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A novel oncogene TRIM63 promotes cell proliferation and migration via activating Wnt/β-catenin signaling pathway in breast cancer

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ABSTRACT

The development of breast cancer is still a relatively unclear biological process, and there is currently no consensus on the occurrence of breast cancer and the process of tumor metastases. This study was to reveal a correlation between TRIM63 and the development of breast cancer. In this study, we found that the expression of TRIM63 was significantly increased in breast cancer tissues and closely related to pathological differentiation and TNM stage of breast cancer. Overexpression of TRIM63 could significantly promote proliferation and migration of breast cancer cells, while TRIM63 knockdown significantly inhibited the proliferation and migration of breast cancer cells. In addition, TRIM63 could activate Wnt/β-catenin signaling pathway in breast cancer cells. Further study found that TRIM63 could regulate β-catenin degradation by promoting GSK3β phosphorylation. Our study revealed that TRIM63, as an oncogene, involved in breast cancer progression by activating the Wnt/β-catenin signaling pathway, suggesting that the potential applicability of TRIM63 as a target for breast cancer treatment.

1. Introduction

Breast cancer is one of the most malignant tumors with the highest morbidity and mortality in women [10]. The incidence of breast cancer is increasing year by year, and the age of onset is younger worldwide [9,11]. There are nearly 1.4 million new cases of breast cancer every year worldwide, meanwhile, about 460,000 patients died of breast cancer and its complications [20,27], which seriously threatened women's health and quality of life, leading to enormous economic and health pressures to society. Therefore, it is the responsibility of all oncologists to explore the mechanism of breast cancer development and find new targets for breast cancer treatment.

The tripartite motif (TRIM) family proteins have similar structure, which include a RING domain, one or two B-box domains and a coiled-coil domain [7,29]. So far, the human TRIM family has been found to include more than 80 members [31]. A large number of evidences have shown that TRIM proteins play an important role in innate immune responses, cell proliferation, apoptosis and autophagy [7,15]. In addition, studies have found that TRIM family proteins are closely related to the pathogenesis of many diseases, including cancer [12,22]. For example, TRIM44 induces melanoma progression by activating TLR4 to activate the AKT/mTOR signaling pathway [36]. TRIM52 may play a carcinogenic role in CRC by regulating the STAT3 signaling pathway [23]. TRIM29 promotes epithelial-mesenchymal transition and progression of colorectal cancer by activating the Wnt/β-catenin signaling pathway [28]. TRIM11 is up-regulated in prostate cancer, which promotes proliferation of prostate cancer cells [24]. TRIM59 is up-regulated in many tumors such as breast cancer, lung cancer, bladder cancer, kidney cancer, prostate cancer, gastric cancer and ovarian cancer, and is positively correlated with poor prognosis [5]. TRIM14 is up-regulated in hepatocellular carcinoma (HCC) [6], osteosarcoma [37], oral squamous cell carcinoma (OSCC) [33], tongue squamous cell carcinoma (TSCC) [34], breast cancer [14] and glioma [30], which promotes tumor cell proliferation, migration, invasion and chemotherapy resistance. Other family members, such as TRIM2, TRIM15, TRIM17, TRIM23, TRIM24, TRIM28, TRIM36, TRIM52,

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TRIM65, TRIM66, etc., are also closely related to the development of tumors [13,35]. TRIM63 gene is also called MuRF1, which plays an important role in the atrophy of skeletal muscle and myocardium [2], and is required for the degradation of myosin heavy chain protein, myosin light chain, myosin binding protein and muscle creatine kinase [16]. Current studies have shown that upregulated TRIM63 mainly lead to the development of muscle tissue diseases [25]. However, no studies have yet revealed a correlation between TRIM63 and the development of tumors.

In this study, we first explored the association between TRIM63 and the development of breast cancer. We found that the expression of TRIM63 mRNA and protein was significantly increased in breast cancer tissues. In breast cancer cells, TRIM63 can promote the proliferation and migration of breast cancer cells, and its mechanism may be related to Wnt/β-catenin signaling pathway. Our study further reveals the important role of TRIM family proteins in the development of tumorigenesis, and provides a new strategy for targeted therapy of breast cancer.

