ORIGINAL RESEARCH

# RETRACTED ARTICLE: Knockdown of TRIM37 Promotes Apoptosis and Suppresses Tumor Growth in Gastric Cancer by Inactivation of the ERK I/2 Pathway

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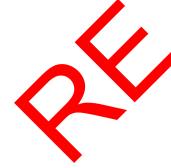
**Objective:** Gastric cancer (GC), a malignant tupor of the gastric purcosa, is the second leading cause of cancer deaths worldwide. Although the acidence and mortality of gastric cancer have been reduced in the US and elsewhere, a staffl a major public health concern. In this study, we attempted to investigate the function of coart of motif-containing protein 37 (TRIM37) in GC cell lines in order of propose a new therepy for GC.

**Methods:** The expression of TRIM37 in GC parts and cell lines was detected by immuno-histochemistry, real-time PCF and Western blotting analysis. After TRIM37 knockdown or overexpression, the cell cycle, proliferation and apoptosis, as well as the expression of related proteins, were detected. In a lition, in vivo experiments on nude mice were performed.

**Results:** We found that TRIM 7 express in was significantly elevated in tumor tissues of GC patients and GC 171. The and patients with high expression of TRIM37 had a poor prognosis. Knockdown of Th M37 is sells significantly inhibited cell proliferation and cell cycle progression promoter poptosis, increased cleaved caspase 3 and decreased c-myc and phosphor ation caprotein mase 1/2 (p-ERK1/2). Effects of TRIM37 overexpression were opposite to at of TR M37 knockdown and were potently attenuated by an ERK1/2 inhibitor. In addition, an EK1 2 agonist increased TRIM37 and p-ERK1/2 in a dose-dependent manner, and TRIM37 knockdown potently attenuated EGF-induced cell proliferation and expression of TRIM37 and p-ERK1/2. Interestingly, we found that TRIM37 overexpression did not affect the mRNA level in dual-specificity phosphatase 6 (DUSP6), but reduced its protein level in GC cells. Co-imageoprecipitation (Co-IP) analyses revealed that TRIM37 interacted with DUSP6, and TRIM37 overexpression enhanced DUSP6 ubiquitination in GC cells. In vivo experiments on nude mice showed the inhibitory effect of TRIM37 knockdown on tumor growth.

**Conclusion:** These findings suggest that TRIM37 may act as an oncogene in the growth of GC cells and illustrate its potential function as a target in the treatment of GC.

Keywords: TRIM37, gastric cancer, ERK1/2 pathway, cleaved caspase 3, C-myc



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#### Introduction

Gastric cancer (GC), a malignant tumor of the gastric mucosa, is the second leading cause of cancer deaths worldwide. 1-3 Data have shown that compared to the southern regions, the incidence of GC in the northwest and eastern coastal areas of China are much higher. 4 GC not only causes damage to the digestive system, but metastasis of GC can affect liver, kidney and respiratory functions. 5 In severe cases, GC may result in cachexia and is ultimately life-threatening. Currently, fiber endoscopy, supplemented with X-ray barium meal examination, abdominal

B-ultrasound, tumor markers, routine blood examination, and gastric juice analysis is the most effective way to diagnose gastric cancer. In recent years, advances in diagnostic techniques have increased early detection of gastric cancer thereby reducing mortality, but recurrent disease often occurs in patients with advanced disease, although aggressively treated, their survival rate is extremely low.<sup>6</sup>

Tripartite motif-containing protein 37 (TRIM37), also known as MUL, contains a RING finger domain, and is identified to be an E3 ubiquitin ligase. TRIM37 is located on the 17q23 chromosome, which is reported to be amplified in up to 40% of breast cancers.8 As a carcinogenic H2A ubiquitin ligase, TRIM37 is overexpressed in breast cancer and can promote transformation by silencing tumor suppressors and other genes. Additionally, studies have reported that TRIM37 is associated with the growth, migration and metastasis in multiple cancers, such as pancreatic cancer, glioma, hepatocellular carcinoma and colorectal cancer. 10-14 Furthermore, knockdown TRIM37 can inhibit human glioma cell proliferation and metastasis via activation of the PI3K/AKT pathway, while in liver cancer, overexpression of TRIM37 can promote cell migration and metastasis through activation of the Wnt/β-catenin pathway. 11,12 In this study, we attempt to explore the function and potential mechanism TRIM37 in human GC cells to put forward a pu al therapeutic target for GC treatment.

Dual-specificity phosphatase 6 (DUSP6), cytosol phatase, is a negative-feedback regulator or the acellularsignal-regulated kinase 1/2 (ERK1/2) Studies have eported that DUSP6 plays a tumor suppressive roomin lung cancer. 16,17 DUSP6 has a neuroprotective effect on Aβ-n luced cytotoxicity via suppression of EP 1/2 activation in neural stem cells, and down-regulation of USP6 Intributes to the progression and differentiation of esoph geal squadous cell carcinoma Athern re, The 17 is reported to promote (ESCC). 18,19 co cell proliferation via the DUSP6/ hepatocellu carcip RIM11 downregulation inhibited DUSP6 p38 pathway, a protein in D-54 g blastoma multiforme (GBM) cells.<sup>20,21</sup> However, the interaction between TRIM37 and DUSP6 in GC cells has not been elucidated.

