

# TRIM8 Inhibits Breast Cancer Proliferation by Regulating Estrogen Signaling

**Zelin Tian**

Zhongnan Hospital of Wuhan University

**Jianing Tang**

Wuhan University Zhongnan Hospital

**Xing Liao**

Wuhan University Zhongnan Hospital

**Qian Yang**

Wuhan University Zhongnan Hospital

**Yumin Wu**

Wuhan University Zhongnan Hospital

**Gaosong Wu** (✉ [wugaosongtj@163.com](mailto:wugaosongtj@163.com))

<https://orcid.org/0000-0001-9296-7080>

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## Research

**Keywords:** Breast cancer, TRIM8, ER $\alpha$ , proliferation, poly-ubiquitination

**Posted Date:** July 17th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-42521/v1>

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# Abstract

**Background:** Breast cancer (BC) ranks first in female malignancies worldwide, 70% of which are estrogen receptor alpha (ER $\alpha$ ) positive. Endocrine treatment, such as tamoxifen, is primary adjuvant therapy for patients with ER-positive BC, while some of them can develop acquired resistance during long-time treatment. Thus, further research on estrogen signaling is of significance to improve the prognosis of BC patients.

**Methods:** In this study, we report that the E3 ubiquitin ligase TRIM8 acts as a novel regulator of ER $\alpha$  signaling. TRIM8 and ER $\alpha$  target genes expression levels were measured by RT-PCR, while protein expression levels were measured by western blot. CCK8 assay, clone formation, and EDU assay were used to measure cells proliferation. Wound healing assay was used to measure cells migration. Protein stability assay and protein ubiquitination analysis were used to detect ER $\alpha$  protein degradation. Co-immunoprecipitation assay was used to detect the interaction domain between TRIM8 and ER $\alpha$ .

**Results:** TRIM8 is downregulated in BC and is associated with poor prognosis. The protein level of TRIM8 is negatively correlated with ER $\alpha$ . RNA-seq result revealed that estrogen signaling maybe a potential target of TRIM8. Knockdown of TRIM8 can significantly enhance BC cell proliferation and migration *in vitro* and *in vivo*. And this effect can be reversed by ER $\alpha$  depletion. Further mechanistic studies have shown that TRIM8 interacts with AF1 domain of ER $\alpha$  via its RING domain in the cytoplasm, and affects poly-ubiquitination of ER $\alpha$  protein.

**Conclusion:** Our study reveals an interesting post-translational mechanism between ER $\alpha$  and TRIM8 in ER-positive BC, TRIM8 may be a potential therapeutic target for BC treatment.

## Introduction

The 2018 GLOBOCAN report revealed that there will be approximately 2.1 million newly diagnosed BC, accounting for one-fourth of all cancer cases among women [1]. Based on the expression levels of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), BC can be broadly classified into four types: Luminal A, Luminal B, HER2-enriched and triple-negative breast cancer (TNBC) [2]. The estrogen receptor alpha (ER $\alpha$ ) was first cloned from MCF-7 cells in 1986 [3]. ER $\alpha$  is a member of the nuclear receptor superfamily, which composed of three domains: activation function domain-1 (AF-1) at N-terminus, activation function domain-2 (AF-2) at C-terminus, and DNA-binding domain (DBD) [2]. AF-2 domain can recognize 17-betaestradiol (E2) and recruit transcriptional coregulators, whereas DBD can specifically bind to the estrogen response elements to regulate gene transcription [4–6].

In ER $\alpha$ -positive BC, the activation of the ER $\alpha$  signaling pathway is essential for proliferation of BC cells [7, 8]. And ER $\alpha$  expression is a crucial indicator for the administration of endocrine therapy: including aromatase inhibitors (anastrozole and letrozole), selective estrogen receptor degraders (SERDs) and selective estrogen receptor modulators (SERMs). Tamoxifen is one of the most common SERMs of

endocrine therapy, which binds to ER $\alpha$ , and recruits transcriptional corepressors to inhibit the induction of target genes [9, 10]. However, many patients will develop an acquired resistance during long time treatment [11]. And the mechanisms underlying endocrine resistance have not been completely elucidated yet. It is important to dig deep into the underlying mechanism of endocrine resistance and seek relevant molecular therapeutic targets for ER-positive BC patients.

ER $\alpha$  protein levels are modified by several post-translational mechanisms, such as phosphorylation, ubiquitination, and acetylation [12, 13]. ER $\alpha$  can be phosphorylated by several kinases such as PKC [14], ERK7 [15], p38 [16], Src [17], and CDK11 [18], which further leads to its poly-ubiquitination and degradation. Previous studies have demonstrated that the ER $\alpha$  is modified by mono-ubiquitination, multi-ubiquitination, and poly-ubiquitination [19]. Ub is covalently or non-covalently bound to ER $\alpha$ , resulting in proteolysis or non-proteolysis of this receptor [20]. The interaction between ER $\alpha$  and ubiquitin protein is very complicated, and does not necessary lead to its degradation. The mono-ubiquitination of ER $\alpha$  increases its stability and transcriptional activity. In contrast, ER $\alpha$  poly-ubiquitination always promotes its degradation via 26S proteasome pathway, and is related to the transcription cycle of ER $\alpha$ . TRIM56 can increase ER $\alpha$  protein stability by targeting K63-linked poly-ubiquitination [21], whereas RNF8, RNF31 and SHARPIN can increase ER $\alpha$  protein stability and enhance ER $\alpha$  signaling by targeting the mono-ubiquitination of ER $\alpha$  [22–24].

Tripartite motif (TRIM) family is mainly composed of the following three primary domains (from the N-terminus to the C-terminus): zinc finger domain (RING finger), B-box domain, and coiled-coil domain. The B-box domain is a characteristic domain of the TRIM family [25–27]. The RING finger domain mediates the ubiquitin transfer of proteins and is a characteristic marker for many E3 ubiquitin ligases [26]. TRIM family proteins are involved in a variety of biological processes, such as regulating transcription, differentiation, immunity, autophagy, and intracellular signaling by different signaling pathways [25, 28]. Some TRIM family proteins are also involved in cancer development [29]. TRIM8 (Tripartite motif 8) is a RING finger family protein consisting of 551 amino acids, and is involved in the process of ubiquitination of proteins [30]. TRIM8 has dual, competing roles in cancers. It activates the nuclear transcription factor  $\kappa$ B (NF- $\kappa$ B) pathway and promotes the occurrence of inflammation-related tumors [31]; however, TRIM8 also has an anti-tumor role by activating the p53 pathway and enhancing the sensitivity of chemotherapy drugs [32]. However, the function of TRIM8 in BC is still unknown. In this study, we found that the E3 ubiquitin ligase TRIM8 acts as a novel regulator of ER $\alpha$  signaling, which affects the degradation and stability of ER $\alpha$  via poly-ubiquitination.

## Materials And Methods

### Cell culture

Human ER $\alpha$  positive breast cancer cell lines (MCF-7 and T47D) and HEK293 cell lines were acquired from American Type Culture Collection (ATCC). DMEM medium (Gibco, China) supplemented with 10% FBS (Gibco, Australia) was used for MCF-7 and HEK293 cells culturing, whereas RPMI-1640 (Gibco, China)

supplemented with 2 mM L-glutamine (25030, Life Technologies) and 10% FBS was used for T47D cells. All the cell lines were cultured in a humidified environment consisting of 95% air and 5% CO<sub>2</sub> at 37°C.

### **Breast cancer tissue acquisition and immunohistochemistry**

BC samples were taken from the remaining specimens of patients undergoing breast surgery at Zhongnan Hospital of Wuhan University and independently confirmed by two pathologists. The ER $\alpha$  status, PR status, and HER2 status of all specimens were counted. Specific primary antibodies against TRIM8 (HPA023561, Sigma) were used for IHC.

### **Plasmid construction and cell transfections**

The Full-length TRIM8 with a Flag tag and TRIM8 deletion constructs (GFP-tag, GFP-TRIM8(1-551), GFP-TRIM8(1-182), GFP-TRIM8(1-351), GFP-TRIM8(182-551), GFP-TRIM8(56-551)) were obtained from Hanbio Biotechnology Co. Ltd (Shanghai, China). HA-Ub, HA-K48, HA-K63 and ER full length and deletion constructs were gifted from Dr. Jian Zhu's Research Group of Molecular Tumors, Xinxiang Medical College [21]. DNA sequencing technology was used for verifying the sequence of all plasmids and Lipofectamine 2000 (1662298, Invitrogen) was used for plasmid transfection according to Instruction & Protocol.

### **siRNA sequence, lentiviral construction, and cell transfection**

The TRIM8 siRNA sequences were: siRNA#1: 5'-GUGGACAACUGUUACU GUUTT-3'; siRNA#2: 5'-CAGACAAGCGCCUGGUGGATT-3'; siRNA#3: 5'-TGATAAGACGGAGGATGTCAGCTTCATGA-3'. TRIM8 siRNAs, lentiviral plasmids were purchased from GeneCopoeia (Guangzhou, China). According to the Instruction & Protocol, siRNAs were transfected into cells using the Lipofectamine RNAiMAX reagent (13778100, Invitrogen) for further experiments.