2. Materials and methods

2.1. Cell culture and tissue microarray

The cell lines used for this study included the human breast cell MCF-10A and breast cancer cell MCF-7, MDA-MB-231, and human epithelial cell line HEK293 T. The cell lines MCF-10A (ATCC® CRL-10317™), MCF-7 (ATCC® HTB-22™), MDA-MB-231 (ATCC® HTB-26™), HEK293 T(ATCC® CRL-11268G-1) were purchased from the China Center for Type Culture Collection (CCTCC, Chinese Academy of Sciences, Shanghai, China). MCF-7, MDA-MD-231 and HEK293 T cells were cultured in medium (DMEM, HyClone, USA, SH30022.01B) supplemented with 10% fetal bovine serum (FBS) (Gibco, Milano, Italy, 10099-141). MCF-10A cells were cultured in Dulbecco's Modifies Eagle Medium (DMEM/F-12; GIBCO, Milano, Italy, 12,400,024) with F-12 supplemented with 5% horse serum, 20 ng/mL EGF, 100 ng/mL cholera toxin, 0.5 mg/mL hydrocortisone and 10 mg/mL insulin.

Breast cancer tissues and cancer adjacent normal tissues were collected from Affiliated Zhongnan Hospital of Wuhan University and diagnosed by the Department of Pathology. All patients were informed and agreed. Our research was approved by the Ethics Board of School of Basic Medical Sciences, Wuhan University and was based on all relevant principles of the Declaration of Helsinki. The human breast cancer tissue microarray (the TMA ID: BC08118a) was purchased from Alenabio company (Xi'an, China).

2.2. Plasmid, siRNA and cell transfection

The TRIM63 overexpression plasmid (flag-TRIM63) and its control plasmid (flag-NC) used in this study were preserved in our laboratory and confirmed by DNA sequencing. The siRNAs of TRIM63 were synthesized by Genepharma Company. The RNA oligos containing 21 nucleotides were synthesized in sense and anti-sense directions corresponding to human TRIM63 (Genbank accession number NM_032588.3) siRNA1 at nucleotides 574 bp (sense: 5′- GCAAGGUGUUUGGGAUC CACA -3′ and anti-sense: 5′- UGUGGAUCCCAAACACCUUGC-3′), siRNA2 at nucleotides 217 bp (sense:5′- GCCUGGAGAUGUUUACCA AGC-3′ and anti-sense: 5′- GCUUGGUAAACAUCUCCAGGC-3′), and siRNA3 at nucleotides 994 bp (sense: 5′- GCAUUGUGGAAGCUUCCA AGG-3′ and anti-sense: 5′−CCUUGGAAGCUUCCACAAUGC-3′) with dTdT overhangs at each 3′ terminus. One negative siRNA control (sense 5′-UUCUCCGAACGUGUCACGU-3′ and anti-sense 5′-ACGUGACACGUU CGGAGAA-3′) was supplied. Each siRNA was verified to confirm the efficiency of knockdown. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen Co., Ltd.). The process of cell transfection is referred to our previous article. siRNA1, siRNA2 and siRNA3 were mixed together to serve as siRNA for TRIM63.

2.3. RT-qPCR

RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the instructions, and then RNA was reverse transcribed into a complementary cDNA using GeneAmpTM RNA PCR Core Kit (Thermo Scientific, USA). cDNA was used as a template to detect the expression level of the target gene by SYBR Premix Ex Taq II kit (Takara, Japan). The primers for mRNA analysis for qPCR are TRIM63: forward primer 5′- CTTCCAGGCTGCAAATCCCTA-3′ and reverse primer 5′- ACACTCCGTGACGATCCATGA-3′, GAPDH: forward primer 5′-GGT GAAGGTCGGAGTCAACG-3′, reverse primer 5′−CCATGTAGT TGAGG TCAATGA AG-3′.

2.4. Western blotting

The protein was extracted with RIPA lysis buffer (biosharp, BL504A), and quantified with BCA Protein Assay Kit (Beyotime Biotechnology Co, Jiangsu, China, P0010S). The process of Western blotting is referred to our previous article [18]. The antibodies used in this study include TRIM63 (Proteintech, USA), flag (Abclonal, China), GSK3β (Proteintech, USA), p-GSK3β (Cell Signaling Technology, USA),□β-catenin (Proteintech, USA), p-β-catenin(S33/S37) (Cell Signaling Technology, USA), c-myc (Proteintech, USA), and GAPDH (Proteintech, USA),and the secondary antibodies (Proteintech, USA).

2.5. Immunohistochemistry

Immunohistochemistry of breast cancer tissue microarrays was used to detect the expression level of TRIM63 protein. The process of immunohistochemistry is referred to our previous article [19]. The Pannoramic MIDI automatic digital slide scanner (3DHISTECH Ltd., Budapest, HUNGARY) was used for image collection. Quantification of TRIM63 expression levels was detected by IHC profiler in ImageJ [32].

