

## Original Article

# TRIM8 inhibits breast cancer proliferation by regulating estrogen signaling

Zelin Tian<sup>1</sup>, Jianing Tang<sup>1</sup>, Xing Liao<sup>1</sup>, Yan Gong<sup>2</sup>, Qian Yang<sup>1</sup>, Yumin Wu<sup>1</sup>, Gaosong Wu<sup>1</sup>

<sup>1</sup>Department of Thyroid and Breast Surgery, Zhongnan Hospital of Wuhan University, Wuhan, China; <sup>2</sup>Department of Biological Repositories, Zhongnan Hospital of Wuhan University, 169 Donghu Road, Wuhan, China

Received July 13, 2020; Accepted September 8, 2020; Epub October 1, 2020; Published October 15, 2020

**Abstract:** Breast cancer (BC) is the most common female malignancy worldwide, and 70% of which are estrogen receptor  $\alpha$  (ER $\alpha$ ) positive. Endocrine treatment, such as tamoxifen, is a primary adjuvant therapy for patients with ER-positive BC. However, some patients will develop acquired resistance following long-time treatment. Further research on estrogen signaling is important to improve the therapy of these patients. In this study, we report that the E3 ubiquitin ligase tripartite motif 8 (TRIM8) acts as a novel regulator of ER $\alpha$  signaling. TRIM8 is downregulated in BC and is associated with poor prognosis. In addition, the protein level of TRIM8 is negatively correlated with ER $\alpha$ . RNA sequencing revealed that estrogen signaling maybe a potential target of TRIM8. Moreover, knockdown of TRIM8 can significantly enhance BC cell proliferation and migration both *in vitro* and *in vivo*. And this effect can be reversed by ER $\alpha$  depletion. Further mechanistic studies showed that TRIM8 interacts with AF1 domain of ER $\alpha$  via its RING domain in the cytoplasm and increases poly-ubiquitination of the ER $\alpha$  protein. In conclusion, our study reveals an interesting post-translational mechanism between ER $\alpha$  and TRIM8 in ER-positive BC, which suggests that TRIM8 may be a potential therapeutic target in the treatment of BC.

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

## Introduction

The 2018 GLOBOCAN report identified 2.1 million newly diagnosed cases of BC, accounting for one quarter of all cancer cases among women worldwide [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 is composed of three domains: activation function domain-1 (AF-1) at N-terminus, activation function domain-2 (AF-2) at C-terminus, and a DNA-binding domain (DBD) [2]. AF-2 domain can recognize 17-betaestradiol (E2) and recruit transcriptional coregulators, whereas DBD can specifically bind to estrogen response elements to regulate gene transcription [4-6].

In ER $\alpha$ -positive BC, activation of ER $\alpha$  signaling pathway is essential for proliferation of BC cells [7, 8]. 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 that 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 are not completely understood. It is important to elucidate the underlying mechanisms of endocrine resistance and to 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 protein kinase C (PKC) [14], ERK7 [15], p38 [16], Src [17], and cyclin-dependent kinase 11 (CDK11) [18], which further leads to its poly-ubiquitination and degradation. Previous studies have demonstrated that 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 complicated and does not necessarily 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 the 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].

Members of the tripartite motif (TRIM) family are mainly composed of the following 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 is a RING finger family protein consisting of 551 amino acids, which is involved in 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 development of inflammation-related tumors [31]. 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 cell culture, whereas RPMI-1640 (Gibco, China) supplemented with 2 mM L-glutamine (25030, Life Technologies) and 10% FBS was used for T47D cells. All cell lines were cultured in a humidified environment consisting of 95% air and 5% CO<sub>2</sub> at 37°C.

### *BC 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, progesterone receptor (PR) status, and HER2 status of all specimens were counted. Specific primary antibodies against TRIM8 (HPA023561, Sigma) were used for immunohistochemistry (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, Xixiang 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 the manufacturer's instructions.

### *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 and lentiviral plasmids were pur-

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chased from Gene Copoeia (Guangzhou, China), and siRNAs were transfected into cells using the Lipofectamine RNAiMAX reagent (1377-8100, Invitrogen) according to the manufacturer's instructions.

### *Tumor formation assays*

Female BALB/c nude mice (4 weeks old) 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 injected into the mammary fat pad (5 mice per group). MCF-7 tumor volume was measured every 10 days, and the mice were euthanized 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 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. Nuclease-free water was added to a 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 normalized to 36B4 levels. Primer sequences for qPCR used in this study were provided below: TRIM8-F: GAGCGAGAGCAGGACATTGAG; TRIM8-R: CAGTTGGTTCACCTTC TCCTCCA; GREB1-F: GGGATCTGTGAGTAGCACTGT; GREB1-R: AATCGG TCCACCAATCCAC; PS2-F: GTCCCTCCAGAAGAGGAGTG; PS2-R: AGCCGA GCTCTGGGACTAAT; CCND1-F: GCTGCGAAGTGGAACCATC; CCND1-R: CCTCCTTCTGCACACATTTGAA; 36B4-F: GGCGACCTGGAAGTCCAAC; 36B4-R: CCATCAGCACCACAGCCTTC.