Here, elevated TRIM37 expression was observed in GC tumor tissues and cell lines, and elevated expression of TRIM37 in patients correlated with a poor prognosis. Knockdown of TRIM37 in GC cells significantly inhibited cell proliferation and cycle progression, promoted apoptosis, increased cleaved caspase 3 and decreased c-myc and phosphorylated ERK1/2 (p-ERK1/2). Effects of TRIM37

overexpression were opposite to TRIM37 knockdown and was potently attenuated by an ERK1/2 inhibitor, U0126. In addition, ERK1/2 activator, EGF, increased TRIM37 and p-ERK1/2 levels in a dose-dependent manner, while TRIM37 knockdown potently attenuated EGF-induced cell proliferation and expression of TRIM37 and p-ERK1/2. Co-Immunoprecipitation (Co-IP) analyses revealed that TRIM37 interacted with DUSP6, and that TRIM37 overexpression enhanced DUSP6 ubiquitination in GC cells. In vivo experiments showed the inhibitory effect of TRIM37 knockdown on turn growth. These findings suggested that TRIM37 y act as oncogene in GC to regulate c-myc, clean caspase and the ERK1/2 pathway.

#### Patients and Math

#### Patients and issues

Ninety-five pants aged 40 60 years old, who were treated at Renji Hospi. School of Medicine, Shanghai Jiao oniversity (Shanga, China) were enrolled in this . After informed consent was obtained, 30 paired cantissues from 30 GC patients were cold paracanc lected detect RIM37 expression. After 80 months, the tian follow-up time, samples of 65 GC patients were ecce to Kaplan-Meier survival analysis and Log rank test. This study was performed in accordance with the rules f the Declaration of Helsinki and approved by the Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University (Shanghai, China).

#### Cell Culture

Five human GC cell lines AGS, HGC27, MKN28, MKN45, SNU719 and a gastric mucosa cell line, GES-1, were purchased from the Cell Bank of Chinese Academy of Science (Shanghai, China). The above cells were cultured with RPMI-1640 medium (SH30809.01B, HyClone), containing 10% fetal bovine serum (FBS; 16,000-044, GIBCO) and 1% penicillin and streptomycin (P1400-100, Solarbio) at 37°C in a 5% CO2 incubator.

#### Construction of Lentivirus

Targeting three different sites of TRIM37 (shown in Table 1), interference sequences were synthesized (T1300-100, Solarbio) and shRNA was constructed by double chain annealing. Subsequently, each of the shRNAs was inserted into the AgeI/EcoRI sites of the pLKO.1-puro vector to construct pLKO.1-puro-shTRIM37-1, -2, and -3. For

Table I TRIM37 Interference Sequences

Name	Sequences	
TRIM37 target site I (1799–1817)	GGAGAAGATTCAGAATGAA	
TRIM37 target site 2 (2119–2137) TRIM37 target site 3 (2730–2748)	CCAGTAGTTTACTAGACAT	
TRIM37 target site 3 (2730–2748)	GCCTTGATACATGGCAGTA	

TRIM37 overexpression plasmids, the full length 2895 bp coding DNA sequence (CDS) region of TRIM37 (BC036012.1) was synthesized by Genewiz Company (Shanghai, China). Then, TRIM37 CDS was inserted into a pLVX-puro vector at the EcoR I/BamH I sites. After sequence confirmation, (Invitrogen, Thermo Fisher Scientific, Inc., USA), pLKO.1-puro-shTRIM37 (1000 ng) or pLVX-puro-TRIM37 (1000 ng) was co-transfected with viral packaging plasmids, psPAX2 (100 ng) and pMD2G (900 ng) (Addgene, Inc., Cambridge, MA, USA) using Lipofectamine 2000<sup>TM</sup> into 293T cells. After 48 hours of transfection, viral particles in supernatants were obtained by ultracentrifugation.

#### Cell Proliferation Assay

GC cells (HGC27, MKN45 and AGS) were grown to the logarithmic phase, trypsinized (T1300-100, Solarbio and counted under a microscope to prepare a cell suspensor with  $3 \times 10^4$  cells/mL. Then, 100  $\mu$ L or cell aspensor was taken to seed in 96-well plates are cultured avernight. After treatment at 0, 24, 48 and 72 tours, Sc. Count Kn-8 (CCK-8; CP002, SAB) and server-free media, were mixed at a volume ratio of 1:10 and then 1.0  $\mu$ L was acced to each group and incubated for chour at 37°C. The the absorbance value (OD) at 450 nm, was evaluated on a dicroplate reader.

#### Cell Cycle Detc 10n

GC cells (GC2) MKN (AGS) were grown to the logarit vic phase tempsinized (T1300-100, Solarbio) and then seed (AGS) as (AGS) were grown to the seed (AGS) as (AGS) were grown to the seed (AGS) as (AGS) were for overnight cure to a first treatment for 48 hours, the cells were collected to carry out cell cycle detection. In brief, cells were trypsinized and centrifuged for 5 min at 1000 g. Cells were then washed with pre-cooled PBS and then resuspended in 300  $\mu$ L FBS-containing PBS (16,000—044, GIBCO), followed by 24 hours of immobilization in 700  $\mu$ L of (AGS) pre-cooled absolute ethanol at (AGS) After removing RNA with a 1 mg/mL RNase A solution (100  $\mu$ L; R8020-25, Solarbio) at 37°C in the dark for 30 min, cells were stained in 400  $\mu$ L Propidium Iodide (PI,

 $50 \mu g/mL$ ; C001-200, 7Seabiotech, Shanghai, China) in the dark for 10 min. Within 24 hours, the DNA at each cell cycle was detected by a flow cytometer (Accuri C6, BD Biosciences) at a wavelength of 488 nm of the excitation wavelength. Cell cycle analysis was then performed using FLOWJO software.