### **Tumor formation assays**

Female BALB/c nude mice (4 weeks of age) were purchased from the Central Laboratory of Animal Science, Wuhan University (Wuhan, China). BALB/c nude mice were estrogen-supplemented by implantation of slow-release 17 $\beta$ -estradiol pellets one day before injection. MCF7 cells were stably transfected with lentivirus-based NC or TRIM8, which suspended in DMEM medium ( $2 \times 10^6$  cells / 100  $\mu$ l DMEM) and then injection into the mammary fat pad, 5 mice per group. MCF-7 tumor volume was measured every 10 days, and the mice were killed after two months.

### **Total RNA extraction and qPCR analysis**

RNeasy plus mini kits (74134, Qiagen) were used to extract total RNA according to the manufacturer's protocol. qRT-PCR was performed using 10  $\mu$ L of the 2  $\times$  SYBR Master Mix (TOYOBO) and 2  $\mu$ L of diluted RT product with 1  $\mu$ L each of the forward and reverse primers, add nuclease-free water to the final volume of 20  $\mu$ L. qRT-PCR was conducted in triplicate, including the nontemplate controls. 36B4 was used as

internal control, with the  $2^{-\Delta\Delta Ct}$  values being normalized to 36B4 levels. Primer sequences for qPCR used in this study were provided below

TRIM8-F:GAGCGAGAGCAGGACATTGAG; TRIM8-R:CAGTTGGTTCACTTTC TCCTCCA; GREB1-F: GGGATCTTGTGAGTAGCACTGT; GREB1-R: AATCGG TCCACCAATCCCAC; PS2-F: GTCCCTCCAGAAGAGGAGTG; PS2-R: AGCCGA GCTCTGGGACTAAT; CCND1-F: GCTGCGAAGTGGAAACCATC; CCND1-R: CCTCCTTCTGCACACATTTGAA; 36B4-F: GGCGACCTGGAAGTCCAAC; 36B4-R: CCATCAGCACCACAGCCTTC.

### **Western blotting analysis**

RIPA buffer (Beyotime, China) was used to lysed cells, and proteins were separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electro-transferred to 0.45 $\mu$ m PVDF membrane. After sealed in TBST (Tris buffered saline Tween) buffer containing 5% skim milk for 2 h at room temperature, the membrane was incubated with the primary antibody (1: 2000) at 4 ° C overnight. After three times washes, the membrane was then incubated with the secondary antibody (1: 5000) for 1 hour at room temperature. Finally, Western blot analysis was performed on PVDF membranes using ECL immunoblotting kits. Each band is normalized relative to its corresponding  $\beta$ -actin band. The antibodies were provided below:

Anti-TRIM8 (HPA023561, Sigma); Anti-ER $\alpha$  (#8644, CST); Anti-ER $\alpha$  (SC-56833, Santa Cruz) (for immunofluorescence); Anti-Flag-tag (20543-1-AP, Proteintech); Anti-GFP-tag (ab290, Abcam); Anti-HA-tag (MMS-101R, COVANCE); Anti- $\beta$ -actin (20543-1-AP, Proteintech); Anti- $\beta$ -Tubulin (#2146, CST); Anti-Histone 3 (#4499, CST).

### **Proliferation Analysis**

MCF7 and T47D cells were transfected into 6-well plates with siTRIM8 or siControl. 24 hours after transfection, cells were washed with PBS, the number of cells was counted. Firstly, we seeded cells in 96-well plates, and CCK8 kit was used to measure the relative cell viability per 24h. Then, EDU staining was performed according to the instructions of the reagent vendor for statistical analysis. Finally, we plated cells at low density into 6-well plates to form clone. After two weeks, we used crystal violet to stain the colonies and the number of clones in each plate was counted with ImageJ software.

### **Wound healing Analysis**

MCF-7 and T47D cells were transfected with control siRNA or siTRIM8#1. After the cells have covered the field of vision, scratch the cells with a yellow pipette tip (200 $\mu$ l). Cell healing condition was observed at the indicated time points. Healing distance was measured using ImageJ software.

### **Luciferase assay**

Dual-Luciferase Reporter kit (Promega, Germany) was used to detect the luciferase activity of estrogen signaling activity according to the manufacturer's protocol. Together with the Renilla plasmid, the ERE luciferase reporter was transfected into MCF7 cells. After 24h, luciferase activity was measured according to the protocol.

### **Cell immunofluorescence assay**

MCF-7 cells planted on a glass slide were fixed with 4% paraformaldehyde. Then the cells were permeabilized with 0.2% Triton X-100 for 10 minute, and blocked by normal Goat serum for 1h. After washes, slides were incubated with primary antibody including rabbit anti-TRIM8 (HPA023561, Sigma) polyclonal antibody and mouse anti-ER $\alpha$  monoclonal antibodies (SC-56833, Santa Cruz) at 4°C overnight. Then the slides were washed and incubated with secondary antibodies including Alexa Flour 647 anti-rabbit antibody and FITC-conjugated anti-mouse antibody. DAPI was used for nucleus staining, Phalloidin was used for cytoskeleton staining and samples incubated with the secondary antibodies without primary antibodies were used as negative control. Images were got from Nikon A+ laser scanning confocal system.

### **Co-immunoprecipitation assay**

The total cell lysis of MCF7 were precleared with rabbit IgG and agarose beads P2051 Beyotime for 2 h and immunoprecipitated with ER $\alpha$  (Cell Signaling Technology, #8644) or TRIM8 (HPA023561, Sigma) antibody overnight, while rabbit IgG (Beyotime) was used as the negative control. The bound protein was analyzed by Anti- ER $\alpha$  or Anti-TRIM8 antibody.

### **Protein stability assay**

HEK293 cells were transfected in 24-well plate with HA-ER $\alpha$  and Flag-TRIM8 or Flag-vector. After 48 hours, cells were treated with 100  $\mu$ M cycloheximide (HY-12320, MedChemExpress) for 0h, 4h, 8h, 12h, and 16h. The rate of ER $\alpha$  degradation was determined by Western blot analysis. MCF7 cells were transfected with siTRIM8 or siControl in a 24-well plate for 48 hours, cells were treated with 100  $\mu$ M cycloheximide (HY-12320, MedChemExpress) for 0h, 4h, 8h, 12h, and 16h. The rate of ER $\alpha$  degradation was determined by Western blot analysis.

### **Protein ubiquitination analysis**

HEK293 cells were seeded in 24-well plates and transfected with HA-ER $\alpha$  plasmid and Flag-TRIM8 or Flag-vector. After 48 hours, the cells were treated with 10 $\mu$ m MG132 (HY-13259, MedChemExpress) for 8 hours and the cells were harvested. Western blot analysis was used to observe the ER $\alpha$  protein degradation.

### **Poly-ubiquitination detection assay**

HEK293 cells were seeded into 6-well plates and transfected with HA-Ub/ HA-K48/ HA-K63 plasmid, Flag-ER $\alpha$ , and GFP-TRIM8/GFP-vector. The total cell lysis of MCF7 were precleared with rabbit IgG and agarose beads (P2051 Beyotime) for 2 h and immunoprecipitated with Anti-Flag antibody (20543-1-AP, Proteintech) at 4 °C overnight, with IgG as a negative control. Western blot was performed with Anti-HA antibodies to detect ER $\alpha$  total, K48-linked, or K63-linked ubiquitination.

## Statistics analysis

Data values in this study were expressed as mean  $\pm$  standard deviation (SD). The comparisons were performed using Student's t-test, ANOVA, Pearson correlation coefficient, and Cox multivariate regression analysis. It was considered statistically significant when  $P < 0.05$ .

# Results

## Low expression of TRIM8 is associated with poor endocrine treatment and indicates poor prognosis in ER-positive BC

Studies by Richardson and Curtis from the oncomine database (<https://www.oncomine.org/resource/main.html>) showed that TRIM8 is downregulated in BC (Figure 1A). Data from the TCGA database also confirmed that TRIM8 expression is elevated in BC, especially in the luminal subtype (Figure 1B, C). The KMploter (<http://kmplot.com/analysis/index.php?p=service&cancer=breast>) database further revealed that low expression of TRIM8 is associated with poor endocrine treatments and indicated poor prognoses in ER-positive BC (Figure 1D). Multivariate COX regression analysis of TCGA data indicated that TRIM8 can be used as an independent factor for ER-positive BC prognosis (Supplementary Figure 1). To better analyze the correlation between TRIM8 expression and BC molecular markers (ER, PR, and Her2), we collected 91 BC patient specimens for TRIM8 immunohistochemistry assays and performed statistical analyses. IHC results showed that the expression of TRIM8 was negatively correlated with ER, PR and pathological grades (Figure 1E, Table 1).

