM47 promotes tumour growth in gastric cancer in vivo

To further investigate the role of TRIM47 in tumour progression, we employed an in vivo model. Specifically, we performed a subcutaneous injection of HGC-27 cells that either stably overexpressed TRIM47 or that were transfected with an empty vector control into the flanks of nude mice. We meticulously measured the tumour volume and weight. The results showed that TRIM47 overexpression significantly promoted tumour growth in this subcutaneous xenograft model (Fig. 9A and B), and both tumour weight and tumour volume were significantly different (Fig. 9C and D). IHC and Western blot confirmed the effective overexpression of TRIM47 in the xenograft tumours. Notably, TRIM47 overexpression also led to significant downregulation of CYLD expression in xenograft tumours. Ki-67 staining revealed a marked increase in the proliferation index of tumour cells in the TRIM47 overexpression group, while the expression level of E-cadherin was decreased (Fig. 9E-G). In summary, these in vivo experiments further support the conclusion that TRIM47 overexpression promotes tumour growth and proliferation and induces EMT in tumour cells. This

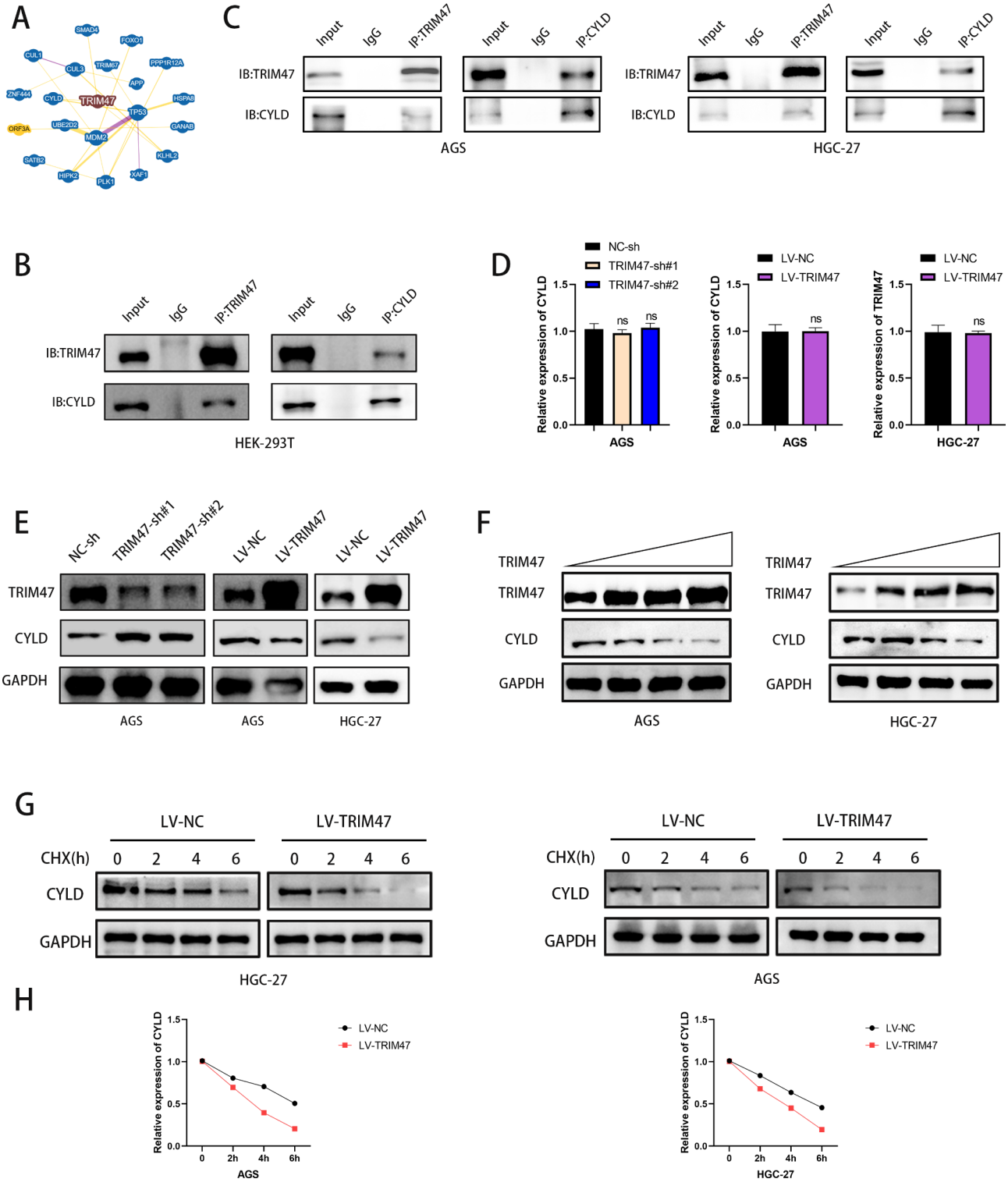

Fig. 6 TRIM47 interacts with CYLD. (**A**) Exploration of proteins possibly interacting with TRIM47 using the BioGRID database. (**B**) Co-immunoprecipitation (Co-IP) assay confirming the interaction between TRIM47 and CYLD in HEK HEK-293T cells. (**C**) Co-IP assay confirming the interaction between TRIM47 and CYLD in AGS and HGC-27 cells. (**D**) mRNA expression of CYLD in TRIM47 knockdown and overexpressing gastric cancer cells. (**E**) Protein expression of CYLD in TRIM47 knockdown and overexpressing gastric cancer cells. (**F**) Expression of CYLD protein in gastric cancer cells transfected with different concentrations of TRIM47 overexpression plasmid. (**G**, **H**) Expression of CYLD protein in gastric cancer cells transfected with specified plasmids and treated with cycloheximide (CHX) for specified durations