#### Cell Apoptosis Assay

GC cells (HGC27, MKN45 and AGS) were grown to the logarithmic phase, trypsinized (T1300-100, Solarbio) and cultured overnight in 6-well plates  $1.2 \times 10^5$  cells/well. After treatment for 48 hours, we cells were trypsinized to collect in a centrifuge tube for coptosis deterion (Annexin V-FITC Apoptosis Deteration Kit, 1063, Payotime). About  $1 \times 10^5$  resuspendencells were incurred with 195  $\mu$ L of Annexin V-FITC tradition solution, followed by 15 min incubation with 5  $\mu$ L. Annexia V-FITC at 4°C in the dark. The care at 4°C in 12 dark, cells were incubated for 5 min in 5  $\mu$ L PI staining solution. A tube without Access V-FITC and PI was used as a negative control, subsequently flow cytometry was used to detect cell apoptosis (FCM; Eccuri C6, BD Biosciences).

# Real-Time Polymerase Chain Reaction (RI-PCR) Assay

After treatment or without treatment, GC tissues or cells were collected to isolate the total RNA by Trizol Reagent (1596-026, Invitrogen). After quantification and confirmation of RNA integrity and purity, about 1 µg of RNA was reverse transcribed into complementary DNA (cDNA) by a Reverse Transcription Kit (#K1622, Fermentas, USA). Using a SYBRGreen PCR Kit, RT-PCR reactions using the cDNA as a template, were performed on the 7300 SDS Software of a Real-time detector (ABI-7300, Applied Biosystems, USA) with the following program: 95°C, 10min; (95°C, 15 sec; 60°C, 45 sec)  $\times$  40. After that, the expression of TRIM37 mRNA, normalized to GAPDH, was analyzed using the  $2^{-\Delta\Delta Cq}$  method.<sup>22</sup> The primers are as listed: TRIM37, Forward: 5'-TGGACTTACTCG CAAATG-3', Reverse: 5'-ATCTGGTGGTGACAAATC -3'; GAPDH, Forward: 5'-AATCCCATCACCATCTTC -3', Reverse: 5'-AGGCTGTTGTCATACTTC-3'.

# Western Blotting Analysis

After treatment or without treatment, GC cells were lysed in RIPA Lysis Buffer (P0013B, Beyotime), containing protease and phosphatase inhibitor, and then centrifuged at 12,000 g for

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15 min and supernatant-containing protein was collected. Proteins were quantified with a BCA Kit (PICPI23223, Thermo Fisher Scientific, Inc., USA) prior to separation by electrophoresis using a 15% PAGE-SDS gel, and then transferred onto polyvinylidene fluoride (PVDF) membranes using a semi-dry transfer. Membranes were then blocked for 1 hour at room temperature in skim milk (BD Biosciences, Franklin Lakes, NJ, USA), followed by incubation at 4°C with primary antibodies against TRIM37 (1:1000; Ab95997, Abcam), c-myc (1:1000; Ab32072, Abcam), cleaved caspase 3 (1:500; Ab2302, Abcam), ERK1/2 (1:1000, #9102, Cell Signaling Technology [CST]), p-ERK1/2 (1:1000, #9101, CST) and GAPDH (1:2000, #5174, CST) overnight with gentle shaking. Thereafter, membranes were washed with TBST 3 times and incubated at 37°C for 1 hour with HRPconjugated goat anti-rabbit secondary (1:1000; A0208, Beyotime, Haimen, China). After washing, a chemiluminescent reagent (WBKLS0100, EMD Millipore) was used to develop the membranes. Membranes were then

exposed on an ECL imaging system (Tanon-5200, Tanon, Shanghai, China). Protein levels of TRIM37, c-myc, cleaved caspase 3, ERK1/2 and p-ERK1/2 were normalized to GAPDH and were then analyzed using ImageJ of version 1.47 (Bethesda, MD, USA).

#### Hematoxylin-Eosin (HE) Staining

About 0.3 cm of tumor tissues of a nude mouse were collected, fixed, embedded and cut into 4 µm slices by a paraffin slicer. After that, the slices were stained hematoxylin and eosin (BASO). In brief were baked for 30 min in an oven at 65°C are then dewaxt in xylene I and II (10,023,418, Sinopharm) or 15 mir in turns. Rehydration was carried at in 100 75% ethyl alcohol (10-2,680 nophan) for 5 min in turns followed by rin th tap ater for 10 min. Sections were en stained rith matoxylin (714,094, BASO) for min, llowed by secs of color separation in amino water (10, 2,118, Sinopharm) and 15 min