## TRIM8 knockdown promotes ER-positive BC proliferation

MCF7 and T47D cell lines were used to further determine the underlying mechanism of TRIM8 in BC. Firstly, we used MCF7 cells to verify the knockdown efficiency of TRIM8 at protein and transcription levels, siRNA-TRIM8#1 and siRNA-TRIM8#2 were used for subsequent experiments (Figure 2A-B). All phenotypic experiments were validated with two ER $\alpha$ -positive BC cell lines, MCF7 and T47D. CCK8 and plate cloning experiments showed that TRIM8 depletion enhanced the cell proliferation ability of BC (Figure 2C, D). EDU is a thymidine analog that can be inserted into DNA during cell proliferation and can reflect cell proliferation level. The results of EDU staining indicated that DNA synthesis was increased after TRIM8 depletion (Figure 2E). Wound-healing assay results demonstrated that TRIM8 knockdown remarkably improved cell migration capacity of MCF7 and T47D (Figure 2F). In addition, we constructed an xenograft mice models to investigate the role of TRIM8 *in vivo*. The result indicated that lentivirus-based TRIM8 overexpression inhibited breast tumor growth (Figure 2G). Furthermore, the result of CCK8

assay and plate cloning experiments showed that overexpressing TRIM8 via lentivirus-based plasmid significantly inhibited MCF7 cell proliferation capacity (Supplementary Figure 2A, B). The wound-healing assay indicated that the overexpression of TRIM8 reduced MCF7 cell migration capacity (Supplementary Figure 2C).

### **TRIM8 knockdown promotes estrogen signaling activity**

Estrogen signaling plays a major role in ER $\alpha$ -positive BC. The RNA sequence analysis was performed to investigate whether TRIM8 affects the ER $\alpha$  pathway. The results indicated that TRIM8 knockdown significantly increased ER $\alpha$  target gene expression in MCF7 cells (Figure 3A), and activated the estrogen signaling at the same time (Figure 3B). TRIM8 knockdown reduced the sensitivity of MCF7 and T47D to tamoxifen (Figure 3C). In comparison with siControl group, TRIM8 depletion significantly upregulated ER $\alpha$  protein level (Figure 3D). qRT-PCR results showed that after depleting TRIM8, downstream target genes of ER $\alpha$ , such as GREB1, PS2, and CCND1, were significantly upregulated, and the ER $\alpha$  signaling pathway was activated (Figure 3E). In addition, TRIM8 knockdown downregulated ER $\alpha$  protein levels in both Estradiol (E2) and ethanol groups (Figure 3F). Consistent with these results, lentivirus-based TRIM8 overexpression reduced the expression of ER $\alpha$  target genes and reduced ER $\alpha$  protein levels (Supplementary Figure 3A, B). To determine whether TRIM8 depletion affected ER $\alpha$  transcriptional activity, we measured the activity of ERE-luciferase after TRIM8 depletion. The results indicated that TRIM8 knockdown enhanced the activity of the ER $\alpha$  reporter gene (Figure 3G). Furthermore, we performed a rescue experiment by ER $\alpha$  knockdown in TRIM8 knockdown cells to verify whether the functions of TRIM8 require ER $\alpha$ . The results revealed that the further depletion of ER $\alpha$  can save cell proliferation and migration in TRIM8 knockdown cells (Figure 4A-C).

### **TRIM8 interacts with ER $\alpha$ in the cytoplasm and decreases ER $\alpha$ protein stability**

Immunofluorescence results indicated that TRIM8 and ER $\alpha$  localized in both the nucleus and cytoplasm (Figure 5A). Endogenous co-immunoprecipitation assay (COIP) results in MCF7 cells indicated that TRIM8 can interact with ER $\alpha$  (Figure 5B). A nuclear and cytoplasmic separation co-immunoprecipitation assay confirmed that the interaction of TRIM8 and ER $\alpha$  occurred in the cytoplasm (Figure 5C). MG132 is a common proteasome inhibitor, which can inhibit protein degradation by inhibiting the proteasome-dependent degradation pathway. In the presence of MG132, TRIM8 overexpression did not reduce ER $\alpha$  protein levels (Figure 5D). To further confirm the direct effect of TRIM8 on ER $\alpha$ , we performed a protein stability assay. After cycloheximide (CHX) treatment to inhibit protein synthesis, overexpressed TRIM8 reduced the stability of ER $\alpha$  protein in HEK293 cells (Figure 5E). Similarly, TRIM8 knockdown in MCF7 cells increased the stability of endogenous ER $\alpha$  protein (Figure 5F).

### **TRIM8 interacts with ER $\alpha$ AF1 Domain via its RING Domain**

To better describe the interaction between TRIM8 and ER $\alpha$ , we constructed the following ER $\alpha$  and TRIM8 deletion constructs: for ER $\alpha$ , 1. HA-vector, 2. HA-ER $\alpha$ -1-595 (full length), 3. HA-ER $\alpha$ -1-180 ( $\Delta$ AF1 domain), 4. HA-ER $\alpha$ -1-300 ( $\Delta$ AF1 domain +  $\Delta$ DBD domain), 5. HA-ER $\alpha$ -180-595 ( $\Delta$ DBD domain +  $\Delta$ AF2 domain), and

6. HA-ERα-300-595 (ΔAF2 domain) (Figure 6A); and for TRIM8: 1. GFP-vector, 2. GFP-TRIM8-1-551 (full length), 3. GFP-TRIM8-1-182 (ΔRING domain+ΔB1/B2 domain), 4. GFP-TRIM8-1-351 (ΔRING domain+ΔB1/B2 domain + ΔCC domain), 5. GFP-TRIM8-56-551 (ΔB1/B2 domain + ΔCC domain + ΔRFP-Like domain), and 6. GFP-TRIM8-182-551 (ΔCC domain + ΔRFP-Like domain) (Figure 6B). COIP results indicated that the AF1 domain was necessary for interactions with TRIM8 (Figure 6C-D). The TRIM8 RING domain retained the ability to interact with ERα (Figure 6E).

### **TRIM8 affects ERα protein stability through poly-ubiquitination**

As an E3 ubiquitin ligase, TRIM8 is likely to function based on ubiquitin levels. TRIM8 overexpression significantly enhanced the endogenous poly-ubiquitination of ERα protein in HEK293 cells (Figure 7A). The ubiquitin-based COIP analysis indicated that TRIM8 overexpression significantly increased ERα total ubiquitination level and K48-linked poly-ubiquitination, while decreased K63-linked poly-ubiquitination (Figure 7B-D). However, lentivirus-based TRIM8 overexpression did not affect the mono-ubiquitination of ERα (Supplementary Figure 4). To determine the functional domain of TRIM8 domain to modulate ERα poly-ubiquitination, TRIM8 deletion constructs were transfected into HEK293 cells together with ERα and Ub / K48 / K63 plasmids. Interestingly, TRIM8 promoted ERα K48-linked poly-ubiquitination, and inhibited K63-linked poly-ubiquitination only in the presence of the RING domain (Figure 7E-G).

The above experiments indicated that the RING domain of TRIM8 plays an indispensable role in promoting ERα poly-ubiquitination. TRIM8 is mainly composed of 551 amino acid sequences, of which 16-56 amino acids are RING domain. Double mutating Cys30 and Cys38 amino acids to Ser30 and Ser38 can inactivate RING domain function (Figure 8A). TRIM8-C30/38S lost the ability to upregulate ERα protein level and to reduce its stability (Figure 8B,C). Collectively, COIP results based on poly-ubiquitination showed that mutant TRIM8 no longer affected ERα poly-ubiquitination (Figure 8D-F).

