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RESEARCH ARTICLE

TRIM36 suppresses cell growth and promotes apoptosis in human esophageal squamous cell carcinoma cells by inhibiting Wnt/β-catenin signaling pathway

Bin Zhao[1] · Gaofeng Qiao[1] · Jianhua Li[2] · Yukun Wang[3] · XiaoDong Li[1] · Hua Zhang[1] · Lu Zhang[4]

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Abstract

Our recent study has shown that TRIM36, a member of tripartite motif-containing (TRIM) family proteins and tumor suppressor and β-catenin may serve as a prognostic biomarker for esophageal squamous cell carcinoma (ESCC). Here, we sought to examine functional roles of TRIM36 and β-catenin in ESCC cells. TRIM36 was overexpressed or silenced by lentivirus transduction. Cell proliferation was examined by Cell Counting Kit (CCK)-8 assay, while cell cycle distribution and cell apoptosis was assessed via flow cytometry analysis. Xenograft mouse model was applied for in vivo analysis. Overexpression of TRIM36 inhibited cell proliferation in human ESCC cells, and silencing of TRIM36 led to opposite effects. We also found that ectopic expression of TRIM36 enhanced the ratio of G0/G1 phase cells and induced apoptosis in ESCC cells. Our data further revealed that TRIM36 stimulated the ubiquitination of β-catenin, and in turn, its inactivation. Finally, we confirmed these in vitro results in a xenograft mouse model and clinical specimens post-operatively obtained from patients of ESCC. In summary, these data support that TRIM36 can effectively inhibit tumorigenesis of ESCC by repressing Wnt/β-catenin signaling pathway, which suggest that selectively repressing this signaling pathway in ESCC may lead to development of a novel therapeutic approach for controlling this disease.

Keywords Esophageal cancer · TRIM36 · β-Catenin · Apoptosis · Ubiquitination

Introduction

Esophageal cancer (ESCC) represents the 6th leading cause of mortality associated with cancer, affecting approximately 450,000 people worldwide each year [1, 2]. As one of the two major pathological subtypes of esophageal cancer, esophageal squamous cell carcinoma (ESCC) is remarkably more common in South and Central Asia, compared with esophageal adenocarcinoma (EAC) that has the highest prevalence in Northern and Western Europe [3]. Recent advances in molecular biology have ascribed several genetic and epigenetic alterations as important factors contributing to tumorigenesis and progression of esophageal cancer, which are exemplified by genetic alterations such as loss of heterozygosity and gene mutations of genes, as well as epigenetic changes including DNA hypermethylation, histone modifications, and dysregulated expression of microRNAs [4, 5]. Despite that, some achievements have been made in treatment of ESCC including targeted therapies based on these findings [6, 7], the overall prognosis of ESCC has remained unsatisfactory. To this end, discoveries of novel molecular mechanisms and biomarkers

Bin Zhao and Gaofeng Qiao contributed equally to this work.

✉ Hua Zhang
95192789@qq.com

✉ Lu Zhang
zhangl_u868@163.com

1 Department of Thoracic Surgery, Shandong Provincial Public Health Clinical Center, Jinan, China

2 Department of Thoracic Surgery, Qingdao ChengYang People's Hospital, Qingdao, China

3 Department of Internal Medicine, The Third Affiliated Hospital of Shandong First Medical University (Affiliated Hospital of Shandong Academy of Medical Sciences), Taian, China

4 Department of Cardiothoracic Surgery, Affiliated Kunshan Hospital of Jiangsu University, Zhenjiang, China

are in an urgent need to further improve clinical outcomes for this disease.

The Wnt/β-catenin signaling could play a critical role in cell growth and differentiation in embryonic development [8, 9]. To date, there are at least 19 Wnt molecules known to exist in mammals. In a quiescent status, an intracellular degradation complex, comprised of glycogen synthase kinase 3 beta (GSK3β), Axin proteins and adenomatous polyposis coli (APC), phosphorylates/ubiquitinates β-catenin to cause its degradation. In an activated status, extracellular Wnt molecules crosslink with membrane receptors including lipoprotein receptor-related protein (LRP) to induce activation of the intracellular molecule Dishevelled (Dvl), which in turn represses the formation of this degradation complex, resulting in the nuclear translocation of β-catenin [10, 11]. An activated Wnt/β-catenin signaling pathway might also contribute to the progression of ESAC. For example, a previous study has revealed that Wnt/β-catenin was aberrantly up-regulated coupled with a down-regulation of GSK3β in ESCC tissues [12]. Our recent study has further demonstrated that ESCC tissues had a significantly higher expression of β-catenin than that of normal healthy controls [13].

Similar to other signaling pathways, Wnt/β-catenin activation is also tightly regulated at multiple molecular levels, which can be exemplified by the tripartite motif-containing (TRIM) family proteins [14]. TRIM family proteins are characterized by a domain containing three zinc-binding regions, one or two B-boxes, one RING finger combined with a coiled-coil domain [14]. Numerous studies have uncovered that TRIM family proteins may contribute to varied types of physiological and pathophysiological processes, such as cell proliferation, invasion, or apoptosis [15–17]. For example, TRIM29 can stimulate Dvl2 to inhibit the activity of GSK3β, thereby resulting in activation of Wnt/β-catenin signaling in pancreatic cancer [16, 18]. In addition, interactions of Wnt/β-catenin and other TRIM proteins, including TRIM28 and TRIM33, have also been implicated with a role that contributes to pathogenesis of human cancers [19, 20]. Our recent results showing an association between a down-regulation of TRIM36 coupled with a high expression of β-catenin in clinical specimens and poor prognosis in ESCC patients further supported a role by TRIM proteins in contributing to human carcinogenesis [13].

In this study, we sought to examine the functional role of TRIM36 in inhibition of Wnt/β-catenin signaling in ESCC with the use of human ESCC cell lines as an in vitro model, and we further evaluated these results in vivo based on a xenograft mouse model.

Materials and methods

Cell culture

Five esophageal cancer cell lines (TE-1, TE-10, TE-11, KYSE140 and KYSE510), and human esophageal epithelial cell line (HEEC) were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). The cells were cultured with DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin, at 37 °C in a humidified atmosphere with 5% CO_2 as previously described [21].