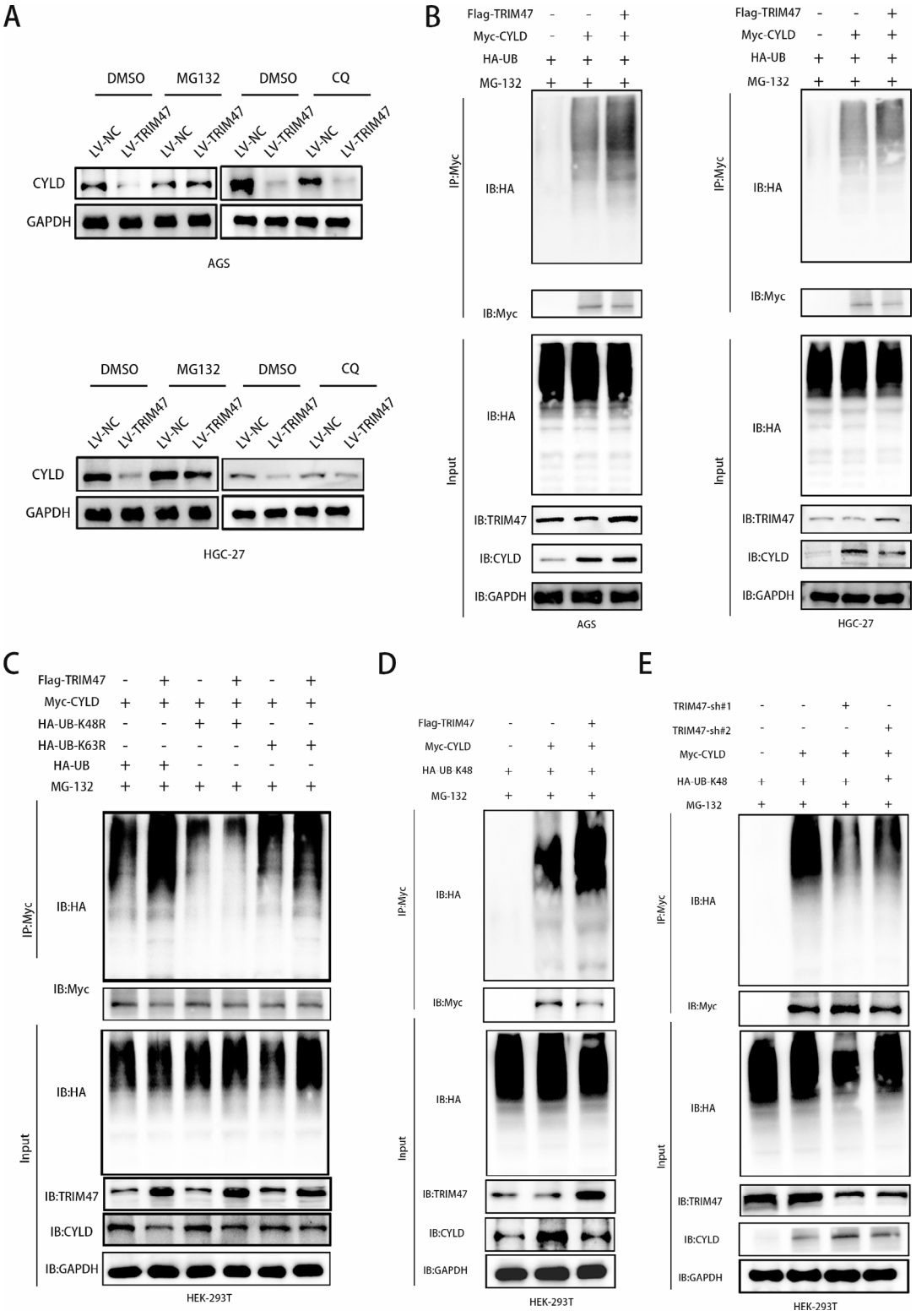

Fig. 7 TRIM47 Induces Lys48-Linked Polyubiquitination of CYLD. (**A**) Expression of CYLD in DMSO, CQ, or MG-132-treated stable TRIM47-transfected gastric cancer cells. (**B**) Verification of CYLD protein ubiquitination levels after LV-NC or LV-TRIM47 transfection in gastric cancer cells. (**C**) Ubiquitylation of CYLD detected in HEK-293T cells transfected with Flag-TRIM47, Myc-CYLD, and Ub-k48R or Ub-63R mutant plasmids. (**D**) Ubiquitylation of CYLD detected in HEK-293T cells transfected with Flag-TRIM47, Myc-CYLD, and Ub-k48 plasmids. (**E**) Ubiquitylation of CYLD detected in HEK-293T cells transfected with TRIM47-SH #1 or 2, Myc-CYLD, and Ub-k48 plasmids