Table 1  
Statistics of clinical features and molecular characteristics

Clinical features and molecular characteristics (cases)	TRIM8			P value
	+	-		
<b>ERα</b>	<b>+</b>	12	43	<b>0.043</b>
	<b>-</b>	15	21	
<b>PR</b>	<b>+</b>	9	37	<b>0.033</b>
	<b>-</b>	18	27	
<b>HER2</b>	<b>+</b>	18	38	0.514
	<b>-</b>	9	26	
<b>Lymph node metastasis</b>	<b>+</b>	12	33	0.535
	<b>-</b>	15	31	
<b>Pathological grade</b>	Low	0	3	<b>0.040</b>
	Medium	21	32	
	High	6	29	

## Discussion

BC is the most common cancer among women in the world, 70% of patients are ERα-positive [33]. Endocrine therapy is an important treatment strategy for ERα-positive patients [33]. However, endocrine resistance is a concern in BC therapy; a number of confirmed and hypothetic mechanisms about ERα resistance have been developed [11]. In addition to ERα gene mutation in the AF-2 domain and the low proportion of ERα gene amplification, endocrine resistance is mainly associated with two mechanisms [34, 35]. One example is that ERα could crosstalk with EGFR/HER2, MAPK and NFκB signaling pathways, which could promote BC cells proliferation and lead to sustained signaling. In clinics, the crosstalk between ERα and EGFR/HER2 provide a good explanation for why ER-positive and HER2-positive patients always show lower efficacy of tamoxifen [36, 37]. Another is that the activity of ERα signaling pathway could be modified via several mechanisms. Many post-translational-modified proteins have been reported to be involved in ERα modification, some of which can affect breast cancer endocrine resistance. For example, the phosphorylation of the S118 and S167 sites of ERα indicates that the ERα signaling pathway is activated and may be used as a clinical indicator for breast cancer endocrine therapy [38]. P300 functions to promoted the acetylation of ERα in 302/303 site, which may be one reason for the reduced sensitivity of ERα to tamoxifen [39]. Moreover, the ubiquitination of ERα is a critical factor in endocrine resistance [20]. ERα protein ubiquitination does not necessary lead to its degradation. Some E3 ligases, such as RNF8, RNF31 and SHARPIN, promote the mono-ubiquitination of ERα to increase its

stability [22–24]. A study indicated that TRIM56 could increase ER $\alpha$  protein stability and facilitate estrogen signaling activity via K63-linked poly-ubiquitination [21].

E3 ubiquitin ligase imparts ubiquitin specificity via specifically recognized substrates and subsequently mediates the transfer of ubiquitin molecules from E2 ubiquitin-binding enzyme substrates [40]. There are three main types of E3 ubiquitin ligase: Homologous to the E6AP Carboxyl Terminus (HECT) family, Really Interesting New Gene (RING) family, and RING-between RING-RING (RBR) family [41, 42]. The HECT family has more than 30 members, whereas the RING family has more than 700 members most of which have not been studied [40, 43]. TRIM8 is located on chromosome 10q24.3 and has a molecular weight of 61.5 kD [30]. The relationship between TRIM8 and cancer was observed for the first time in glioblastoma [44]. TRIM8 acts as a tumor suppressor gene in most tumors, such as clear cell renal cell carcinoma (ccRCC), osteosarcoma, glioblastoma multiforme (GBM), colorectal cancer (CRC), anaplastic thyroid cancer (ATC) and chronic lymph in cellular leukemia (CLL) [44–47]. As a target gene of P53, TRIM8 can play a tumor-suppressive role by promoting P53 activation and MDM2 degradation [32]. Interestingly, TRIM8 has also been reported as a new oncogenic factor to promote the regulation of TNF-induced NF- $\kappa$ B pathway by promoting PIAS3 nuclear translocation [48, 49]. However, the mechanisms underlying of TRIM8 in BC has not been completely defined yet.

In this study, we investigated the biological functions of TRIM8 in ER-positive BC. TRIM8 associates with the AF1 domain of ER $\alpha$  via its RING domain, promotes the degradation of ER $\alpha$  via poly-ubiquitination, and subsequently inhibits ER $\alpha$  signaling activity. TRIM8-C30/38S lost the ability to upregulate ER $\alpha$  protein level and to reduce its stability. Further COIP results based on poly-ubiquitination showed that mutant TRIM8 could not facilitate K48-linked ubiquitination, and also could not inhibit K63-linked ubiquitination. There are three possible explanations for the effect of TRIM8 on ER $\alpha$ : I) TRIM8 promotes ER $\alpha$  degradation in a K48-dependent manner. K48-linked ubiquitination is the most classic poly-ubiquitination modification and plays a role in proteasome degradation and regulating protein stability [50]; II) TRIM8 decreases ER $\alpha$  protein stability in a K63-dependent manner; III) K48 and K63 polyubiquitination may compete for ER $\alpha$  modification at the same site. TRIM8 binds to ER $\alpha$  protein and catalyzes its K48-linked ubiquitination to block the K63-linked ubiquitination, thus promotes ER $\alpha$  degradation.

## Conclusion

Since ER $\alpha$  signaling plays a central role in BC proliferation, a better understanding of the relationship between ER $\alpha$  and TRIM8 may help to reveal the underlying mechanism of ER $\alpha$  signal regulation and improve the resistance of endocrine therapy. In this study, we report that the E3 ubiquitin ligase TRIM8 interacts with the AF-1 domain of ER $\alpha$  via its RING domain, which subsequently inhibits breast cancer cells proliferation *in vitro* and *in vivo*. Interestingly, the competition between K48 and K63 poly-ubiquitination may exist. K48-linked poly-ubiquitination enhance ER $\alpha$  protein degradation, which further inhibits K63-linked polyubiquitination. As an ER $\alpha$  regulator, targeting TRIM8 may provide a feasible treatment strategy for ER $\alpha$ -positive BC.

## Abbreviations

BC: Breast cancer ; ER $\alpha$ : Estrogen receptor alpha ; AF-1: Activation function domain-1 ; AF-2: Activation function domain-2; DBD: DNA-binding domain ; SERDs: Selective estrogen receptor degraders ; SERMs: Selective estrogen receptor modulators ; TRIM: Tripartite motif;

## Declarations

### Ethics approval and consent to participate

All studies involving human samples have received the patient's informed consent and the approval of the Wuhan University Ethic;s Committee. The Wuhan Animal Experiment Administration Committee reviewed and approved all animal experiments.

### Consent for publication

Not applicable.

### Availability of data and materials

The data and materials are available from the corresponding authors on request.

### Competing interests

The authors declare no conflict of interest.

### Funding

Not applicable.

### Author's contribution

ZL.T participated in most experiments and manuscript writing, and JN.T. and X.L. participated in the modification and prognosis analysis of the manuscript. Q.Y. and YM.W. participated in the collection of clinical specimens. GS.W oversees the research process.

### Acknowledgments

Thanks to Professor Zhu Jian of the Laboratory of Molecular Oncology, Xinxiang University for sharing plasmids and providing research support.