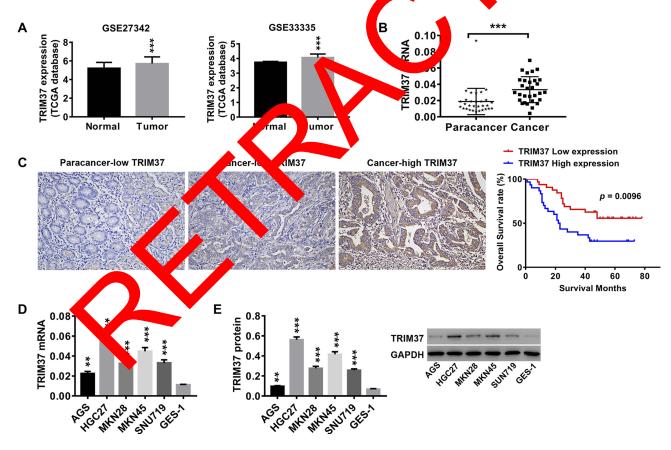


Figure 1 TRIM37 was highly expressed in tumor tissues of GC patients and GC cell lines. Notes: (A) Analysis of TRIM37 expression in normal and tumor samples from TCGA database. Thirty paired cancer and paracancer tissues were collected from GC patients. (B) The mRNA expression of TRIM37 in cancer and paracancer tissues was detected by RT-PCR. (C) Detection of TRIM37 expression by IHC, and Kaplan-Meier survival analysis and Log rank test of 65 GC patients (TRIM37 low expression: 32; TRIM37 high expression: 33). TRIM37 expression in cancer and paracancer tissues was detected by IHC. (D, E) The mRNA and protein expression of TRIM37 in GC cells AGS, HGC27, MKN28, MKN45, SNU719 and gastric mucosa cells, GES-1, was detected by RT-PCR. \*\*p < 0.01, \*\*\*p < 0.001 vs Normal, Paracancer, paracancer-low TRIM37, or GES-1.

**Table 2** Relationship Between TRIM37 Expression and Clinicopathological Features of Gastric Cancer

Clinicopathological	TRIM37		P value
Features	Low (n=32)	High (n=33)	
Gender			0.1648
Male (n=42)	18	24	
Female (n=23)	14	9	
Age (years)			0.1698
≤60 (n=40)	17	23	
>60 (n=25)	15	10	
Location			0.7824
Cardia (n=32)	15	17	
Corpus (n=21)	10	П	
Antrum (n=12)	7	5	
Tumor size (cm)			0.1072
≤3 (n=29)	17	П	
>3 (n=36)	15	22	
Tumor differentiation			0.9379
Well (n=17)	9	8	
Moderate (n=21)	10	П	
Poor (n=27)	13	14	
Lymph node metastasis			0.0178
No (n=29)	18	9	
Yes (n=36)	14	24	
TNM stage			0.0194
I (n=20)	15	5	
II (n=14)	6		
III (n=31)	11	20	

Note: Differences between groups were by the Chi-squarest.

rinsing with tap water ections were ben dehydrated in 70% and 90% ethy acohol for 10 min in turns and then stained with eosin (A400), BASO) for 2 min, followed by dehydrati with solute nanol. 3 sections were I twice, sealed with neutral hyalinize in xyl e for s gum (**5**90, **S** and placed in a 65°C oven for 15 min. Suc quently, sections were imaged using a microscopic nage analysis system (DS-Ri2, NIKON), and the relevant parts of the samples were collected and analyzed.

#### **TUNEL Fluorescence**

About 0.3 cm of tumor tissues of a nude mouse were collected, fixed, embedded and cut into 4  $\mu m$  slices by a paraffin slicer. Later, sections were used to detect cell apoptosis of tumor tissues by a TUNEL Apoptosis Detection Kit (FY600017-

20T, Fuyuanbio) following the manufacturer's instructions. Finally, the slides were imaged on a fluorescence microscope.

#### Immunohistochemical (IHC) Detection

About 0.3 cm of cancer and paracancer tissues of GC patients were collected, fixed, embedded and cut into 4 um slices by a paraffin slicer. After being baked for 30 min in an oven at 65°C, sections were dewaxed in xylene I and xylene II (Shanghai Sinopharm) for 15 min in order, and then rehydrated sequentially in gradient concentrations of 100%, 95%, 85%, and 75% et 5 min, and then rinsed for 10 min with tar water. An that, antigen min in 0 retrieval was carried out for 1 M sodium citrate buffer (pH6.0) and section were ten blocked in a wet-box with  $0.3^{\circ}$   $A_2O_2$ . Following ubation with Rb-TRIM37 antibody is a boar at room temperature, sections were incubated with he 2-conjugated secondary antibody. Sections then subject to DAB staining, 3 min staining of hem. xylin and alcohol differentiation with nystochloric act. Following a 10 min rinse with tap vater, sections were hyalinized in xylene for 3 min  $\times$  2 mes, sealed with neutral gum (G8590, Solarbio), and ed in a C oven for 15 min. Subsequently, pictures were taken using a microscopic image analysis system i2, NIKON), and the relevant parts of the samples were collected and analyzed. According to the positive areas, the specimens were categorized into two groups: TRIM37 low expression: < 25% of the tumor cells showed positive TRIM37; TRIM37 high expression: > 25% of the tumor cells showed positive TRIM37. For evaluation criteria of IHC results, refer to the report of Chen et al.<sup>23</sup>

# Co-Immunoprecipitation (Co-IP)

Briefly, whole-cell extracts were isolated after infection with vector or oeTRIM37 lentivirus. Then, all samples were incubated with the appropriate antibodies plus Protein A/G beads (Santa Cruz Biotechnology, USA) overnight. Beads were washed five times and separated by SDS-PAGE. Western blot was performed as indicated above.