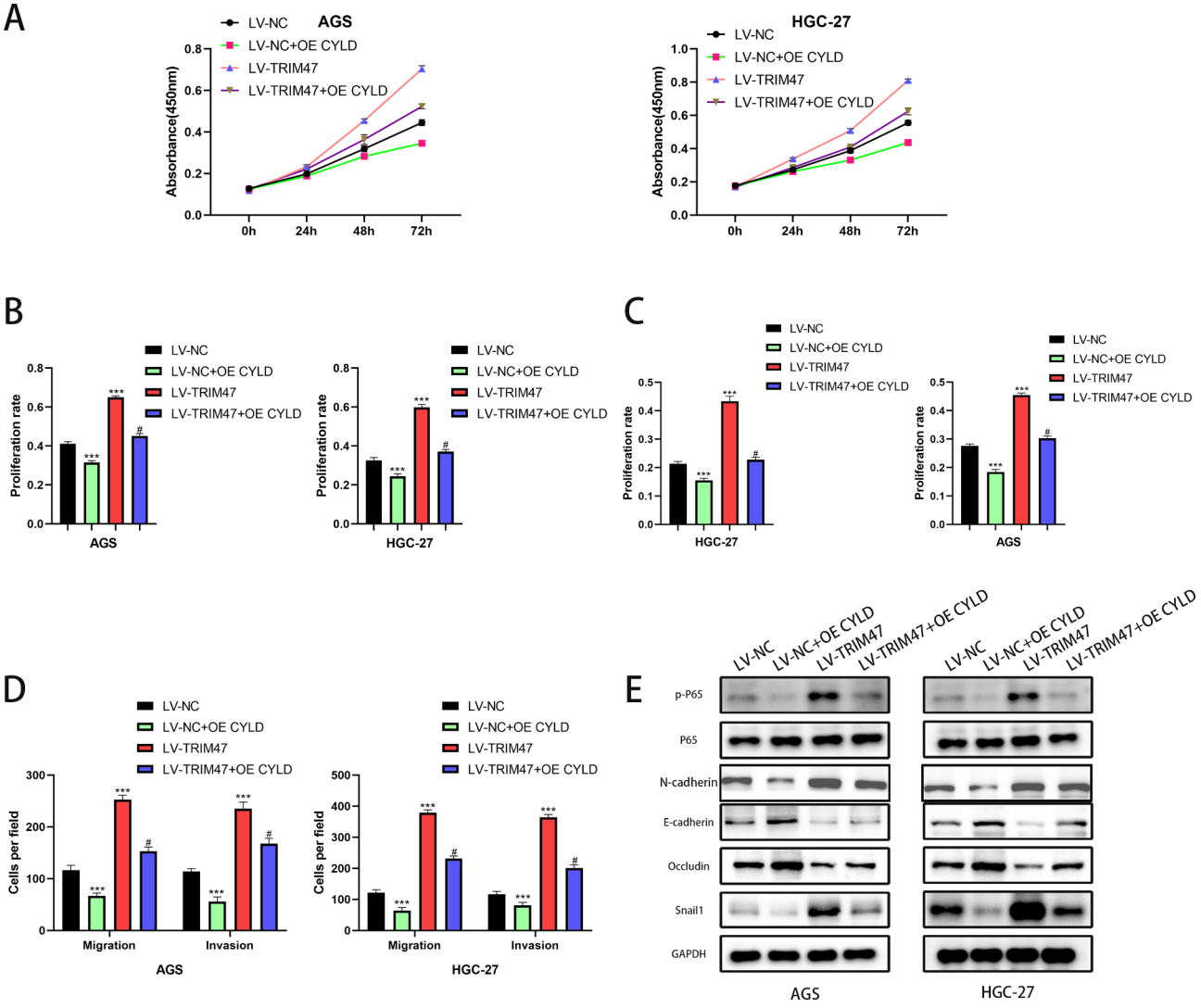

Fig. 8 TRIM47 promotes gastric cancer cell proliferation, invasion, and migration through CYLD-Mediated NF-κB pathway activation. (**A**) Treatment of AGS and HGC-27 cells transfected with TRIM47 or empty vector control with CYLD overexpression plasmid for 48 h, followed by CCK8 assay to assess cell proliferation. (**B**) Edu assay to evaluate cell proliferation. (**C**) Wound healing assay to determine cell migration. (**D**) Transwell assay to assess cell migration and invasion. (**E**) Western blot analysis of p-P65, P65, E-cadherin, N- cadherin, Occludin, and Snail1 protein expression in cells. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, t-test

study demonstrated that TRIM47 modified CYLD protein by ubiquitination, promoted CYLD degradation through the ubiquitin-proteasome system, and activated the NF-κB pathway to regulate the biological behavior of gastric cancer(Fig. S3).