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## Figures

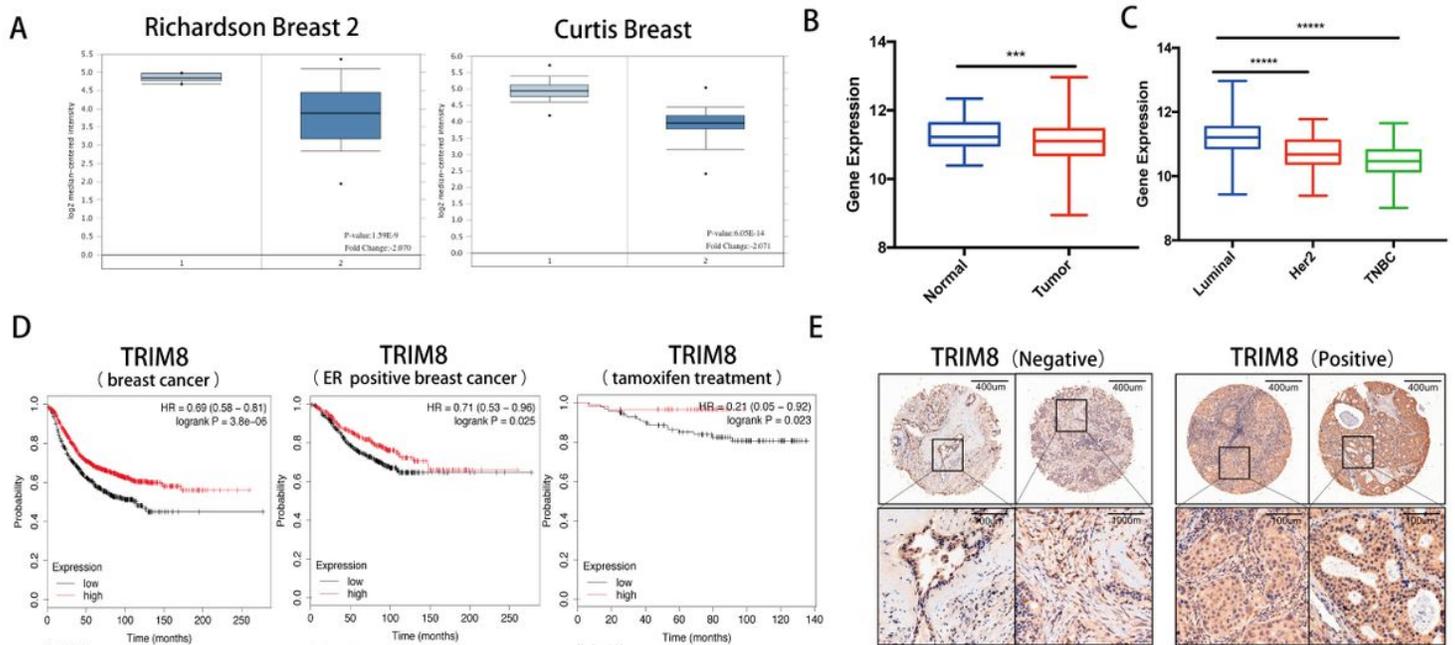
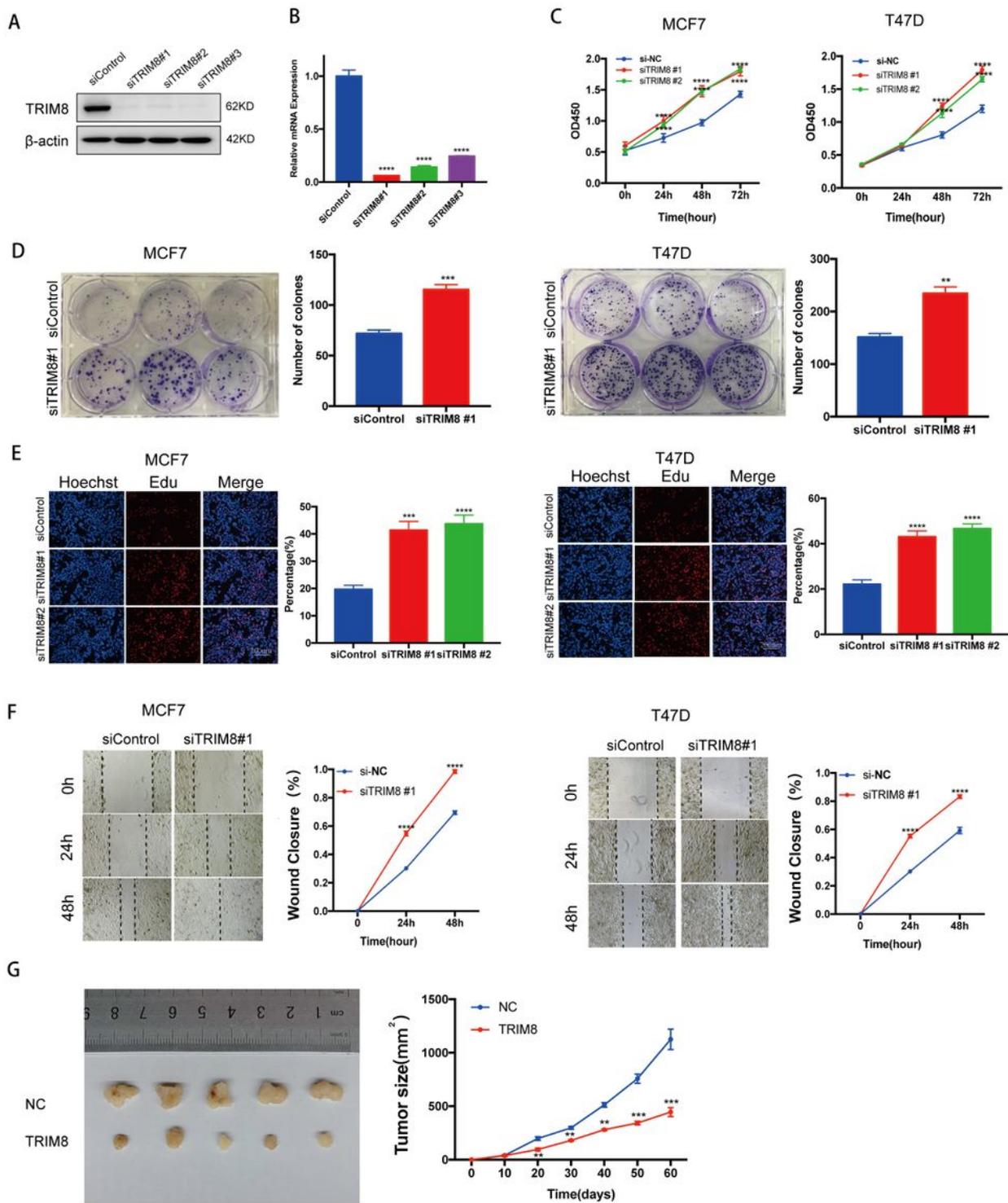


Figure 1

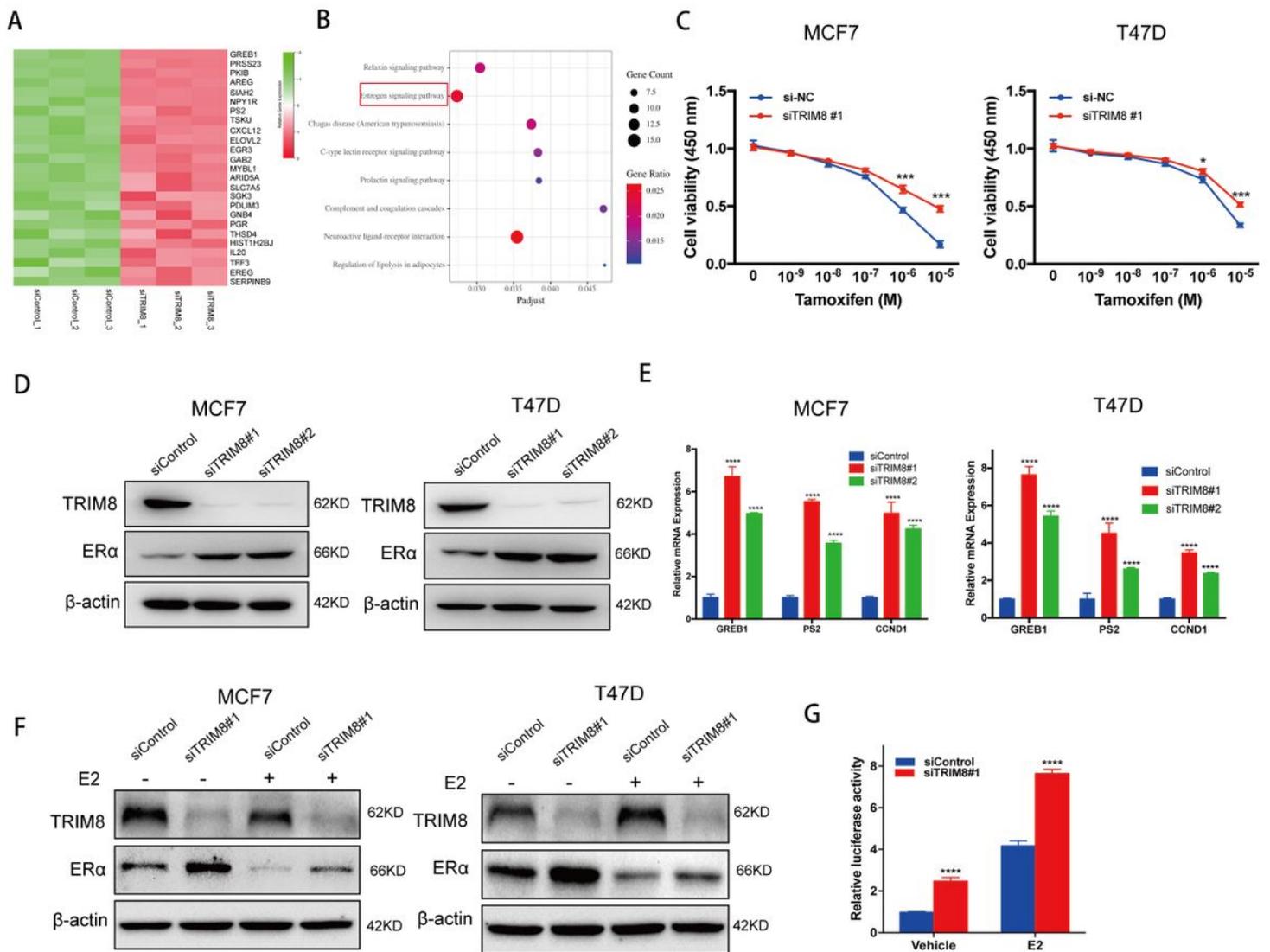
TRIM8 is downregulated in BC and associated with poor prognosis. (A, B). TRIM8 is downregulated in BC samples in Oncomine (A) and TCGA (B) databases. (C). TRIM8 is upregulated in luminal subtype of BC in TCGA database. (D). TRIM8 downregulation correlates with poor endocrine treatment outcomes from KMploter database. (E). Immunohistochemical results of patients with down- or up-regulated of TRIM8. \*, P value < 0.05; \*\*, P value < 0.01; \*\*\*, P value < 0.001; \*\*\*\*, P value < 0.0001.