2.6. TOP/FOP flash

The process is referred to our previous article [19]. The TOP plasmid and the FOP plasmid, as well as the plasmid of the target gene, were transfected into HEK293 T cells, and the cells were collected 48 h later, and then the reporter gene activity was detected using a Dual Luciferase Reporter Assay Kit (Promega).

2.7. Detection of cell proliferation and migration

Colony formation assay and CCK-8 assay were used to detect the ability of cell proliferation. For the colony formation assay, cells (2×10^2 cells/well) were seeded in 6-well plates. About one week later, the cells were fixed 20 min with 4% paraformaldehyde and stained 30 min with 5% crystal violet, then counted the colony number under a camera. For the CCK-8 assay, cells (3×10^3 cells/well) in the logarithmic growth phase were cultured in 96-well plates and incubated for 0–48 hours. After the treatment, 10 μl CCK-8 (Cell counting kit-8, Dojindo Laboratories, China) was added and continued to culture 2 h. The value of OD450 was measured by microplated reader (BD, USA).

Transwell assay was used to detect the ability of cell migration. 200 cells were seeded in upper transwell chamber insert (Corning, USA). The upper transwell chamber insert contained culture medium without FBS, while the lower chamber with complete culture medium containing 10% FBS. After 24–36 hours, migrated cells were stained and counted by crystal violet staining. Five random fields were counted per experiment.

2.8. Statistical analysis

All experiments were repeated at least three times and representative images are shown. The two-tailed Student's *t*-tests were

used for comparisons between two groups. The significance of relationship between expression of TRIM63 and clinicopathologic features in breast cancer patients were determined by χ^2-tests. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. The expression level of TRIM63 is increased in breast cancer, which is closely related to the clinicopathologic feature of breast cancer

To investigate the expression levels of TRIM63 in breast cancer tissues and normal breast tissues, we collected 20 pairs of breast cancer tissues and their matched paracancerous tissues, and then found that the mRNA expression level of TRIM63 was significantly increased in 16 cases of breast cancer tissues, which was detected by RT-qPCR (Fig. 1A). Furthermore, we found that the protein expression levels of TRIM63 in breast cancer tissues were significantly higher than that in normal breast tissues by immunohistochemistry of breast cancer tissue microarray (Fig. 1B). In addition, we found that the protein expression level of TRIM63 was negatively correlated with the pathological differentiation of breast cancer (Fig. 1C), while positively correlated with the TNM stage of breast cancer (Fig. 1D). These results indicated that TRIM63 was upregulated during the development of breast cancer.

3.2. TRIM63 promotes cell proliferation and migration in breast cancer

We have found that the expression level of TRIM63 is significantly up-regulated during the development of breast cancer. Does the dysfunction of TRIM63 have an effect on the biological behavior of breast cancer cells? We first examined the mRNA and protein expression levels of TRIM63 in normal breast cell MCF-10A, as well as in breast cancer cells MCF-7 and MDA-MB-231. The results showed that the mRNA and protein expression levels of TRIM63 were significantly increased in MCF-7 and MDA-MB-231 cells relative to MCF-10A cells, while even higher in more malignant MDA-MB-231 cells than MCF-7 cells with lower malignancy (Fig. 2A). We then used gain- and lose-of-expression strategy to study the function of TRIM63 in breast cancer cells (Fig. 2B). In breast cancer MCF-7 cells, overexpression of TRIM63 significantly enhanced the cell proliferation (Fig. 2C, E). In contrast, knockdown of TRIM63 significantly inhibited proliferation of MDA-MB-231 cells (Fig. 2D, F). In breast cancer cell MCF-7, overexpression of TRIM63 significantly enhanced cell migration (Fig. 2G), while knockdown of TRIM63 significantly inhibited migration of MDA-MB-231 cells (Fig. 2H). These results indicated that TRIM63 acted as an oncogene to promote proliferation and migration of breast cancer cells.