Cell treatment

Short hairpin RNA (shRNA) oligos targeting TRIM36 (shTRIM36-1, 5′-GCATGCAAGGAGCTGTTTA-3′; shTRIM36-2, 5′-GCAGCTCCACCTCAGAATA-3′; and shTRIM36-3, 5′-GGTTCAATCTGTAGTCCTT-3′) were constructed into pLKO.1 (Addgene, USA) as previously described [22]. In addition, the full-length of human TRIM36 coding sequence and β-catenin coding sequence, lentivirus of shTRIM36, control shRNA (shNC), pLVX-TRIM36 (oeTRIM36) pLVX-β-catenin (oeβ-catenin) or pLVX-puro (Vector) were all generated following the procedures as detailed previously [22]. TE-11 and TE-10 cells were infected with lentivirus for TRIM36 overexpression. KYSE510 cells with infected with lentivirus with shRNA for TRIM36 gene silencing. TE-11 cells were also simultaneously infected with lentivirus for TRIM36 and β-catenin overexpression.

Preparation of total lysates, cytosolic fraction and nuclear extracts, and Western blot analysis

Whole cell lysates, and cytosolic and nuclear extracts were prepared with radioimmunoprecipitation buffer containing proteinase inhibitor (Beyotime) and NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific), respectively, as previously described [23, 24]. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Millipore, Billerica, WI, USA). Western blot analysis was performed with primary antibodies (Table S1) as per the manufacturer's instructions. The signal was detected with enhanced chemiluminescence system (ECL) (Millipore).

Cell proliferation assay

Cell proliferation was determined using Cell Counting Kit-8 (Dojindo Laboratories, Japan) as previously described [25]. Cell viability was assessed by the absorbance at 450 nm.

Evaluation of cell cycle distribution and apoptosis by flow cytometry

We assessed cell cycle distribution and apoptosis as previously described [26]. Briefly, cells were harvested, and washed with PBS. For evaluation of cell cycle distribution, the cells were fixed in ice-cold ethanol, and stained with propidium iodine (PI). For cell apoptosis analysis, the cells were stained with Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kits (KeyGEN Biotech, China). DNA content and cell apoptosis was determined on a flow cytometer (BD Biosciences, USA).

TOP/FOP flash assay

TE-11 and TE-10 cells infected with lentivirus for TRIM36 overexpression, and KYSE510 cells infected with lentivirus with shRNA for TRIM36 gene silencing, were transfected with TOP/FOP Flash (Beyotime, Shanghai, China). The Firefly Luciferase Reporter Gene Assay Kit (Beyotime) was applied at 48 h after transfection to monitor the luciferase activity of TOP Flash or FOP Flash. The TCF reporter plasmid/Mutant TCF binding sites (TOP/FOP) ratio was then calculated to assess the activity of Wnt/β-catenin pathway.

Coimmunoprecipitation (Co-IP) assay

Cell lysates were immunoprecipitated with anti-TRIM36 (Santa Cruz Biotech, USA, Sc-100881), anti-β-catenin (Abcam, Ab16051) or control IgG (Santa Cruz Biotech, sc-2027) for 1 h at 4 °C, which was followed by protein A/G-agarose beads (150 µg protein A) for 3 h at 4 °C. Immunoprecipitates were then subjected to Western blot analysis.

Ubiquitination analysis

Plasmids overexpressing Myc-TRIM36, His-ubiquitin and FLAG-β-catenin (Wild-type, K19R, K49R or K625R) were constructed with pCMV-c-Myc vector (Beyotime), pCMV-C-His vector (Beyotime) and pCMV-Tag 2 (Addgene, USA), respectively, by Genwiz Company (Suzhou, China). We performed the ubiquitination assay as previously described [23]. In brief, the 293 T cells were transfected with plasmids overexpressing Myc-TRIM36, His-ubiquitin and FLAG-β-catenin. After 2 days, cells were harvested and lysased. Cell lysates were incubated with nickel nitrilotriacetic acid beads (Qiagen) at room temperature for 1 h. After washing, the immunoprecipitated proteins were analyzed by Western blotting analysis with use of anti-FLAG (Abcam) and anti-His antibodies (Abcam).

Xenograft mouse model

Animal experiments were approved by the Animal Care Committee of Shandong Provincial Public Health Clinical Center (Approval number: 2021XKYYEC-37) and followed the guidelines of the Animal Care Committee. A total of 20 male nude mice, aged 4 weeks (SLRC Laboratory Animal Co., China) were randomly placed into four groups ($n=5$ per group), and subcutaneously injected with TE-11 cells stably expressing Vector, TRIM36 (oeTRIM36), β-catenin (oeβ-catenin) or TRIM36 plus β-catenin (oeTRIM36 + oeβ-catenin) (5×10^6 cells per mouse). Tumor volume was calculated as previously described with following formula: volume = 1/2 × (largest diameter) × (smallest diameter)^2 [27]. After 33 days, the mice were sacrificed followed by xenografts recovery. The xenografts were processed to hematoxylin and eosin (HE) staining, TUNEL (TdT-mediated DUTP nick end labeling, Roche, USA) assays, and Western blotting assays.

Tissue specimens

This study was approved by Ethics Research Committee of Shandong Provincial Public Health Clinical Center (Approval number: 2021XKYYEC-37). Twenty patients who underwent surgical resection at Shandong Province Chest Hospital were enrolled in this study. Written informed consents were gathered from all the participants before the study. A total of 20 pairs of ESCC and normal esophageal mucosa samples were collected, and stored at − 80 °C.

Statistical analysis

Statistical analysis was carried out using Graphpad Prism software (version 6.0, San Diego, CA, USA). For statistical evaluation of two groups or more, Student's *t* test (two-tailed) and one-way analysis of variance (ANOVA) were used, respectively. In vitro experiments were conducted three times. *P* values less than 0.05 were considered significant.