# Ubiquitination Assay

AGS cells that were infected with vector or oeTRIM37 lentivirus were lysed by sonication in 1% SDS-containing radio immunoprecipitation assay (RIPA) buffer on ice. Next, lysates were treated with Protein A/G PLUS-Agarose (sc-2003, Santa Cruz Biotechnology, USA) for 1 h. Then, the samples were incubated with IgG (sc-2027,

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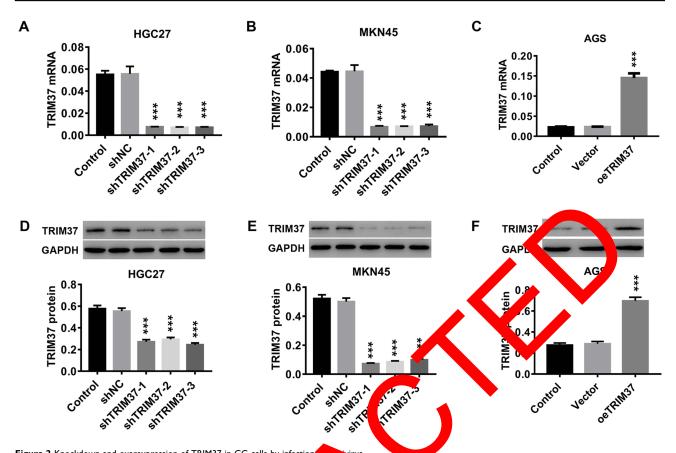


Figure 2 Knockdown and overexpression of TRIM37 in GC cells by infection Notes: In vitro, GC cells (HGC27, MKN45 and AGS) were infected with lentive ses on NM37 or oeTRIM37. (A–C) The knockdown or overexpression efficiency of shTRIM37 or oeTRIM37 in GC cells was determined by RT-PCR. (D–F) The knockdown or overexpression efficiency of shTRIM37 or oeTRIM37 or oeTRIM37 in GC cells was also detected by Western blotting. \*\*\*\*p < 0.001 vs shNC or Vector.

Santa Cruz Biotechnology, USA) overnight at 4°C After centrifugation for 5 min at 3000 rpm 4 4 the nuclear pellet was gathered and washed four ames with votein A/G Plus-Agarose beads. The purited pateins were an on a 4–20% gradient SDS-PAGE. Anti-LASP6 antibody (ab76310, Abcam, UK) and anti-Ubique an antibody (ab7780, Abcam, UK) are used for immunoblotting.

#### In vivo Ex erin ents

ice were divided into two groups: The BALK nude and HGC27-shNC (normal control). 0.1 mL of PBS s pended with 1×10<sup>6</sup> cells was subcutaneously injected into nude mice (BALB/c, male, 4 weeks old), respectively. Tumor volume was measured every third day. The mice were euthanized 4 weeks later. The tumor growth and apoptosis was detected by HE staining and TUNEL fluorescence. All animal protocols were carried out following the Guidelines for Care and Use of Laboratory Animals (Ministry of Science Technology of China) and authorized by the Institutional Animal Ethics Care and Use Committee of the Renji ospital, School of Medicine, Shanghai Jiao Tong University.

#### Statistical Analysis

Statistical analysis in this research was conducted on the GraphPad prism 7.0 software (GraphPad Software, USA). With three repeated experiments, all graphed results are presented as mean  $\pm$  SD. Paired Student's *t*-test was used to determine the significance between two groups, while multiple groups were determined by One-way analysis of variance (ANOVA) with Tukey's post hoc test. A value of p < 0.05 was considered statistically significant.

#### Results

# TRIM37 Was Highly Expressed in Tumor Tissues of GC Patients and GC Cell Lines

Analysis of normal and tumor samples from TCGA database showed that expression of TRIM37 in GC tumors was much higher than that in normal tissues (Figure 1A). In our study, thirty paired cancer and paracancer tissues

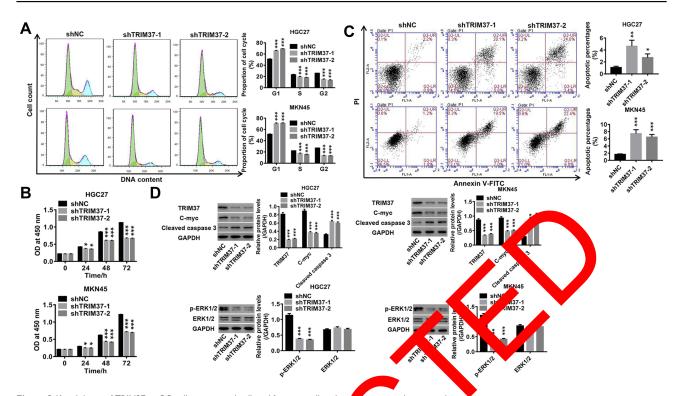


Figure 3 Knockdown of TRIM37 in GC cells suppressed cell proliferation, cell cycle pression and promoted apoptosis.