3.3. TRIM63 activates Wnt/β-catenin signaling pathway in breast cancer cells

Some studies have found that TRIM family proteins are closely related to the Wnt/β-catenin signaling pathway [3,8,28,39]. Overactivation of the Wnt/β-catenin signaling pathway promotes uncontrolled proliferation of cells, leading to tumorigenesis and progression. However, the correlation between TRIM63 and Wnt/β-

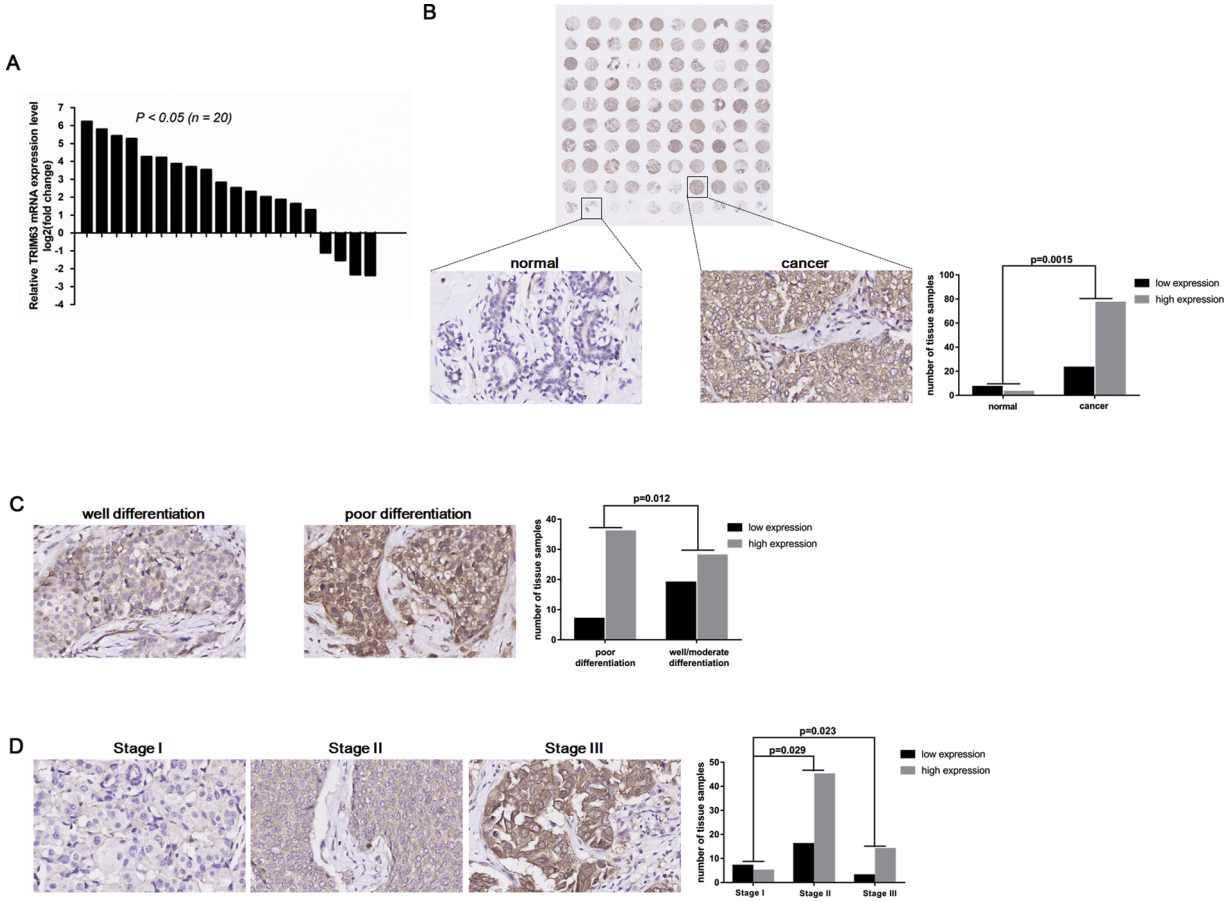

Fig. 1. TRIM63 is significantly upregulated in human breast cancer tissues and cells. ***A,*** TRIM63 mRNA expression was upregulated in breast cancer tissues (16/20) than paired normal breast tissues which was analyzed by RT-qPCR. ***B,*** Compared to normal breast tissues, TRIM63 protein expression was increased in breast cancer, which was detected by immunohistochemistry. ***C,*** The protein levels of TRIM63 in poor differentiation of breast cancer tissues were higher than that in well differentiation of breast cancer tissues. ***D,*** The protein levels of TRIM63 were higher in stage II and stage III than that in stage I, according to TNM staging of breast cancer. Values represent the mean ± SD from three independent measurements. *P < 0.05, **P < 0.01, ***P < 0.001.

catenin signaling pathway is unclear. In breast cancer cell MCF-7, overexpression of TRIM63 significantly up-regulated the protein expression levels of β-catenin, p-GSK3β (S9) and c-myc, and inhibited the expression level of p-β-catenin (S33/37), while had no effect on the expression level of GSK3β (Fig. 3A). In MDA-MB-231 cells, knockdown of TRIM63 significantly inhibited the expression levels of β-catenin, p-