Results

TRIM36 inhibits proliferation of ESCC cells

To explore the correlation between TRIM36 expression levels and cell proliferation in ESCC, we first used Western blot to assess TRIM36 protein abundance in four human ESCC cell lines including TE-1, TE-10, TE-11, KYSE140 and KYSE510 with human esophageal epithelial cell line (HEEC) as a control (Fig. 1A). TE-10 and -11 cell lines were selected for further overexpression study because of their relatively lower levels of TRIM36 compared to others (Fig. 1B). In Fig. 1C, D, we found that an overexpression of TRIM36 led to a significant inhibition of cell proliferation in TE-10 and TE-11 cell lines. Because KYSE510 cell line showed a relatively higher level of TRIM36, we then chose it for gene silencing study (Fig. 1D). We found that a depletion of TRIM36 significantly promoted cell proliferation in this ESCC cell line. These data suggest that TRIM36 possesses an

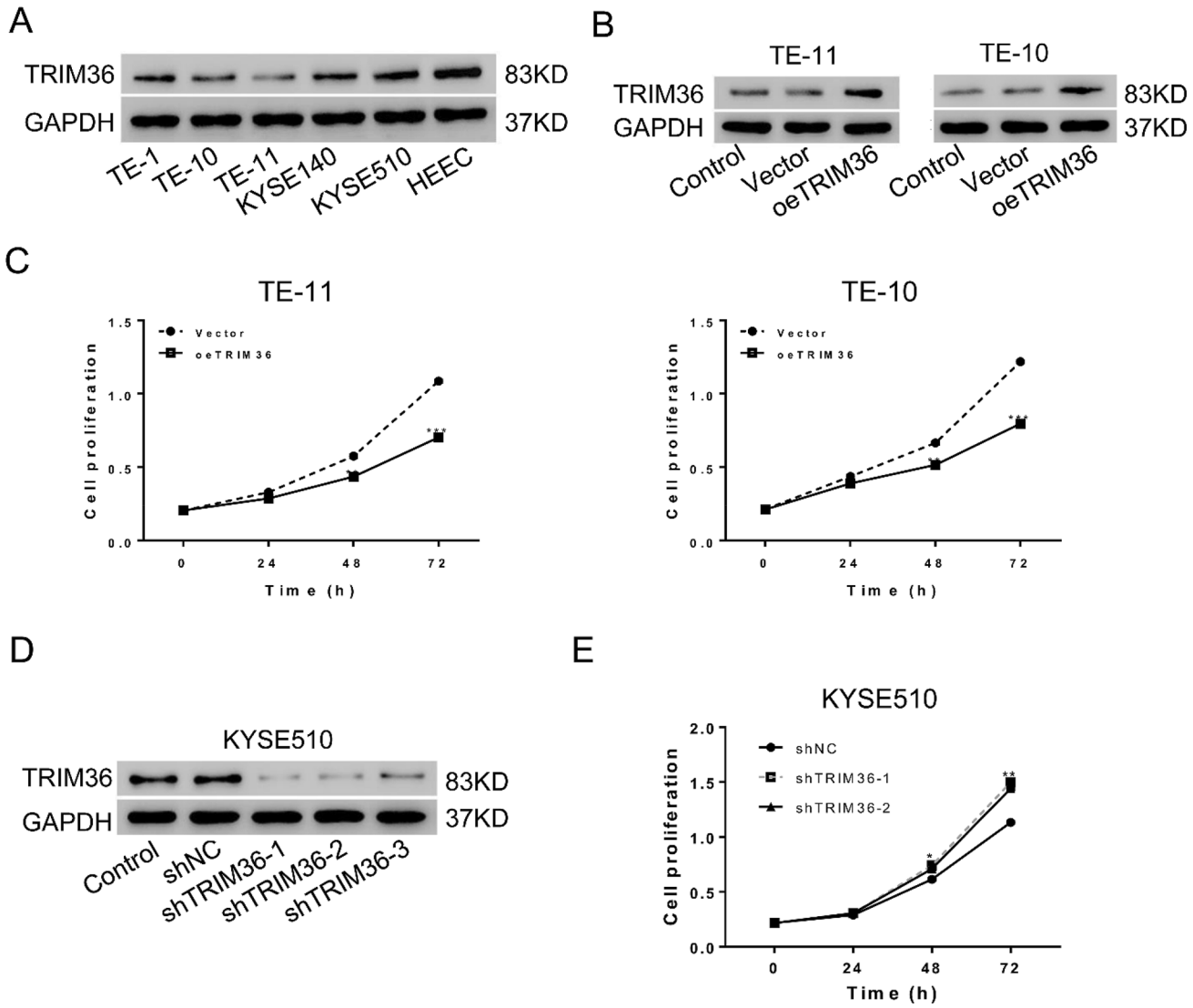


Fig. 1 TRIM36 expression affected the proliferation of ESCC cells. **A** TRIM36 protein expression in ESCC lines and a human esophageal epithelial cell line (HEEC) was detected by Western blot. **B**, **C** TE-11 and TE-10 cell lines were infected with lentivirus for TRIM36 overexpression (oeTRIM36) or Vector. Western blot experiment was performed to detect TRIM36 expression (**B**). CCK-8 assay was used to determine cell proliferation (**C**). $^{**}P<0.01$, $^{***}P<0.001$ vs Vector. **D**, **E** KYSE510 cell line was infected with lentivirus expressing shRNA for TRIM36 gene silencing (shTRIM36-1 or 2) or control shRNA (shNC). TRIM36 expression and cell proliferation was assessed by Western experiment (**D**) and CCK-8 assay (**E**), respectively. $^{**}P<0.01$, $^{***}P<0.001$ vs shNC

inhibitory effect on cell proliferation in human esophageal cancer cells.

TRIM36 induces cell cycle arrest and apoptosis in ESCC cells

Here, we used flow cytometry to analyze the effects of TRIM36 on cell cycle progression and apoptosis in human esophageal cancer cells. As shown in Fig. 2A, an overexpression of TRIM36 resulted in an increase in the ratio of G0 to G1 phase cells coupled with a significant decrease in S phase and G2/M ratio in TE-10 and TE-11 cell lines. However, a silencing of TRIM36 in KYSE510 cells significantly enhanced the abundance of S phase cells (Fig. 2B). Furthermore, we found that an increased TRIM36 promoted cell apoptosis as evidenced by significantly reduced viable TE-10 and 11 cells (Fig. 2C). As expected, a decreased expression of TRIM36 significantly inhibited apoptosis in KYSE510 cells (Fig. 2D).

Our Gene Set Enrichment Analysis (GSEA) analysis in clinical ESCC specimens showed a negative correlation between TRIM36 and β-catenin (Fig. S1). We then determined the effects of TRIM36 on expression of β-catenin and three major factors involved in regulation of cell cycle progression and apoptosis including Survivin, cyclin D1, and c-Myc [28] in human esophageal cancer cells. In Fig. 2E, F, we found that an overexpression of TRIM36 inhibited nuclear accumulation of β-catenin, but its depletion had an opposite effect. The TOP/FOP-Flash reporter activity was significantly decreased in TRIM36-overexpressed cells, but remarkably increased in TRIM36-knockdown cells (Fig. 2G, H). Moreover, the same results were observed for the changes of protein levels of Survivin, cyclin D1, and c-Myc in response to TRIM36 overexpression or inhibition (Fig. 2I, J). These data suggest that TRIM36-induced cell cycle arrest and apoptosis in ESCC are likely dependent on repression of the activity of β-catenin.