Notes: GC cells HGC27 and MKN45 were infected with shTRIM37-1 and shTRIM37-1 entiviruses. (A) cell cycle proportions were assessed by flow cytometry at 48 hours after infection. (B) Cell proliferation was calculated at 0, 24, 48 and 72 hours are infection by flow cytometry. (D) The expression levels of related-proteins TRIM37, c-myor paved caspases p-ERK1/2 and ERK1/2 were analyzed by Western blotting. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 vs shNC.

from GC patients were collected to analyze the expres of TRIM37. As shown in Figure 1B, cancer tissues, the mRNA expression tissues of GC patients was sign cantly creased. IHC staining of 65 GC patients showed gh protein expression of TRIM37 in ancer sues. In CC tissues, the staining intensity TRIM37 is enificantly higher than that of the responding adjacent tissue, and the expression of TRN 37 found in both cytoplasm and mon s, 37 g o5 patients died of GC. nucleus. Aft Kaplan-Vier surival and s and Log rank test demonrepression was significantly correverall survival, and patients with high lated with RIM37 had a poor prognosis (Figure 1C). expression of Relationship between TRIM37 expression and clinicopathological features of gastric cancer was shown in Table 2. Consistent with the above observation, we found significantly higher expression of TRIM37 in GC cell lines (AGS, HGC27, MKN28, MKN45 and SNU719) compared with the gastric mucosa cell line, GES-1 (Figure 1D and E). Furthermore, compared with other cell lines, TRIM37 is relatively high in HGC27 and MKN45, and relatively low in AGS. These findings suggested that TRIM37 may act as an oncogene in GC.

# Knockdown and Overexpression of TRIM37 in GC Cells by Infection with Lentivirus

In vitro, two GC cell lines, HGC27 and MKN45, were infected with shTRIM37 lentiviruses (shTRIM37-1, -2 and -3), while AGS cells were infected oeTRIM37 lentivirus. After RT-PCR and Western blotting analysis, the results showed that all three shTRIM37 lentiviruses significantly down-regulated the expression of TRIM37 mRNA in HGC27 (Figure 2A and D) and MKN45 (Figure 2B and E) cells, while oeTRIM37 lentivirus significantly up-regulated TRIM37 expression in AGS cells (Figure 2C and F). In addition, compared to shTRIM37-3, lentiviruses of shTRIM37-1 and -2 had a more profound effect. Therefore, due to the effectiveness of the knockdown or overexpression, lentivirus of shTRIM37-1, -2 and oeTRIM37 were used for follow-up experiments.

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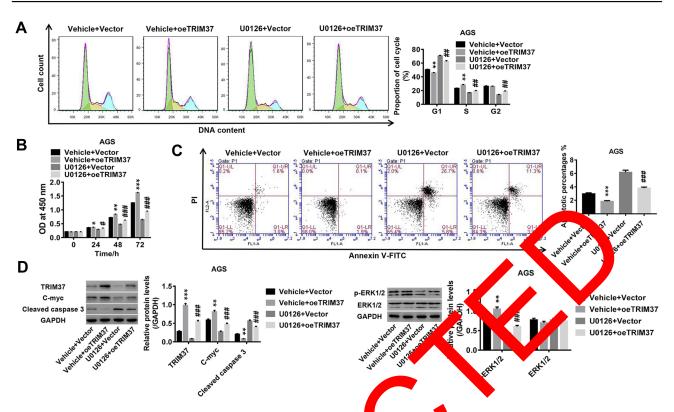


Figure 4 TRIM37 regulation of proliferation, cell cycle progression and apoptosis in GC cel may be mediated FRK1/2 signaling pathway. Notes: AGS cells were treated with oeTRIM37 lentivirus and ERK I/2 inhibitor, U0126. (A) cell cycle prop tions were calculated at 48 hours after infection by flow cytometry. (B) Cell proliferation was assessed at 0, 24, 48 and 72 hours after infection by CCKsay. (**C**) C apoptosis was assessed at 48 hours after infection by flow e 3, p-ERK 17. 1/2 were analyzed by Western blotting. \*p < 0.05, \*\*p < 0.01, cytometry. (D) The expression levels of related-proteins TRIM37, c-myc, cleaved \*\*\*p < 0.001 vs Vehicle + Vector; p < 0.05, p < 0.01, p < 0.001 vs Vehicle

# Knockdown of TRIM37 in GC Cells Suppressed Cell Proliferation, Cell 9 Progression and Promoted Apoptaisis

To understand the function of \( \text{M37} \) in \( \text{V} proliferation, cell cycle and apoptosic were evaluated after TRIM37 knockdoy. The results Figure 3A showed that knockdom of TM37 significantly prevented cell cycle progression by arresting cells in the G1 phase, the oy duch cell entry into the S/G2 phase. Furthermore in TRIM /-silenced HGC27 and eration was significantly decreased (Figu. 3B), whereas apoptosis was increased (Figure 3C), concurrent with increased cleaved caspase 3 and decreased c-myc, as well as decreased ERK1/2 phosphorylation, while ERK1/2 remained unchanged (Figure 3D). These results indicated that knockdown of TRIM37 could suppress cell proliferation in GC cancer, likely by blocking cell cycle progression and promoting apoptosis.

# TRIM37 Regulation of Proliferation, Cell Cycle Progression and Apoptosis in GC Cells May Be Mediated by ERK 1/2 Signaling Pathway

We also investigated the underlying mechanism of TRIM37 in regulating GC cells. As shown in Figure 4A, overexpression of TRIM37 in AGS cells significantly promoted the progression of the cell cycle from the G1-phase to the S-phase. Concurrently, cell proliferation in AGS was significantly increased by TRIM37 overexpression (Figure 4B), while apoptosis was decreased (Figure 4C). This was accompanied by decreased cleaved caspase 3 and increased c-myc and phosphorylated ERK1/2, while ERK1/2 remained unchanged (Figure 4D). In addition, the induction of TRIM37 overexpression was potently attenuated by the ERK1/2 inhibitor, U0126. Taken together, we speculated that overexpression of TRIM37 may contribute to GC progression, possibly through the activation of the ERK1/2 signaling pathway.