Discussion

Gastric cancer remains one of the most common malignant tumours and a leading cause of cancer-related deaths worldwide. In recent years, the incidence and mortality rate of gastric cancer remain high, which brings serious economic pressure and social burden [1]. Although the overall incidence of gastric cancer is declining, significant variations in incidence and mortality rates persist across different countries. However, the lack of early diagnostic markers, limited treatment options, and delays in the development of targeted therapies emphasize the urgent need for more reliable diagnostic indicators and more effective treatment strategies.

Tripartite Motif-Containing 47 (TRIM47) is a member of the TRIM protein family [11]. Previous studies have shown that TRIM47 is involved in the pathogenesis and progression of a variety of diseases, especially its abnormal expression in a variety of tumors [14–16]. In this study, we used bioinformatics methods to verify that TRIM47 is highly expressed in most cancers. We then analysed TRIM47 expression levels in 20 pairs of gastric cancer and adjacent noncancer tissues collected from patients at our centre. Both the immunohistochemistry and Western blot results indicated that TRIM47

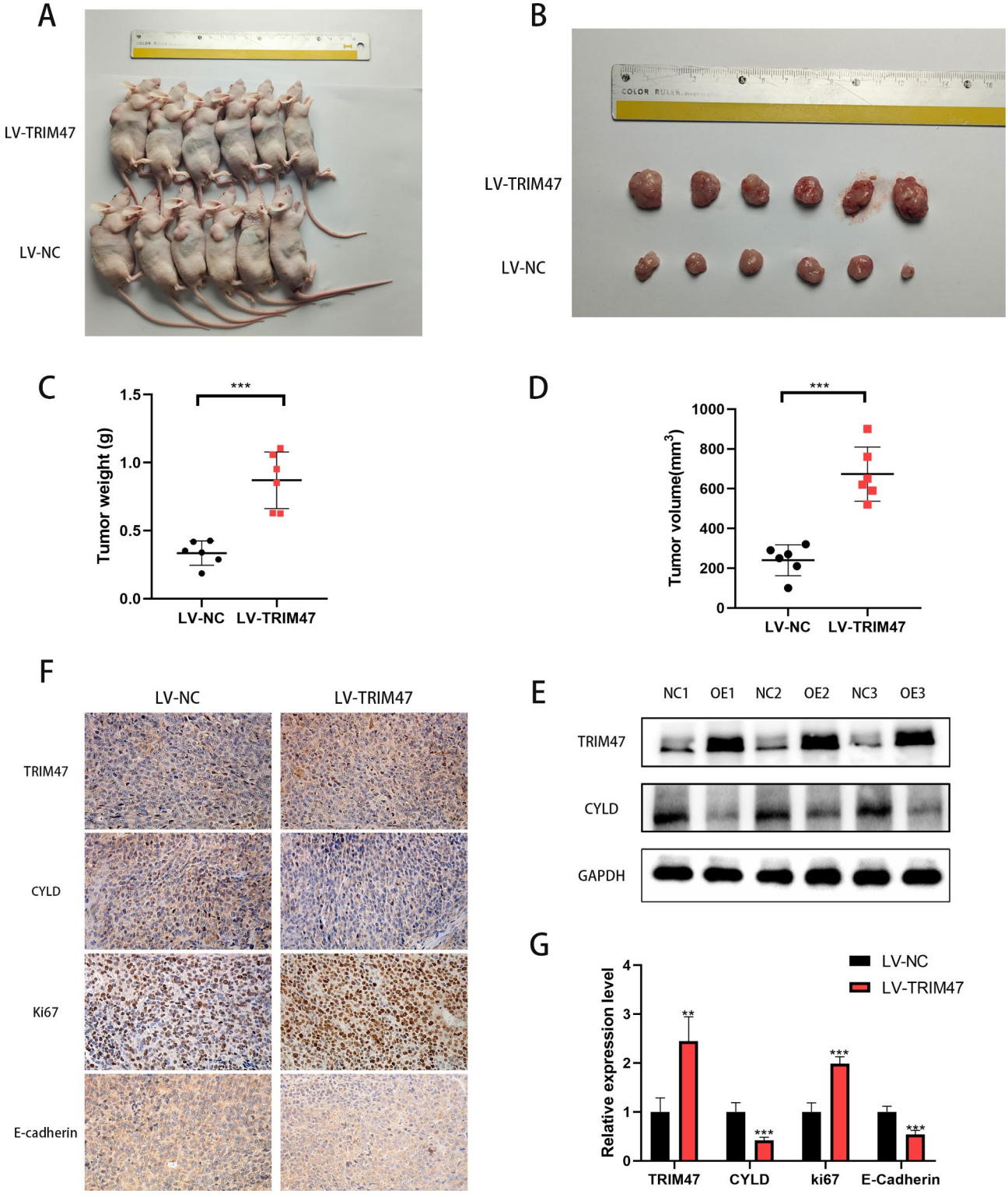

Fig. 9 In vivo experiments validate the role of TRIM47 in tumor progression. (**A**) Injection of stable TRIM47-overexpressing HGC-27 cells or control HGC-27 cells into the flanks of nude mice, $N=6$ per group. Photographs of nude mice after 14 days. (**B**) Photographs of xenograft tumors dissected from nude mice. (**C**) Measurement of the weight of xenograft tumors. (**D**) Calculation of the volume of xenograft tumors. (**E**) Western blotting to detect TRIM47 and CYLD protein levels in the two groups of tumors. (**F**) Immunohistochemistry (IHC) staining to determine the expression of TRIM47, CYLD, Ki67, and E-cadherin in the two groups of tumors(400×). (**G**) Quantitative analysis of immunohistochemistry staining. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, t-test