TRIM36 promotes ubiquitination of β-catenin in human esophageal cancer cells

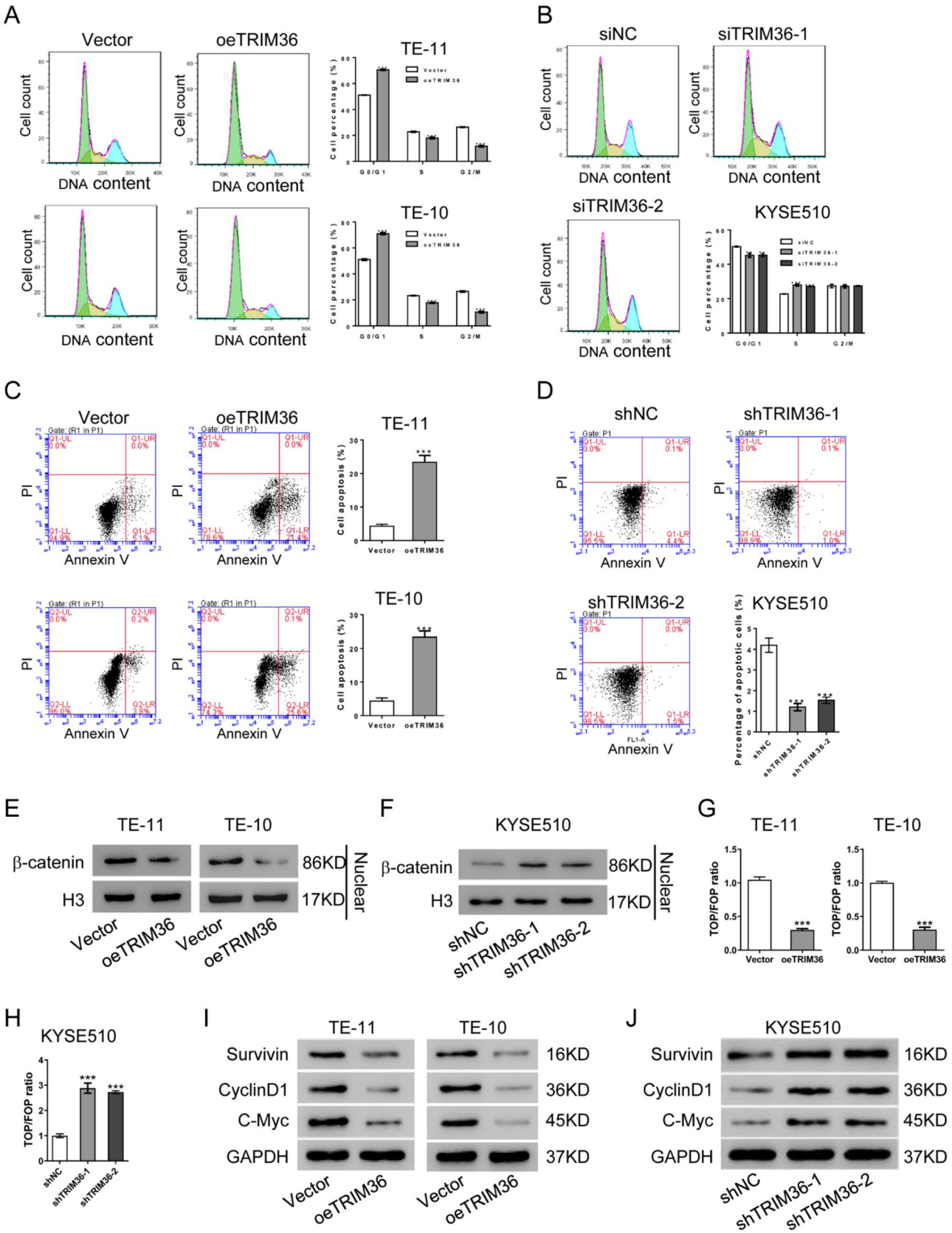
β-Catenin can be inactivated by its ubiquitination and subsequent degradation [29]. To test whether TRIM36 is able to ubiquitinate β-catenin in human esophageal cancer cells, we first used co-immunoprecipitation assay to examine the interaction between them. In Fig. 3A, we found that the use of TRIM36 antibody led to an efficient pull-down of β-catenin in TE-11 cells; conversely, β-catenin antibody also efficiently captured TRIM36. In addition, administration of proteasome inhibitor, MG132, abolished the effects of TRIM36 overexpression in β-catenin protein level (Fig. 3B), which suggested the involvement of proteasome. In addition, an overexpression of TRIM36 in this cell line resulted in a remarkable increase in ubiquitination levels of β-catenin (Fig. 3B). To further explore the ubiquitination sites in β-catenin protein, we created three mutant versions of β-catenin protein: K19R, K49R, and K625R. HEK293T cells were transfected with plasmids for overexpression of His-tagged ubiquitin along with those for overexpression of TRIM36 or FLAG-tagged mutant β-catenin protein as indicated. As shown in Fig. 3C, the overexpression of TRIM36 increased the ubiquitination levels on β-catenin of wild-type, K19R, or K49R, but had no effects on the ubiquitination of mutant β-catenin K625R. These data suggest that TRIM36 interacts with β-catenin to promote its ubiquitination at lysine 625 in human esophageal cancer cells.

TRIM36-induced cell proliferation inhibition is dependent on its inhibition of β-catenin activity in ESCC

We sought to further examine whether TRIM36 inhibits cell proliferation by its inhibitory effects on β-catenin activity in ESCC cells. ESCC cells were treated with XAV939, a potent and selective Wnt/β-catenin signaling inhibitor [30], along with shRNAs targeting for TRIM36 silencing or overexpression plasmids for an ectopic expression of TRIM36 or β-catenin as indicated. Figure 4A–C shows that the inhibition of β-catenin activity reversed TRIM36 depletion-mediated effects of enhancing cell proliferation. However, Fig. 4D–F reveals that the overexpression of β-catenin abolished effects of the overexpressed TRIM36-induced effects of cell cycle arrest. Of note, the overexpression of β-catenin in TE-11 cells was further confirmed by Western blot (Fig. S2). These data further support that TRIM36 inhibits β-catenin activity to lead to cell proliferation inhibition in human esophageal cancer cells.