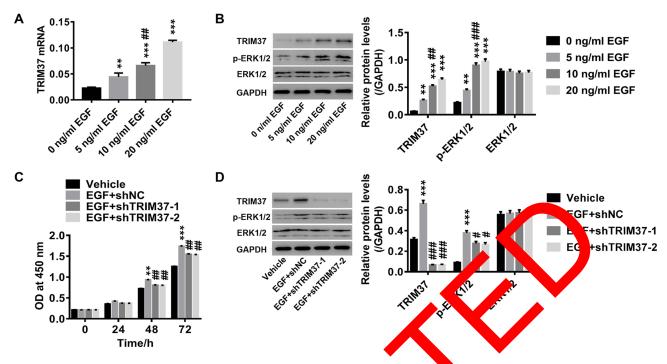


Figure 5 Knockdown of TRIM37 potently attenuated EGF-induced cell proliferation in GC Notes: AGS cells were treated with a series of concentrations of EGF recombinant prote EKK 172 agonist; 0, 5, 10 20 ng/mL EGF). (A) Expression of TRIM37 mRNA analyzed by Western blotting. AGS cells were treated with 10 ng/mL EGF and was detected by RT-PCR. (B) The protein levels of TRIM37, p-ERK1/2 and ERK1/2 wy shTRIM37 lentivirus. (C) Cell proliferation was assessed at 0, 24, 48 and 72 hours after eatment by CCK assay. (**D**) The protein levels of TRIM37, p-ERK1/2 and ERK1/2 were analyzed by Western blotting. \*\*p < 0.01, \*\*\*p < 0.001 vs 0 ng/mL EGF or Vehic 01, ###p < 0.001 vs 5 ng/mL EGF or EGF + shNC.

# Knockdown of TRIM37 Potently Attenuated EGF-Induced Cell Proliferation in GC Cells

ombin A series of concentrations of EGF (ERK1/2 agonist) were used to track AGS ıls. As shown nbinant pre in Figure 5A and B, EGF ins significantly increased TRIM37 express. and ERK1/2 phosphorylation in a dos dependent mayer. Furthermore, rg/mL) GC cell proliferation was EGF-induced (10 potently attenuated v T M37 knockdown (Figure 5C). duce TRIM? expression and ERK1/2 Likewise, E ntly decreased by TRIM37 phospho ation as sign. 1/2 expression was unchanged knocka n, y These data further prove that the ERK1/2 y is involved in the regulation of GC signaling path cells by TRIM37.

# TRIM37 Interacted with DUSP6 and Enhanced Its Ubiquitination in GC Cells

Next, we examined the mRNA and protein levels of DUSP6 in GC cells infected with oeTRIM37. Interestingly, the relative DUSP6 mRNA showed no significant change (Figure 6A), however, the DUSP6 protein

was significantly down-regulated in oeTRIM37-infected GC ells (Figure 6B). This demonstrated that overexpression of TRIM37 did not affect DUSP6 transcription, but instead, suppressed its translation in GC cells. In addition, Co-IP assays showed the interaction between TRIM37 and DUSP6 in GC cells (Figure 6C). TRIM37 overexpression significantly enhanced DUSP6 ubiquitination in GC cells, suggesting that TRIM37 might suppress DUSP6 translation by enhancing its ubiquitination in GC cells (Figure 6D).

# Knockdown of TRIM37 in Nude Mice Significantly Inhibited Tumor Growth

In vivo experiments were also performed on nude mice. We found that knockdown of TRIM37 significantly inhibited tumor weight (Figure 7A) and volume (Figure 7B) of nude mice. HE (Figure 7C) and TUNEL (Figure 7D) staining of tumor tissues showed a significant increased in cell apoptosis. Moreover, Western blotting showed that in TRIM37-silenced tumor tissues, protein levels of cleaved caspase 3 were significantly increased, and c-myc and phosphorylated ERK1/2 were decreased, while ERK1/2 was unchanged (Figure 7E). These demonstrated that knockdown of TRIM37 in nude mice

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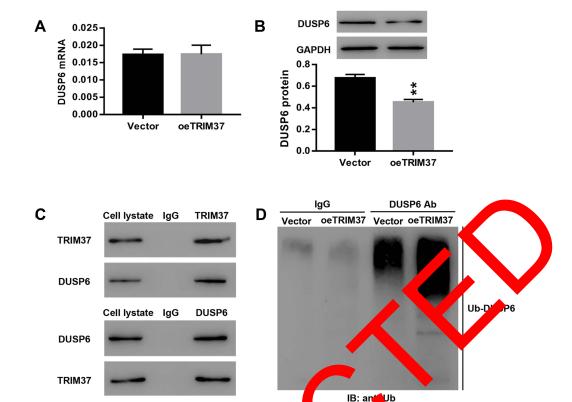


Figure 6 TRIM37 interacted with DUSP6 and enhanced its ubiquitination.

Notes: AGS cells were infected with vector or oeTRIM37 lentivirus. (A, B) The relative mRNA a protein to also of DUSP6 were examined. (C) The interaction between TRIM37 and DUSP6 in GC cells was analyzed. (D) Ubiquitin-mediated degrada and SSP6 was examined.

significantly inhibited tumor growth, which form indicated the inhibitory effect of TRIM37 knock down at the ERK 1/2 signaling pathway in GC progres on.