is highly expressed in gastric cancer tissues. Analysis of clinical data from the TCGA database further revealed that TRIM47 is associated with lymph node metastasis in gastric cancer.

To further investigate the role of TRIM47 in gastric cancer, we constructed stable cell lines with TRIM47 overexpression or knockdown and conducted a series of functional assays. TRIM47 overexpression significantly promoted the proliferation, invasion, and migration of gastric cancer cells, whereas TRIM47 knockdown had the opposite effects. Additionally, our in vivo experiments confirmed that the subcutaneous tumour volume and weight in the TRIM47-overexpressing group were significantly greater than those in the control group. Immunohistochemical staining of tumour tissues from nude mice also indicated increased expression of proliferation-related markers in the TRIM47 overexpression group. These findings suggest that TRIM47 plays a crucial role in promoting the proliferation, invasion, and migration of gastric cancer cells. Previous studies have demonstrated that TRIM47 overexpression promotes tumour proliferation, invasion, and migration in renal cell carcinoma, breast cancer, glioma, and pancreatic cancer, while TRIM47 knockdown inhibits these malignant behaviours [15, 19–22]. These results align with our finding, which indicate that TRIM47 plays a vital role in promoting the malignant biological behaviours of gastric cancer. In this study, we used Western Blotting to verify the effect of TRIM47 on EMT [23, 24]. We observed that TRIM47 overexpression enhanced the EMT process in gastric cancer cells. Conversely, TRIM47 knockdown inhibited the ability of gastric cancer cells to acquire an EMT phenotype.