TRIM36 prevents proliferation and promotes apoptosis of ESCC in vivo

Here we used a xenograft mouse model to explore the role of TRIM36 in regulating cell growth and apoptosis of ESCC in vivo. Male nude mice were subcutaneously injected with TE-11 cells along with vectors for a stable overexpression of TRIM36, β-catenin or a combination of them. Then, mice were followed by monitoring of tumor size at an interval of every 3 days. The data of Fig. 5A–C


A
Vector
oeTRIM36
TE-11
TE-10
Cell count
DNA content
Cell percentage (%)
G 0/G 1
S
G 2/M
B
siNC
siTRIM36-1
siTRIM36-2
KYSE510
C
Vector
oeTRIM36
TE-11
TE-10
PI
Annexin V
Cell apoptosis (%)
D
shNC
shTRIM36-1
shTRIM36-2
KYSE510
Percentage of apoptotic cells (%)
E
TE-11
TE-10
β-catenin
H3
86KD
17KD
Nuclear
F
KYSE510
G
TE-11
TE-10
TOP/FOP ratio
H
KYSE510
I
TE-11
TE-10
Survivin
CyclinD1
C-Myc
GAPDH
16KD
36KD
45KD
37KD
J
KYSE510

◂**Fig. 2** TRIM36 expression affected the cell cycle progression and cell apoptosis of ESCC cells. **A**, **B** TE-11 and TE-10 cell lines were infected with lentivirus for TRIM36 overexpression (oeTRIM36) or Vector (**A**), KYSE510 cell line was infected with lentivirus expressing shRNA for TRIM36 gene silencing (shTRIM36-1 or 2) or control shRNA (shNC, **B**), and cell cycle distribution was analyzed by propidium iodine (PI) staining and flow cytometry analysis. **C**, **D** TE-11 and TE-10 cell lines were infected with lentivirus expressing oeTRIM36 or Vector (**C**), KYSE510 cell line was infected with lentivirus expressing shTRIM36-1, shTRIM36-2 or shNC (**D**), and cell apoptosis was analyzed by Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kits and a flow cytometer. **E**, **F** TRIM36 expression affected β-catenin in nuclear fraction. Nuclear extracts were prepared from indicated cells, and β-catenin expression was detected by Western blot with Histone H3 as loading control. **G, H** TRIM36 expression affected the activity of the Wnt/β-catenin pathway as indicated by the TOP/FOP reporter assay. **I, J** TRIM36 expression affected the expression of Survivin, Cyclin D1 and c-myc as indicated by Western blot analysis. $^{**}P < 0.01$, $^{***}P < 0.001$

revealed that while an overexpression of β-catenin significantly enhanced tumor growth compared with empty vector but an overexpressed TRIM36 had totally opposite effects. Furthermore, an elevated TRIM36 significantly reversed β-catenin-induced expansion of tumor volume. In consistent with the in vitro results, furthermore, we found here that: (1) increased TRIM36-induced cell apoptosis; (2) overexpression of β-catenin led to a significant inhibition of apoptosis; (3) Overexpressed β-catenin-mediated inhibition of apoptosis was reversed because of increased expression of TRIM36; and (4) overexpressed TRIM36 efficiently quenched nuclear accumulation of β-catenin (Fig. 5D–G). These in vivo data further support the hypothesis that TRIM36 represses β-catenin signaling pathway to inhibit cell proliferation and promote apoptosis of ESCC.

Downregulation of TRIM36 but upregulation of β-catenin in clinical samples of ESCC

Finally, we used Western blot analysis to measure the protein levels of cytoplasmic TRIM36 and nuclear β-catenin in clinical samples post-operatively obtained from patients of ESCC (Fig. 6A). A quantitative analysis indicated that TRIM36 and nuclear β-catenin showed a significantly lower