#### **Discussion**

Although the incidence and intrality of gardic cancer have been reduced in the US applies where, it is still major public health concern. In recent years, it reased research showed the in a various of human cancers, involvement of TRIM prov expression of TRIM29, including gastr cance For ex TRIM44 ar TRIM2 are associated with the poor prognosis of cancer patients. 24–26 Studies have found and overall sur that TRIM59 and RIM31 are up-regulated in GC, 27,28 and TRIM29 acts as an oxogene in GC and is a new marker for lymph node metastasis.<sup>29,30</sup> In addition, down-regulation of TRIM25 can suppress GC cell migration and invasion.<sup>31</sup> In this study, we found a significant increase in the expression of TRIM37 in tumor tissues of GC patients and GC cell lines, and patients with high expression of TRIM37 had a poor prognosis. Knockdown of TRIM37 significantly inhibited cell proliferation, blocked cell cycle progression at the G1phase and promoted apoptosis, whereas TRIM37 overexpression had the opposite effect. Our results are consistent with previous reports that knockdown of TRIM37 can suppress cell proliferation and tumor growth in breast cancer. These findings suggested that TRIM37 may act as an oncogene in GC, and knockdown of TRIM37 may be considered for GC treatment.

Furthermore, we explored the potential mechanisms of TRIM37 in regulating GC cell proliferation, cell cycle and apoptosis. We found that expression of cleaved caspase 3 was increased, while c-myc was decreased in TRIM37silenced GC cells. C-myc is a pro-oncogene that can regulate cell proliferation, promote cell division and participate in cell apoptosis, and is associated with the development of various tumors. 32,33 Cleaved caspase 3 is a cysteine protease regulating apoptosis or programmed cell death.<sup>34</sup> Thus, we speculated that TRIM37 regulation of cell proliferation, cell cycle progression and apoptosis may be mediated by the modulation of cleaved caspase 3 and c-myc. Previous studies have reported that β-catenin signaling is frequently activated in GC, which is associated with the survival of GC patients. 35,36 The TCF-β signaling pathway is involved in the regulation of TRIM25 in GC cell

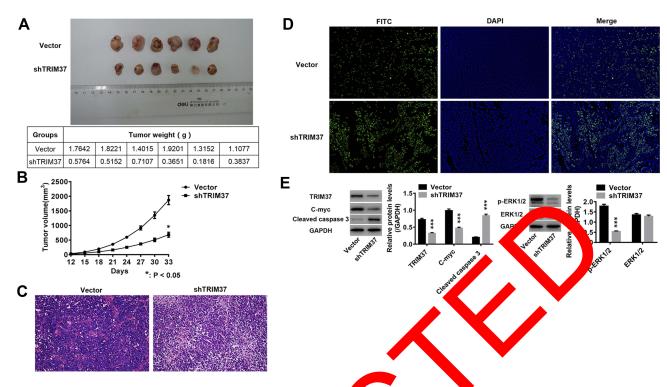


Figure 7 Knockdown of TRIM37 in nude mice significantly inhibited tumor growth.

Notes: shTRIM37 lentivirus and empty control HGC27 cells were used for subcutane as tumors in nude pice. (A and B) The tumor weight and volume of subcutaneous tumors was measured. (C and D) Tumors of nude mice were collected to carry out H and TUNEL stair g. (E) The protein levels of TRIM37, c-myc, cleaved caspase 3, p-ERK1/2 and ERK1/2 were analyzed by Western blotting. \*p < 0.05, \*\*\*p < 0.001 vs or.

migration and invasion.<sup>31</sup> In our study, knockdow TRIM37 significantly reduced ERK1/2 phorphorylat while TRIM37 overexpression had the oppos Furthermore, TRIM37-induced phosp rylation 2 and c-myc was potently decreased by a K1/2 inhibitor, U0126, while cleaved pase 3 w increased. TRIM37 knockdown potenty receded cell piviferation, as well as the phosphylation of K1/2 which was induced by an ERK 2 agonist, EGF recombinant protein. Moreover, we found that XIM37 overexpression did not affect DUSP6 transcription, but so pressed its translation in monstrated the interaction GC cells analys urthe betwee TRIM2 and DUSP6, and TRIM37 overexpresd DUSP6 ubiquitination in GC cells. MM37 overexpression might suppress Therefore, DUSP6 protein Evels by enhancing its ubiquitination and degradation in GC cells. In vivo experiments in nude mice further showed the inhibitory effect of TRIM37 knockdown on tumor growth, accompanied with increased cleaved caspase 3 and decreased c-myc and phosphorylated ERK1/2. Taken together, we inferred that TRIM37 regulated GC cell proliferation, cell cycle and apoptosis likely by activating the ERK1/2 pathway via the modulation of cleaved caspase 3 and c-myc, which is in agreement with reports that the

However, the lack of specific mechanisms of TRIM37-ERK1/2 in regulation of caspase 3 and c-myc in GC is one of the limitations of this study. If possible, further experiments exploring this aspect will be carried out in the future. In summary, this research demonstrates that TRIM37 likely functions as an oncogene in GC. Knockdown of TRIM37 can significantly suppress GC cell proliferation, arrest cell cycle progression at the G1-phase and promote apoptosis, likely mediated by the activation of the ERK1/2 signaling pathway via the modulation of cleaved caspase 3 and c-myc. Thus, targeting TRIM37 may serve as a potential novel therapy for GC treatment.

# **Data Sharing Statement**

All data generated or analyzed during this study are included in this published article.

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#### **Disclosure**

The authors report no conflicts of interest in this work.

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