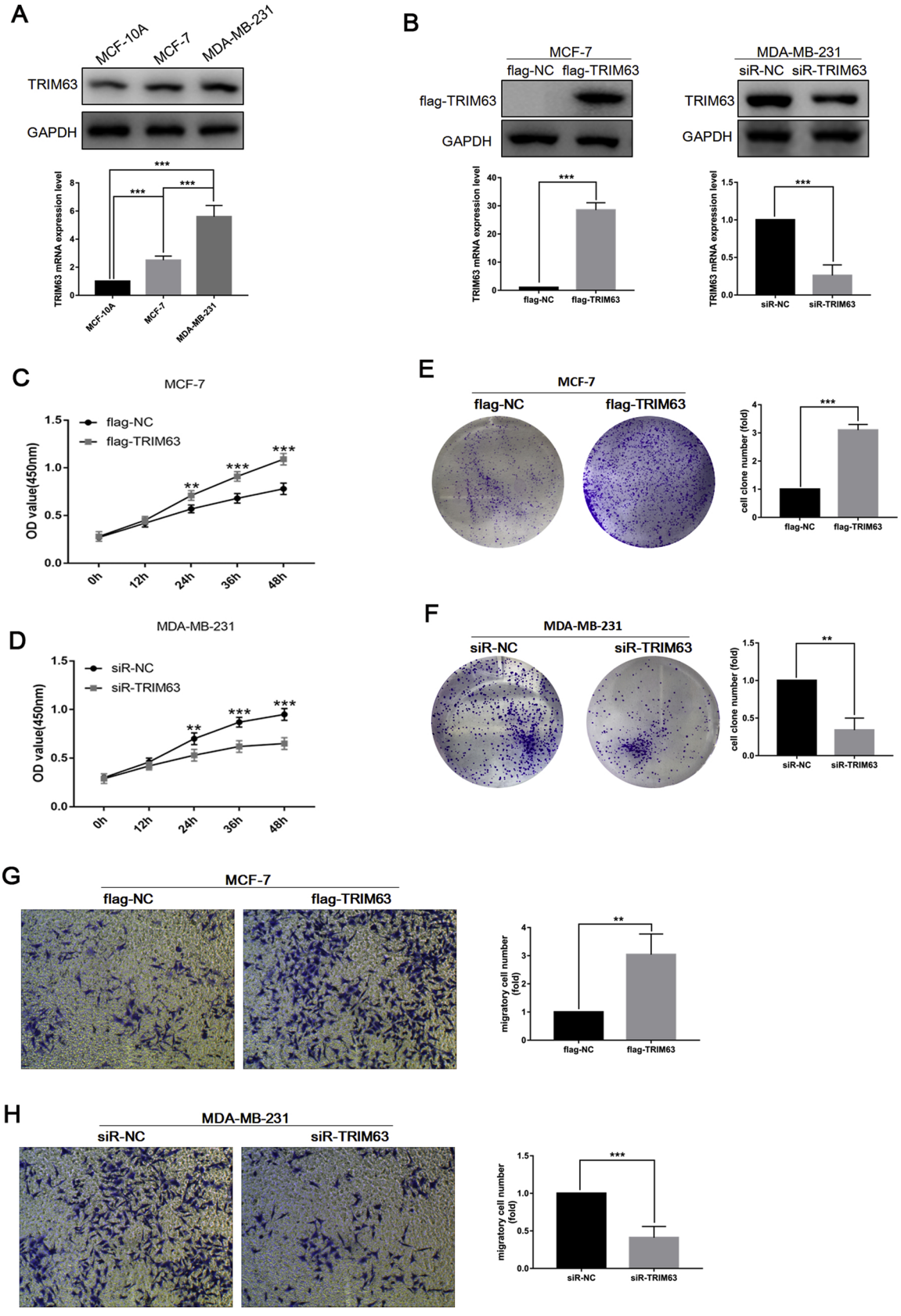

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Fig. 2. TRIM63 promotes proliferation and migration of breast cancer cells. ***A,*** TRIM63 mRNA and protein expression were detected by RT-qPCR and western blotting in normal breast cell line MCF-10A and breast cancer cell lines MCF-7 and MDA-MB-231. ***B,*** MCF-7 and MDA-MB-231 cells were transfected with flag-TRIM63 overexpression plasmid or flag-NC negative control vector, and siRNA of TRIM63 (siR-TRIM63) or negative control (siR-NC), which was detected by RT-qPCR and western blotting. ***C,*** TRIM63 overexpression enhances the proliferation of MCF-7 cells which was detected by CCK-8 assay. ***D,*** TRIM63 knockdown inhibits the proliferation of MDA-MB-231 cells which was detected by CCK-8 assay. ***E,*** TRIM63 overexpression enhances the proliferation of MCF-7 cells which was detected by the colon formation assay. ***F,*** TRIM63 knockdown inhibits the proliferation of MDA-MB-231 cells which was detected by the colon formation assay. ***G,*** TRIM63 overexpression enhances the migration ability of MCF-7 cells which was detected by Transwell assay. ***H,*** TRIM63 knockdown suppresses the migration ability of MDA-MB-231 which was detected by Transwell assay. Values represent the mean ± SD from three independent measurements. *P < 0.05, **P < 0.01, ***P < 0.001.'.

GSK3β (S9) and c-myc, and up-regulated the expression level of p-β-catenin (S33/37), while had also no effect on the expression level of GSK3β (**Fig. 3B**). In addition, the TOP/FOP flash assay was used to detect the activity of Wnt/β-catenin signaling pathway. In breast cancer cell MCF-7, overexpression of TRIM63 significantly increased the activity of TOP flash, but had no significant effect on FOP flash (**Fig. 3C**). In breast cancer cell MDA-MB-231, knockdown of TRIM63 significantly inhibited the activity of TOP flash, but had no significant effect on FOP flash (**Fig. 3D**). These results indicated that TRIM63 activated Wnt/β-catenin signaling pathway in breast cancer cells.