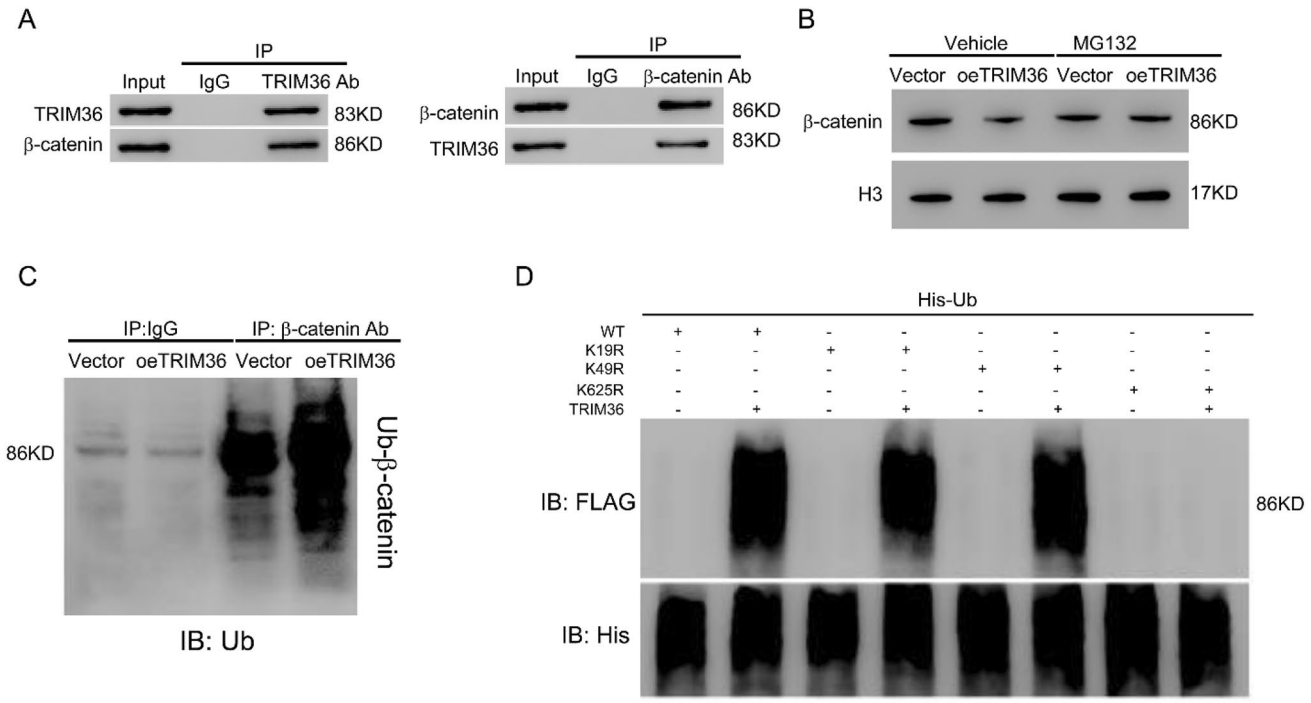
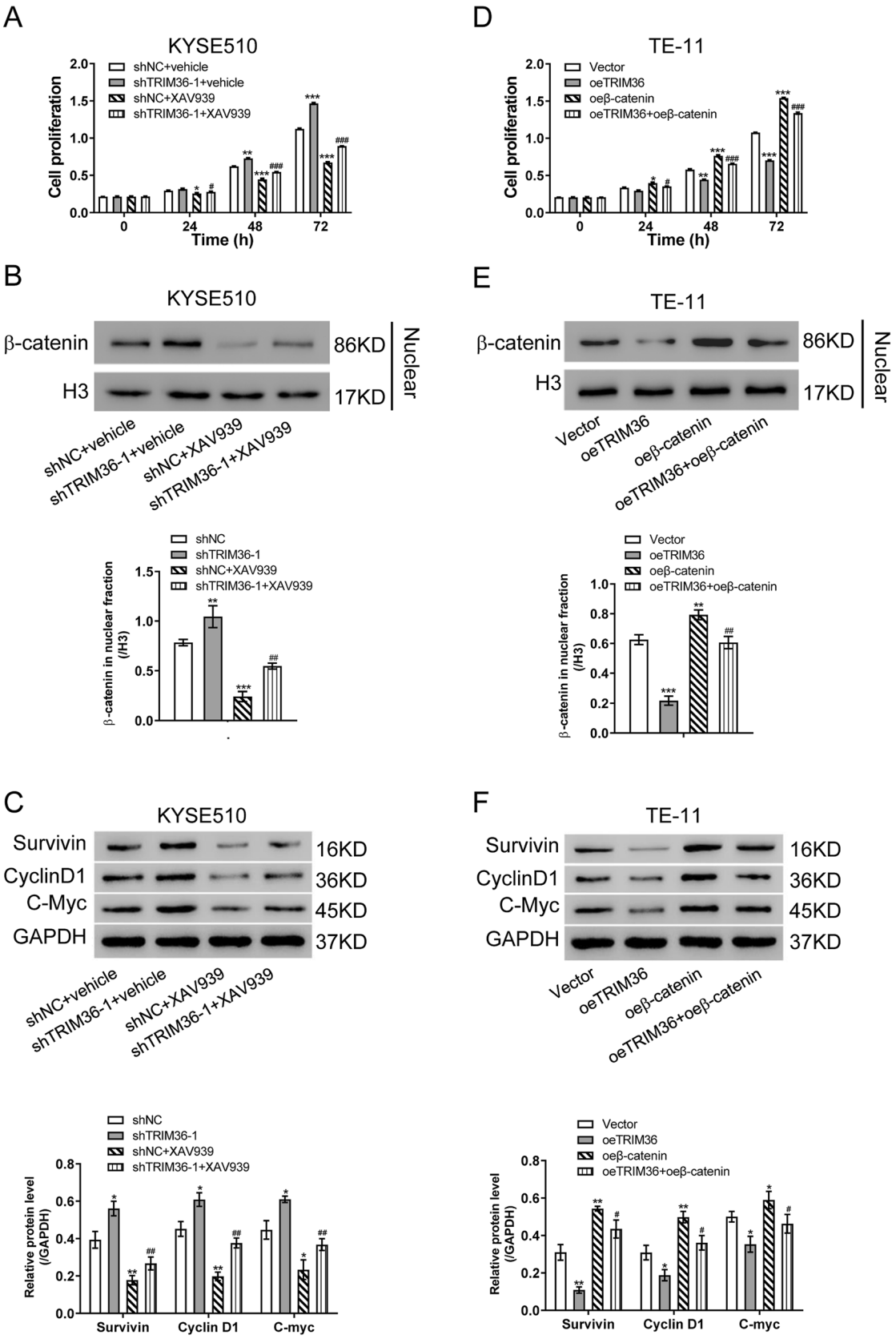


Fig. 3 TRIM36 interacted with β-catenin to promote ubiquitination. **A** Cell lysates of TE-11 cells were immunoprecipitated (IP) with anti-TRIM36, anti-β-catenin or control IgG, and then subjected to Western blot analysis with antibodies against TRIM36 or β-catenin. **B** TE-11 cells overexpressed with TRIM36 were treated with DMSO or 10 μM MG132 for 4 h, nuclear extracts were prepared, and β-catenin in nuclear fraction was detected. **C** TE-11 cells overexpressed with TRIM36 were subjected to IP assay with anti-β-catenin or control IgG, and β-catenin ubiquitination was detected by Western blot analysis with anti-Ubiquitin. **D** Ubiquitination assay was performed in HEK293T cells transfected with plasmids expressing myc-TRIM36, His-ubiquitin and FLAG-β-catenin (WT, K19R, K49R or K625R). Cell lysates were incubated with nickel nitrilotriacetic acid beads and subjected to Western blotting assay with anti-FLAG and anti-His antibodies


A
KYSE510
shNC+vehicle
shTRIM36-1+vehicle
shNC+XAV939
shTRIM36-1+XAV939
Cell proliferation
Time (h)
D
TE-11
Vector
oeTRIM36
oeβ-catenin
oeTRIM36+oeβ-catenin
B
KYSE510
β-catenin
H3
86KD
17KD
Nuclear
shNC+vehicle
shTRIM36-1+vehicle
shNC+XAV939
shTRIM36-1+XAV939
β-catenin in nuclear fraction (/H3)
E
TE-11
C
KYSE510
Survivin
CyclinD1
C-Myc
GAPDH
16KD
36KD
45KD
37KD
Relative protein level (/GAPDH)
Survivin
Cyclin D1
C-myc
F
TE-11

◂**Fig. 4** Wnt/β-catenin mediated the effects of TRIM36 in ESCC cells. **A**–**C** KYSE5#10 cells infected with lentivirus expressing shTRIM36-1 or shNC were treated with XAV939/vehicle (DMSO). Cell proliferation was assessed by CCK-8 assay (**A**). β-catenin in nuclear fraction (**B**), and expression of Survivin, Cyclin D1 and c-myc (**C**) was detected by Western blot analysis. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs shNC + vehicle; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $P<0.001$ vs shTRIM36-1 + vehicle. **D**–**F** TE-11 cells were overexpressed with TRIM36 (oeTRIM36), β-catenin (oe β-catenin) or TRIM36 plus β-catenin (oeTRIM36 + oeβ-catenin). Cell proliferation was assessed by CCK-8 assay (**D**). β-catenin in nuclear fraction (**E**), and expression of Survivin, Cyclin D1 and c-myc (**F**) was detected by Western blot analysis. Representative images and quantification results of Western blot assays are shown. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs Vector; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs oeTRIM36

or high levels in cancerous tissues compared to adjacent normal tissues, respectively (Fig. 6B). These data were consistent with the results observed in vitro and in vivo as described above.