The NF-κB (nuclear factor-kappa B) pathway is a crucial cellular signaling pathway involved in the regulation of various biological processes, such as immune responses, cell survival, and inflammation [25]. The NF-κB family consists of five members: p65 (RelA), RelB, c-Rel, p50, and p52. These members form different dimers, primarily p50/p65 and p52/RelB, which regulate gene transcription and mediate cellular responses to various stimuli [26, 27]. Abnormal activation of NF-κB is closely associated with autoimmune diseases, various inflammatory conditions, and tumorigenesis [28–30]. In this study, we found that TRIM47 may influence gastric cancer progression through the NF-κB pathway. The western blot results indicated that TRIM47 overexpression promoted activation of the NF-κB pathway, while TRIM47 knockdown had the opposite effect. Furthermore, treatment of TRIM47-overexpressing gastric cancer cells (AGS and HGC-27) with the NF-κB pathway inhibitor BAY 11-7085 reversed the TRIM47-induced increase in cell proliferation, migration, and invasion. This finding was further confirmed by the protein levels of EMT-related markers. In summary, TRIM47 regulates the malignant biological behaviour of gastric cancer cells by activating the NF-κB signaling pathway.

Based on previous research, we established that TRIM47 regulates gastric cancer progression by activating the NF-κB signaling pathway. Additionally, we identified a potential interaction between TRIM47 and CYLD. A review of the literature indicated that CYLD downregulation can promote breast cancer metastasis by activating the NF-κB pathway [31]. CYLD is a deubiquitinating enzyme that negatively regulates the NF-κB pathway through TNFR family members 32. Furthermore, the tumour suppressor CYLD interacts with TRIP to coregulate the negative activation of the NF-κB pathway [33]. Therefore, we hypothesized that CYLD might mediate TRIM47-induced activation of the NF-κB pathway. To test this hypothesis, we conducted Co-IP assays and confirmed the interaction between TRIM47 and CYLD.

Ubiquitination is a crucial intracellular mechanism for protein degradation and regulation and is facilitated by the ubiquitin–proteasome system. This system includes ubiquitin proteins, ligases, substrate proteins, and proteasomes, which collectively regulate the content and function of intracellular proteins [34]. Ubiquitination modulates a range of vital biological processes, including cell proliferation, apoptosis, and DNA repair, by regulating key signaling pathway proteins, cell cycle proteins, and inhibitory regulatory proteins [35]. In gastric cancer, POU5F1 promotes cell proliferation, migration, and invasion by reducing the ubiquitination level of TRAF6 [36]. TRIM29 enhances the ubiquitination of IGF2BP1 in gastric cancer, which leads to the downregulation of PD-L1 and the promotion of antitumour immunity [37]. TRIM31 promotes gastric cancer cell proliferation and invasion by regulating Axin1 protein stability and activating the Wnt/β-catenin pathway [38]. Eight types of ubiquitin chains have been identified (K6, K11, K27, K29, K33, K48, K63, and M1), and each leads to different outcomes for the proteins involved [39]. In this study, we found that TRIM47 is involved in the proteasome-mediated degradation of CYLD. Ubiquitination assays showed that TRIM47 overexpression increased the level of ubiquitinated CYLD in the gastric cancer cell lines AGS and HGC-27. Additionally, we discovered that TRIM47 promotes CYLD degradation through the ubiquitin–proteasome system by modifying CYLD with K48-linked ubiquitin chains.

In conclusion, this study demonstrated that TRIM47 promotes gastric cancer cell proliferation and invasion by modifying CYLD with K48-linked ubiquitin chains, thereby activating the NF-κB signaling pathway. Our findings indicate that TRIM47 may act as a novel oncogene and provide a theoretical basis for the diagnosis and treatment of gastric cancer. Although in vitro

experiments have confirmed that TRIM47 promotes gastric cancer progression through the CYLD/NF-κB signaling pathway, further in vivo functional studies are needed to validate these results. Therefore, more comprehensive research is required to address these questions in future studies.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13062-024-00555-1.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

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All authors reviewed and approved the final manuscript.

Author contributions
J.w., c.c. and w.w. designed the study. J.w., j.y., and r.l. performed the experiments. J. w. and j.y. analyzed and counted the experimental data. c.c. and r.l. wrote the article.All authors reviewed the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of Renmin Hospital of Wuhan University and the Animal Research Ethics Committees at Renmin Hospital of Wuhan University.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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