**Figure 2**

TRIM8 inhibits ER-positive BC cell proliferation. (A,B) TRIM8 protein and gene knockdown efficiencies were determined by Western blot assay and qRT-PCR. (C-E) TRIM8 knockdown increases MCF7 and T47D cells proliferation. CCK8 assay of MCF7 and T47D cells (C). Clone formation assay of MCF7 and T47D cells (D). EDU assay of MCF7 and T47D cells (E). (F) Wound-healing assay of MCF7 and T47D cells. TRIM8 knockdown increases MCF7 and T47D cells migration. (G) TRIM8 overexpression decreases BC

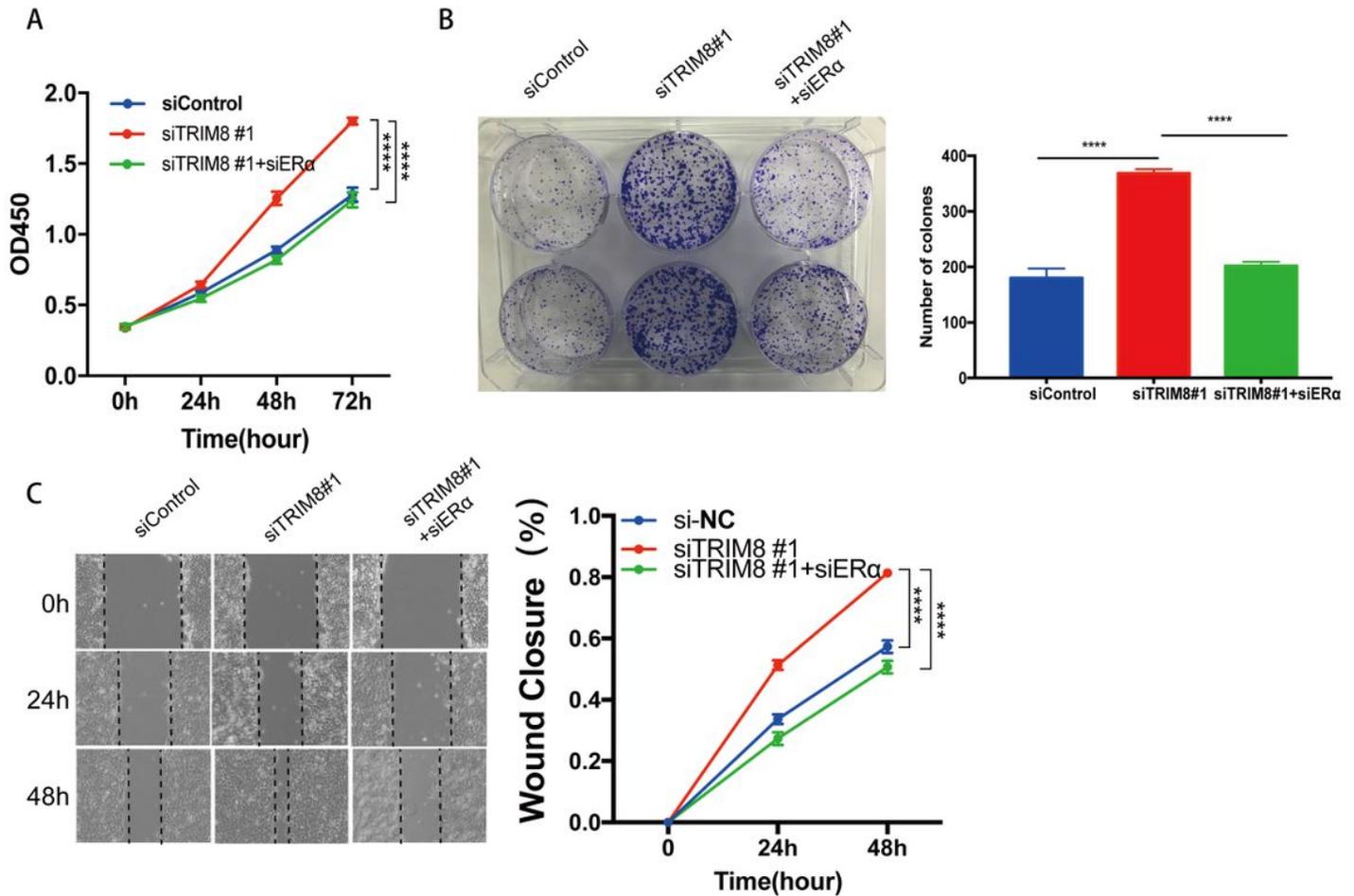
cells proliferation *in vivo*. MCF7 cells were stably transfected with lentivirus-based NC or TRIM8 plasmid, then injected into the mammary fat pad, 5 mice per group. MCF-7 tumor volume was measured every 10 days. \*, P value < 0.05; \*\*, P value < 0.01; \*\*\*, P value < 0.001; \*\*\*\*, P value < 0.0001.



**Figure 3**

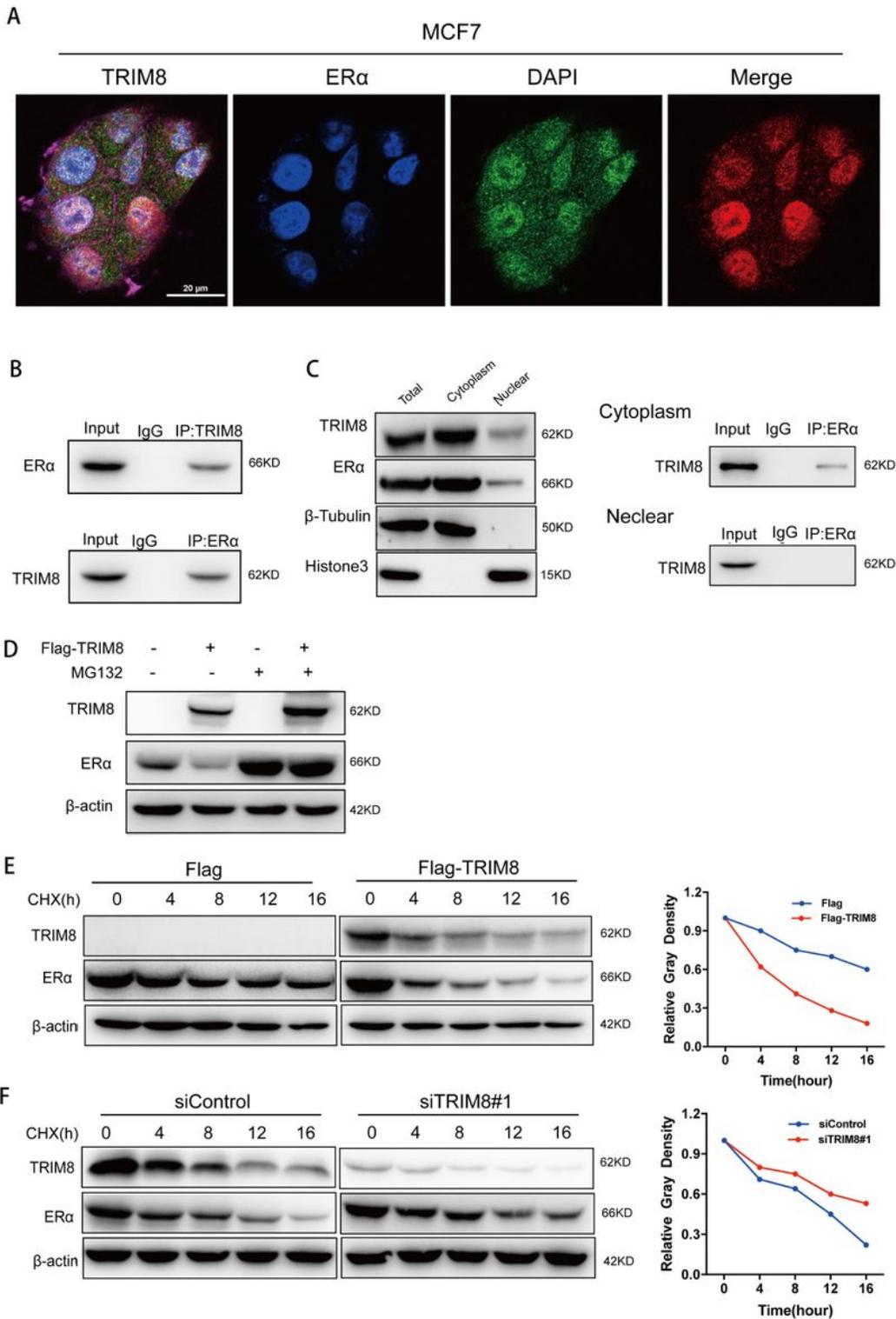
TRIM8 depletion promotes estrogen signaling activity (A) Heatmap indicates TRIM8 knockdown increases ERα target genes in MCF7 cells. (B) TRIM8 depletion promotes estrogen signaling activity. Top signaling pathways significantly upregulated via TRIM8 knockdown in MCF7 cells. (C) TRIM8 knockdown reduced the sensitivity of MCF7 and T47D to tamoxifen. MCF7 and T47D cells were transfected with siTRIM8#1, siTRIM8#2 or control siRNA. Cells were treated with the indicated concentration of tamoxifen. Cell proliferation activity was measured via CCK8 kit. Three replicates were set for each group, and the experiment was repeated three times. (D-F) TRIM8 depletion improved ERα target genes and ERα protein levels. TRIM8 and ERα protein expression were determined by western blot. Actin was used as an internal control (D). ERα target genes expression (GREB1, PS2, and CCND1) were determined by qRT-PCR. Three replicates were set for each group, and the experiment was repeated three times. 36B4 was used as an

internal control (E). MCF7 cells were transfected with siTRIM8#1 or control siRNA. After 48 h, cells were treated with ethanol or 10 nM estradiol for 6 h. TRIM8 and ER $\alpha$  protein levels were determined by western blot assay. Actin was used as an internal control (F). (G) TRIM8 knockdown affected ERE-luciferase activity in MCF-7 cells. MCF7 cells were transfected with siTRIM8#1 or control siRNA with ERE luciferase reporter plasmid. cells were treated with ethanol or 10 nM estradiol for 6h. 48h after transfection, luciferase activity was measured. Three replicates were set for each group, and the experiment was repeated three times. \*, P value < 0.05; \*\*, P value < 0.01; \*\*\*, P value < 0.001; \*\*\*\*, P value < 0.0001.