Discussion

We have recently reported that that TRIM36 expression was significantly down-regulated at both mRNA and protein levels in ESCC tissues compared to normal ones [13]. We further found that the protein expression levels of TRIM36 were negatively associated with tumor size, tumor staging, distant metastasis, and vital status. More importantly, the cumulative survival length of patients with a lower expression of TRIM36 was considerably reduced in comparison with those of patients showing higher expressions of TRIM36. In this continued investigation, we focused on characterization of the molecular mechanisms underlying TRIM36-mediated apoptosis in ESCC. This study has offered evidence supporting that an increased TRIM36 induces cell cycle arrest and apoptosis in ESCC cells and tissues by promoting ubiquitination and degradation of β-catenin. Thus, the current study convincingly provides meaningful results for further identification of potential novel drug targets in treatment of ESCC.

Here, we have for the first time reported that a crosstalk between TRIM36 and β-catenin plays a critical role in cell cycle progression arrest and induction of apoptosis in ESCC. To date, there are at least 80 TRIM proteins discovered in humans, a majority of which are engaged in a wide range of biological and pathophysiological processes, including but not limited, regulation of transcription, cell growth, development and carcinogenesis [16, 17]. A previous study has shown that TRIM29 was positively correlated with the aberrant expression of β-catenin in squamous cancer cell of lung, intriguingly, which was not observed in lung adenocarcinoma [31]. In addition, a functional study by Wang and colleagues revealed that a relation between TRIM29 and β-catenin levels was observed in pancreatic cancer and oncogenic effects of TRIM29 largely depend on β-catenin function [32]. TRIM29 can associate with Dvl2 to inhibit the activity of GSK3β, thereby resulting in activation of Wnt/β-catenin signaling in pancreatic cancer [16, 18]. Furthermore, the correlation between β-catenin and other TRIM proteins including TRIM33, TRIM32, TRIM28, and TRIM44 has also been broadly investigated and found to molecularly account for varied types of carcinogenesis. In addition, interactions of Wnt/β-catenin and other TRIM proteins including TRIM28 and TRIM33 have also been implicated with a role in contributing to human tumorigenesis [19, 20]. Our data identified a role that TRIM36 protein plays and its interaction with β-catenin that contributes to the pathogenesis of ESCC.

Mechanistically, our study further supports that the inactivation of β-catenin is mainly dependent on TRIM36-induced its ubiquitination and subsequent degradation in ESCC. Apart from this study, our recent and other studies have shown that in ESCC tissues, the amount of β-catenin was significantly higher than in normal tissues [13, 33, 34]. The ubiquitination of β-catenin is a sophisticated process that can be either phosphorylation-dependent or independent. In addition, such a post-translational modification could occur in either the nucleus or cytoplasm [29]. At molecular levels, E3 ubiquitin ligases can recognize and ubiquitinate β-catenin, thus leading to its degradation in the proteasome. Our data are consistent with a previous study showing that TRIM36 possesses an intrinsic ubiquitin E3 ligase activity [35].

In summary, we report a TRIM36-β-catenin regulatory pathway likely contributing to cell cycle arrest and apoptosis in ESCC. Our results suggest that selectively controlling this pathway in human esophageal epithelial cells might constitute a novel therapeutic approach for controlling progression of ESCC.