3.4. In breast cancer cells, TRIM63 activates Wnt/β-catenin signaling pathway by promoting GSK3β phosphorylation and inhibiting the degradation of β-catenin protein

In the present study, we first discovered that TRIM63 activated the Wnt/β-catenin signaling pathway, and further explored the molecular mechanism by which TRIM63 activated Wnt/β-catenin signaling pathway. In previous work, we have found that TRIM63 up-regulates the expression level of p-GSK3β and inhibits the expression level of p-β-catenin (S33/37). This result suggests that TRIM63 may have an effect on the degradation of β-catenin protein. MDA-MB-231 cells were treated with the protease inhibitor MG-132 combined with TRIM63 knockdown. The results showed that MG-132 significantly reversed the inhibitory effect of TRIM63 knockdown on β-catenin (**Fig. 4A**). Licl is an inhibitor of GSK3β and promotes GSK3β phosphorylation. We found that Licl reversed the inhibitory effect of TRIM63 knockdown on the expression of p-GSK3β and β-catenin (**Fig. 4B**). In addition, in breast cancer cell MCF-7, we found that the effect of TRIM63 overexpression is similar to that of Licl treatment, which both can up-regulate the protein expression of p-GSK3β and β-catenin (**Fig. 4C**). These results indicated that TRIM63 activated the Wnt/β-catenin signaling pathway by promoting GSK3β phosphorylation and inhibiting the degradation of β-catenin proteins.

4. Discussion

Normal human development is tightly controlled by complex signaling pathways that allow cells to communicate with cells and their surroundings. These signaling pathways are dysregulated or hijacked by cancer cells during tumorigenesis [21]. Essentially, tumors are driven by genetic and epigenetic changes that drive cells to escape the normal