**Figure 4**

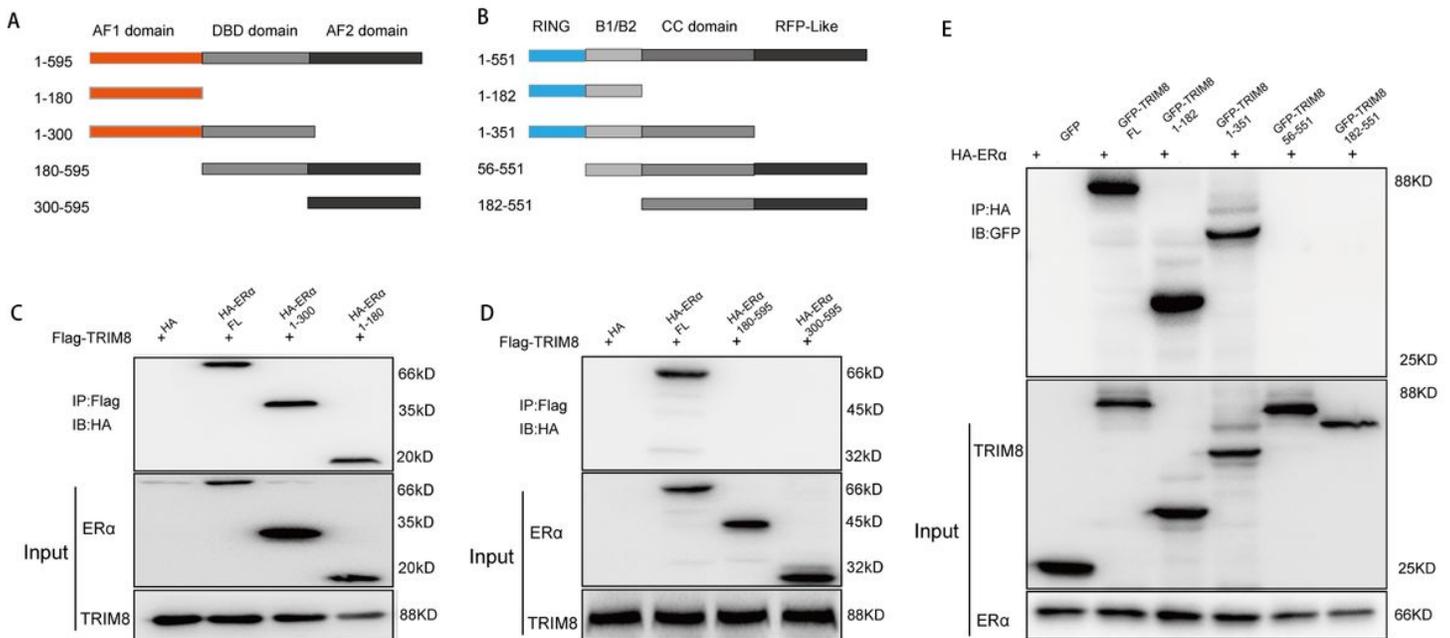
Downregulation of ER $\alpha$  in knockdown TRIM8 cell lines can rescue the effects of TRIM8. (A) CCK8 assay of MCF7 cells. (B) Wound healing assay of MCF7 cells. (C) Clone formation assay of MCF7. \*, P value < 0.05; \*\*, P value < 0.01; \*\*\*, P value < 0.001; \*\*\*\*, P value < 0.0001.



**Figure 5**

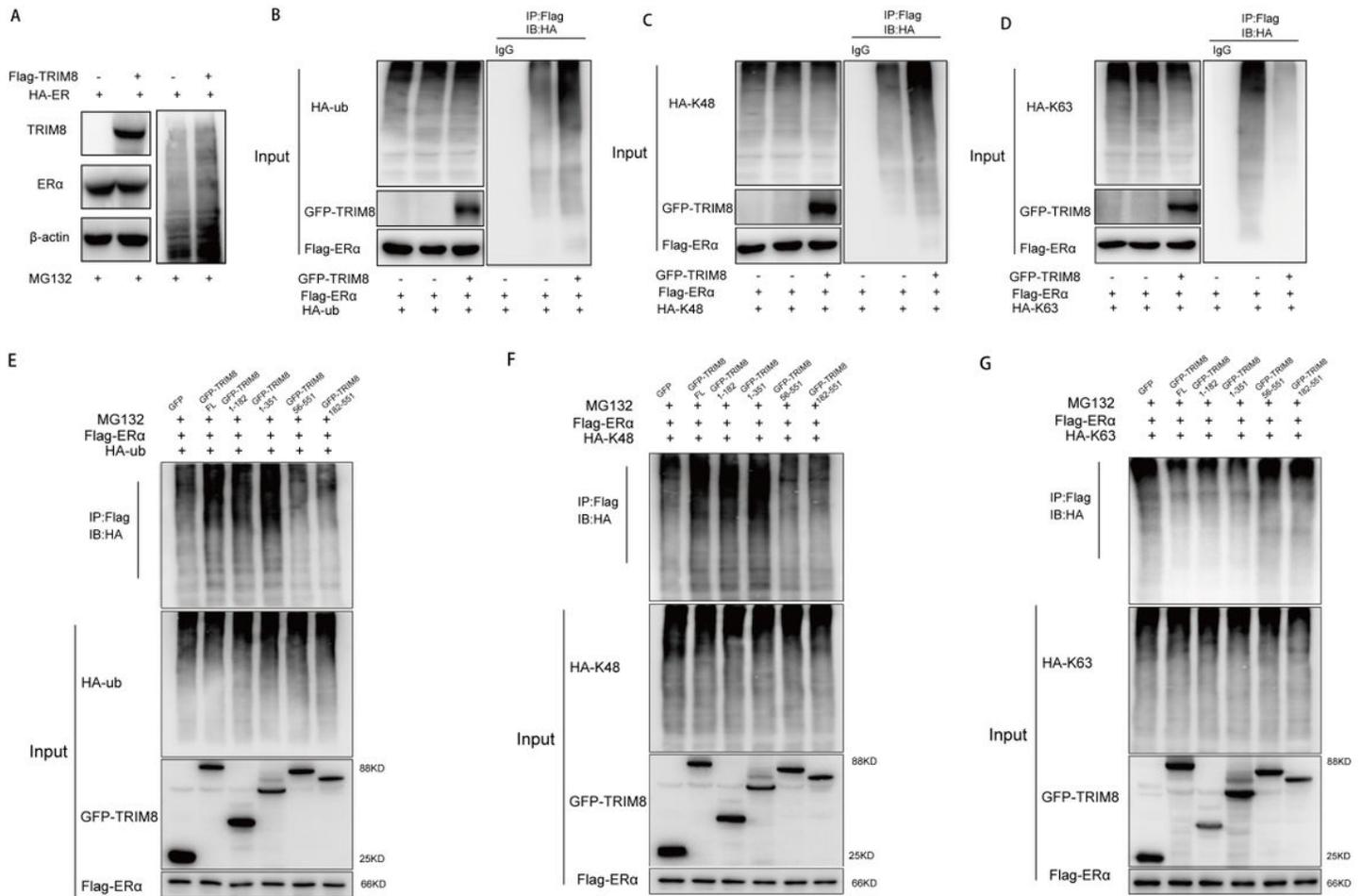
TRIM8 interacts with ER $\alpha$  in the cytoplasm and affects its stability by promoting ER $\alpha$  protein degradation (A) Intracellular localization analysis of TRIM8 and ER alpha by immunofluorescence assay. The results showed that ER $\alpha$  (red) and TRIM8 (green) were distributed both in the nucleus and cytoplasm. (B) COIP results indicated endogenous ER $\alpha$  and TRIM8 interacted. The total cell lysis of MCF7 were precleared with rabbit IgG and agarose beads for 2 h and the immunoprecipitated with ER $\alpha$  or TRIM8 antibody

overnight, while rabbit IgG was used as the negative control. The bounded protein was analyzed by Anti-ER $\alpha$  or Anti-TRIM8 antibody. (C) TRIM8 interacts with ER $\alpha$  in the cytoplasm. The subcellular protein fractionation kit was used for cytoplasmic and nuclear separations.  $\beta$ -Tubulin and Histone 3 were used for cytoplasm and nuclear controls. Based on the separation, COIP was performed as indicated. (D) TRIM8 promoted ER $\alpha$  proteasome pathway degradation. HEK293 cells were transfected with ER $\alpha$  plasmid and Flag-tag or Flag-TRIM8 plasmid. After 24 h, cells were treated with 10 $\mu$ m MG132 or isopropanol for 8 h. Actin was used as a negative control. (E) TRIM8 decreased ER $\alpha$  half-life in HEK293 cells. HEK293 cells were transfected with ER $\alpha$  plasmid and Flag-tag or Flag-TRIM8 plasmid. After 48 hours, cells were treated with 100 $\mu$ M cycloheximide at indicated time points. The rate of ER $\alpha$  degradation was determined by western blot analysis. The relative ER $\alpha$  protein's grayscale value was measured by ImageJ. (F) TRIM8 knockdown increased ER $\alpha$  half-life in MCF7 cells.