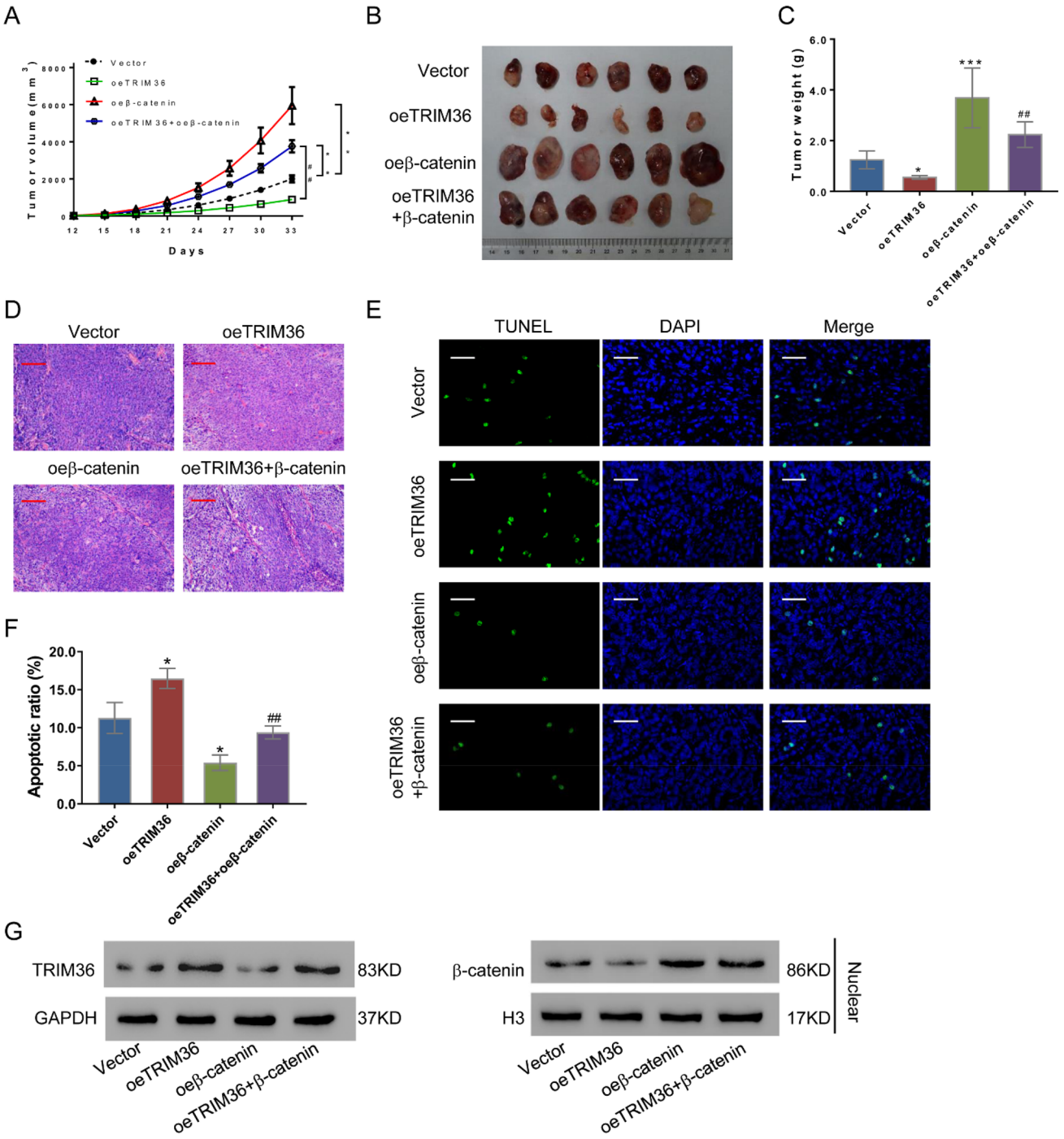


Fig. 5 β-catenin overexpression reversed the effects of TRIM36 overexpression in vivo. TE-11 cells stably expressing Vector, TRIM36 (oeTRIM36), β-catenin (oeβ-catenin), or TRIM36 plus β-catenin (oeTRIM36 + oeβ-catenin) were used to establish subcutaneous tumors in nude mice. Tumor volume (**A**) was measured every three days. 33 days after the treatment, the mice were sacrificed, and the tumors were resected (**B**) and weighed (**C**). Representative images of hematoxylin and eosin (HE)-stained tumor sections are showed (**D**, scale bar: 50 μm). **E**, **F** TUNEL assays were performed to analyze apoptosis (green), and the nuclei were stained with DAPI (bule). Representative images (**E**, scale bar: 50 μm) and quantitative analysis (**F**) are shown. **G** Western blot assays were performed for the detection of TRIM36 and β-catenin in nuclear fraction. $^{*}P < 0.05$, $^{**}P < 0.01$ vs Vector; $^{\#\#}P < 0.01$, vs oeTRIM36

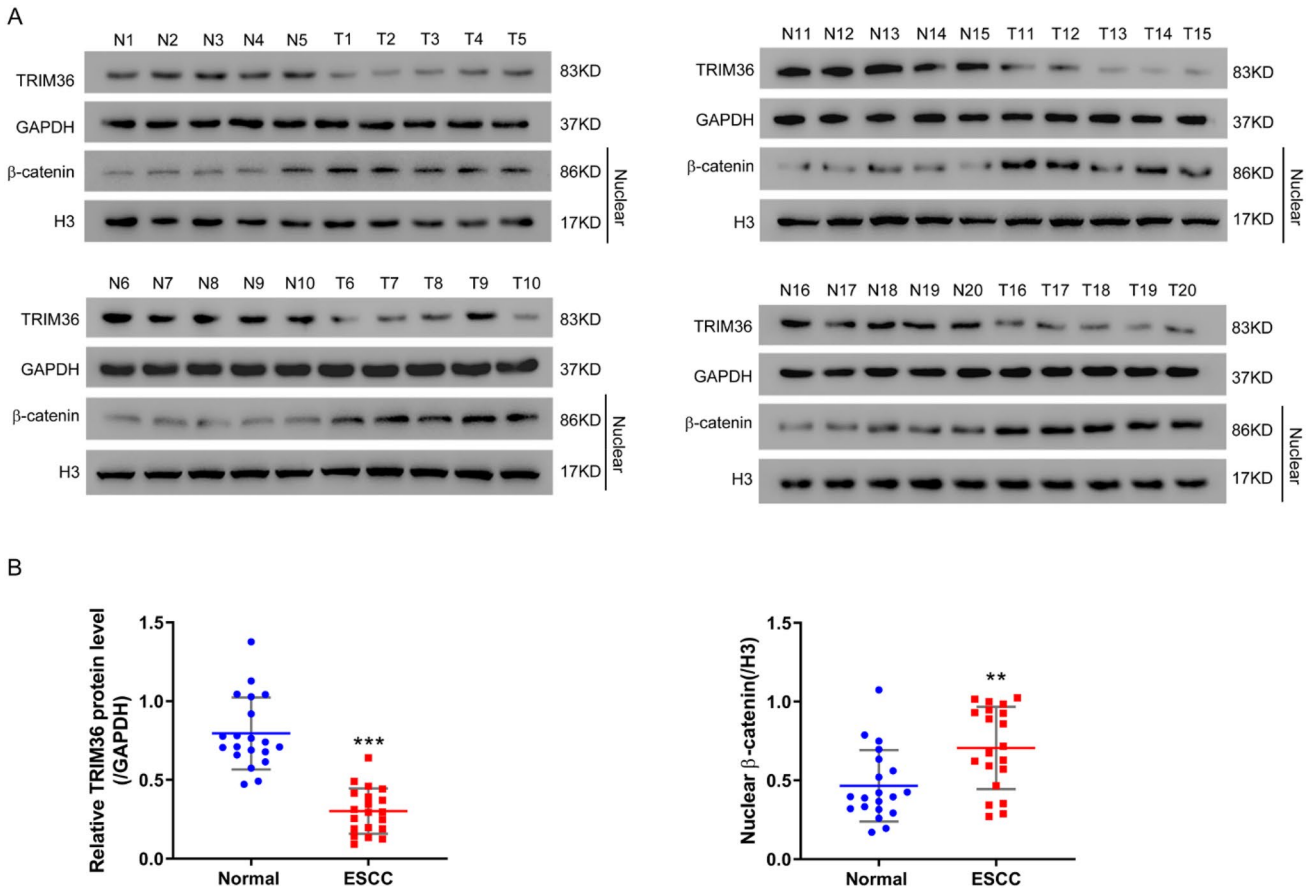


Fig. 6 TRIM36 protein expression and nuclear β-catenin levels in ESCC and normal tissues. Twenty paired sample sets of ESCC (T1–T20) and normal tissues (N1–N20) were obtained and Western blot analysis was used determine levels of TRIM36 and nuclear β-catenin. Images (**A**) and quantification results (**B**) of Western blot assays are shown. $^{**}P < 0.01$, $^{***}P < 0.001$

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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