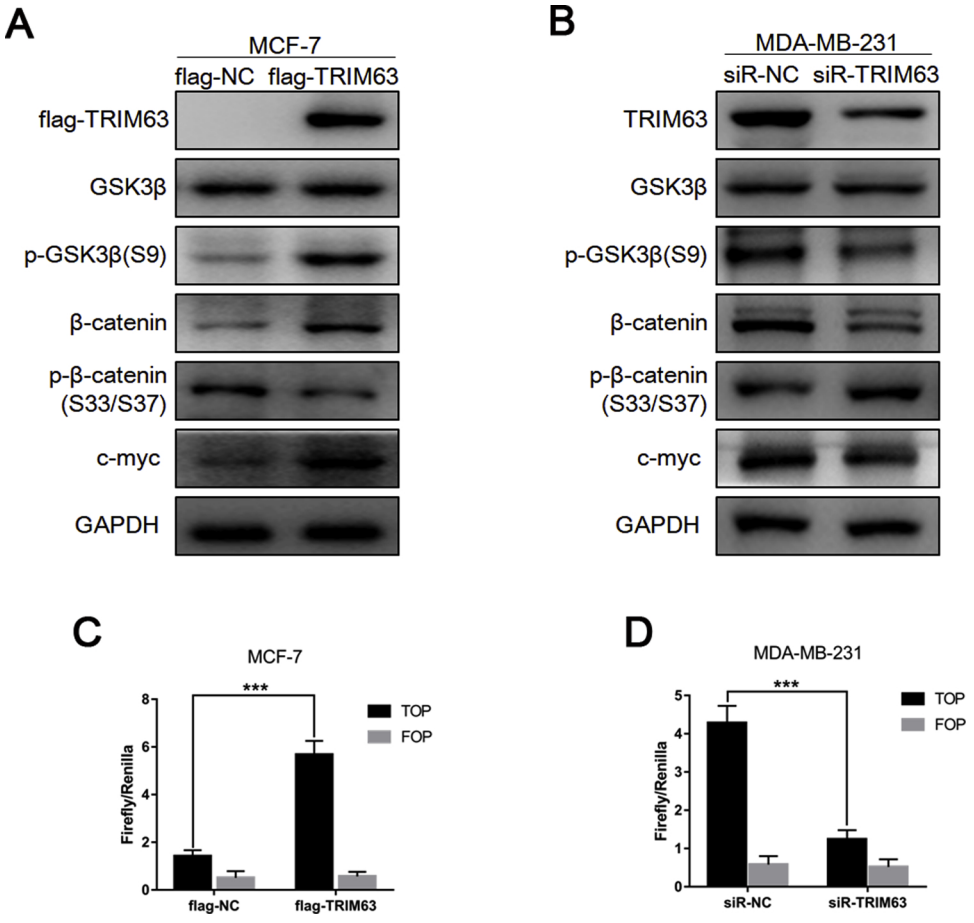

Fig. 3. TRIM63 activates Wnt/β-catenin signaling pathway in breast cancer cells. ***A,*** The effect of transfecting with flag-TRIM63 overexpression plasmid or flag-NC negative control vector on the protein levels of flag-TRIM63, β-catenin, p-β-catenin (S33/S37), GSK3β, p-GSK3β(S9), c-myc in MCF-7 cells. ***B,*** The effect of transfecting with siRNA of TRIM63 (siR-TRIM63) or negative control (siR-NC) vector on the protein levels of flag-TRIM63, β-catenin, p-β-catenin (S33/S37), GSK3β, p-GSK3β(S9), c-myc in MDA-MB-231 cells. ***C,*** TRIM63 overexpression increases the activity of Wnt/β-catenin signaling pathway which was detected by TOP/FOP flash assay. ***D,*** TRIM63 knockdown inhibits the activity of Wnt/β-catenin signaling pathway which was detected by TOP/FOP flash assay. Values represent the mean ± SD from three independent measurements. *P < 0.05, **P < 0.01, ***P < 0.001.

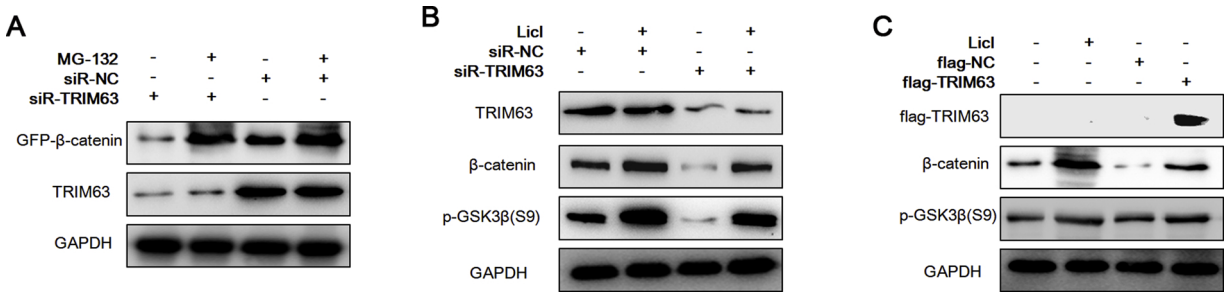

Fig. 4. TRIM63 activates Wnt/β-catenin signaling pathway via GSK3β pathway. ***A,*** Proteasomes inhibitor MG-132 reverses the effect of TRIM63 knockdown on β-catenin degradation. ***B,*** Inhibiting GSK3β kinase activity by LiCl reduces inhibition of β-catenin expression by TRIM63 knockdown. siR-TRIM63 or siR-NC was transfected into MDA-MB-231 cells. 36 h after transfection, the cells were treated with 30 mM LiCl for 12 h and then harvested for western blotting analysis to detect the expression of β-catenin, p-GSK3β (S9) and TRIM63. ***C,*** MDA-MB-231 cells were transfected with flag-NC and flag-TRIM63, treated with LiCl for 48 h and harvested for western blot assay.

mechanisms that normally control their proliferation, survival, and migration [17]. Thus, activation of oncogenes, or inactivation of tumor suppressor genes, can lead to the overactivation of these oncogenic signaling pathways, such as the Wnt/β-catenin signaling pathway [38].