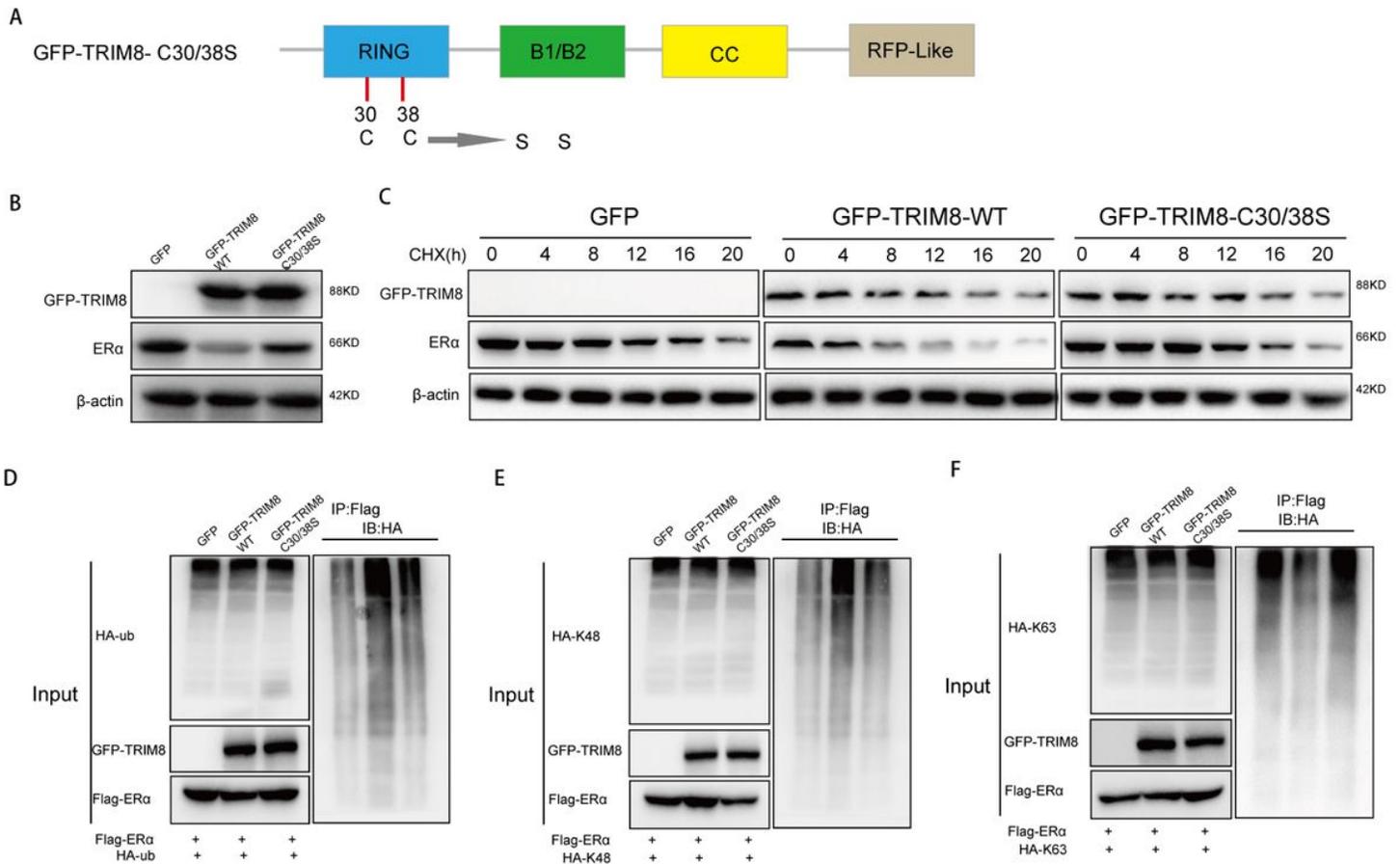
**Figure 6**

TRIM8 interacts with ER $\alpha$  AF1 Domain through Ring Domain (A) ER $\alpha$  domain structure and deletion mutants used in this study: 1. HA-vector, 2.HA-ER $\alpha$ -1-595(Full Length), 3.HA-ER $\alpha$ -1-180( $\Delta$ AF1 domain), 4.HA-ER $\alpha$ -1-300( $\Delta$ AF1 domain +  $\Delta$ DBD domain), 5.HA-ER $\alpha$ -180-595( $\Delta$ DBD domain+ $\Delta$ AF2 domain), 6.HA-ER $\alpha$ -300-595( $\Delta$ AF2 domain). (B) TRIM8 domain structure and deletion mutants used in this study: 1.GFP-vector, 2.GFP-TRIM8-1-551(Full Length), 3.GFP-TRIM8-1-182( $\Delta$ RING domain+ $\Delta$ B1/B2 domain), 4.GFP-TRIM8-1-351( $\Delta$ RING domain +  $\Delta$ B1/B2 domain +  $\Delta$ CC domain), 5. GFP-TRIM8-56-551 ( $\Delta$ B1/B2 domain +  $\Delta$ CC domain +  $\Delta$ RFP-Like domain), 6. GFP-TRIM8-182-551 ( $\Delta$ CC domain +  $\Delta$ RFP-Like domain). (C,D) ER $\alpha$  interacts with TRIM8 via AF1 domain. HEK293 cells were plated in 6-well plates with 1 $\mu$ g of Flag-TRIM8 and HA-ER $\alpha$  full length or mutant. The total cell lysis were precleared with IgG and agarose beads for 2 h and the immunoprecipitated with TRIM8 antibody overnight. The bounded protein was analyzed by Anti-HA antibody. (E) TRIM8 interacts with ER $\alpha$  via RING domain.



**Figure 7**

TRIM8 increases ERα K48-linked poly-ubiquitination via RING domain (A) TRIM8 enhanced ERα endogenous poly-ubiquitination. HEK293 cells were transfected with ERα plasmid and Flag-tag or Flag-TRIM8 plasmid. After 48 hours, the cells were treated with 10μm MG132 for 8 hours. Western blot analysis was used to observe the ubiquitination of ERα protein. (B) TRIM8 enhanced ERα poly-ubiquitination. HEK293 cells were transfected with Flag-ERα plasmid, HA-Ub plasmid, and GFP-TRIM8 plasmid. Anti-Flag antibody was used for COIP, and Anti-HA antibody was used for western blot analysis to detect ERα poly-ubiquitination. (C) TRIM8 enhances K48-linked ERα poly-ubiquitination. (D) TRIM8 decreases K63-linked ERα poly-ubiquitination. (E) TRIM8 increases ERα poly-ubiquitination via RING domain. HEK293 cells were transfected with Flag-ERα, HA-Ub plasmid, and GFP-TRIM8 full-length or deletion mutant. Anti-Flag antibody was used for COIP, and Anti-HA antibody was used for western blot analysis to detect ERα poly-ubiquitination. (F) TRIM8 increases ERα K48-linked poly-ubiquitination via RING domain. (G) TRIM8 decreases ERα K63-linked poly-ubiquitination via RING domain.



**Figure 8**

RING domain is the function domain of TRIM8. (A) The schematic diagram of TRIM8 mutants used in this study. (B) TRIM8-C30/38S mutant lost its ability to upregulation ERα protein in MCF7 cells. (C) TRIM8-C30/38S mutant does not decrease the ERα half-life in HEK293 cells. (D-F) The TRIM8 mutant lost its ability to modulate ERα protein poly-ubiquitination. Total ubiquitination (D). K48-linked poly-ubiquitination (E). K63-linked poly-ubiquitination (F).

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