There was no study to investigate the correlation between TRIM63 and tumorigenesis. In this study, we first found that TRIM63 protein was up-regulated in breast cancer tissues. In breast cancer cells, TRIM63 overexpression promotes the proliferation and migration of tumor cells, which indicate that TRIM63 is involved in the development of breast cancer as an oncogene. However, the mechanism of TRIM63 in breast cancer was unclear.

The structure of the protein determines the function. Due to the large similarity in the structure of TRIM family proteins, there may also be functional similarities between these proteins. It was found that overexpression of TRIM29 protein in colorectal cancer cells promoted Ser9 phosphorylation of GSK3β, inhibited Ser33/37 phosphorylation of β-catenin, thus activated Wnt/β-catenin signaling pathway [28]. In hepatoma carcinoma cells, overexpression of TRIM66 up-regulated the phosphorylation level of GSK3β at Ser9 and the expression level of β-catenin [8]. In neuroblastoma, knockdown of TRIM59 down-regulates the expression levels of β-catenin and the target genes survivin and c-myc, and inhibits tumor cell proliferation. Interestingly, LiCl, an inhibitor of GSK3β, blocks the inhibitory effect of cell proliferation induced by TRIM59 knockdown [3]. In thyroid cancer cells, TRIM44 protein promotes tumor cell proliferation and invasion by activating Wnt/β-catenin signaling pathway [39]. These results suggest that TRIM29, TRIM44, TRIM59 and TRIM66 can activate the Wnt/β-catenin signaling pathway, which may be through promoting phosphorylation of GSK3β, thereby inhibiting the degradation of β-catenin protein.

Wnt/β-catenin signaling pathway is critical in embryonic development and promotes rapid cell division and migration. However, overactivation of Wnt/β-catenin signaling pathway promotes uncontrolled proliferation of cells and leads to tumorigenesis [4]. Under normal conditions, Axin, APC, PP2A, GSK3β, and CK1α in the cytoplasm form degradation complexes [26]. When the degradation complex binds to β-catenin protein, GSK3β can promote the phosphorylation of β-catenin at Ser33 and Ser37, resulting in β-catenin to be ubiquitin-labeled and subsequently degraded by the proteasome. This regulatory mechanism maintains the stability of Wnt/β-catenin signaling pathway. However, when GSK3β is phosphorylated, the kinase activity of GSK3β is inactivated, which can no longer promote β-catenin phosphorylation, leading to the accumulation of β-catenin in the cytoplasm, which eventually leads to β-catenin entering the nucleus and interacts with TCF/LEF protein to forms a transcriptional activation complex that activates a range of target genes involved in cell proliferation and migration such as c-myc and cyclin D1 [1].

In the present study, we found that TRIM63 also up-regulated the protein expression levels of β-catenin and the target gene c-myc, and activated the Wnt/β-catenin signaling pathway. Further studies found that overexpression of TRIM63 also promoted phosphorylation of GSK3β and expression of β-catenin, and down-regulated the expression of p-β-catenin (Ser33/37). MG-132 was able to inhibit the effect of TRIM63 knockdown on the expression level of β-catenin protein, which further demonstrated that TRIM63 can inhibit the degradation of β-catenin protein. We also found that LiCl can inhibit the effect of TRIM63 knockdown on GSK3β phosphorylation and β-catenin expression, suggesting that TRIM63 regulated the degradation of β-catenin protein, which was related to phosphorylation of GSK3β.

In conclusion, we first found that TRIM63 was significantly up-regulated during breast cancer development. Furthermore, we revealed that TRIM63 promoted GSK3β phosphorylation, inhibiting the degradation of β-catenin protein, and activating Wnt/β-catenin signaling pathway, thus promoted the proliferation and migration of breast cancer cells. Our research facilitates an in-depth understanding of the important role of TRIM family proteins in tumorigenesis and the development of breast cancer targeted therapy.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflict of interest

All authors declare that there are no conflicts of interests.

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