### *Western blotting analysis*

RIPA buffer (Beyotime, China) was used to lyse cells, and proteins were separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to 0.45  $\mu$ m PVDF membrane. After sealing in TBST (Tris buffered saline-tween) buffer containing 5% skimmed milk for 2 h at room temperature, the mem-

brane was incubated with the primary antibody (1:2000) at 4°C overnight. After three 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 was 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 (ab-290, Abcam); Anti-HA-tag (MMS-101R, COVANCE); anti-GST-tag (SC-374171, Santa Cruz); 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#1, siTRIM8#2 or siControl. 24 hours after transfection, cells were washed with PBS and counted. Seeded cells in 96-well plates, and a CCK8 kit was used to measure the relative cell viability per 24 h. EDU staining was performed according to manufacturer's protocol. We plated cells at low density into 6-well plates to form clones. After two weeks, crystal violet was used 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, siTRIM8#1 or siTRIM8#2. The cells were then scraped with a yellow pipette tip (200  $\mu$ l). Cell healing was observed at the indicated time points. Healing distance was measured using ImageJ software.

### *Luciferase assay*

A 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 24 h, luciferase activity was measured according to the protocol.

### *RNA sequence analysis*

The RNA sequence analysis (siControl and siTRIM8) was performed by Shanghai Majorbiol

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Institute. The RNA sequence data were deposited in GEO database, which are available at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE157109>.

### *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 minutes and blocked with normal Goat serum for 1 h. After washing, the 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 nuclear staining, and phalloidin was used for cytoskeleton staining. Samples incubated with the secondary antibodies without primary antibodies were used as negative controls. Images were acquired using Nikon A+ laser scanning confocal system.

### *Co-immunoprecipitation assay*

The total cell lysates 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.

### *GST-pulldown assays*

GST and GST-TRIM8 were purified via glutathione-Sepahrose™ 4B beads (GE healthcare). Purified GST and GST-TRIM8 were then incubated with ER $\alpha$  recombinant protein (Ab82606, Abcam) at 4°C for 2 h. The mixture was washed three times with GST-pulldown buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1% Triton X-100) and subject to SDS-PAGE analysis.

### *Protein stability assay*

HEK293 cells were transfected in 24-well plates with HA-ER $\alpha$  and Flag-TRIM8 or Flag-vector. After 48 hours, cells were treated with

100  $\mu$ M cycloheximide (HY-12320, MedChem-Express) for 0 h, 4 h, 8 h, 12 h, and 16 h. 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, and cells were treated with 100  $\mu$ M cycloheximide (HY-12320, MedChemExpress) for 0 h, 4 h, 8 h, 12 h, and 16 h. 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. Denatured loading buffer was used for all ubiquitination assays. Western blot analysis was used to observe 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 lysates 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 analysis was performed with Anti-HA antibodies to detect ER $\alpha$  total, K48-linked, or K63-linked poly-ubiquitination.

### *In vitro ubiquitylation assay*

The ubiquitination reaction contained 10  $\times$  E3 Ligase Reaction Buffer, E1, E2, and purified GST-TRIM8 and purified ER $\alpha$ . The mixture was incubated at 37°C for 1 h and terminated by boiling with SDS-PAGE loading buffer for 10 minutes. Ubiquitin conjugation reaction products were analyzed by Western blotting.

### *Statistical 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.  $P < 0.05$  was considered statistically significant.

**Results**

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

Data from oncomine database (<https://www.oncomine.org/resource/main.html>) and TCGA database indicated that TRIM8 is downregulated in BC (**Figure 1A, 1B**). The TCGA database further confirmed that TRIM8 expression is elevated in luminal subtype tumors compare with other subtypes (**Figure 1C**). The KMplotter (<http://kmplot.com/analysis/index.php?p=service&cancer=breast>) database revealed that low expression of TRIM8 is associated with poor endocrine treatments and indicates poor prognoses in ER-positive BC (**Figure 1D**). Multivariate COX regression analysis of TCGA data indicated that TRIM8 can be used as an independent prognostic factor in 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 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 determine the role of TRIM8 in BC. We used MCF7 cells to verify the knockdown efficiency of TRIM8 at the protein and transcriptional levels; siRNA-TRIM8#1 and siRNA-TRIM8#2 were used for subsequent experiments (**Figure 2A, 2B**). All phenotypic experiments were validated with two ER $\alpha$ -positive BC cell lines, MCF7 and T47D. CCK8 and plate cloning experiments indicated that TRIM8 depletion enhanced the proliferation of BC cells (**Figure 2C, 2D**). EDU is a thymidine analog that can be inserted into DNA during cell proliferation that 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 enhanced the migration of MCF7 and T47D cells (**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 overexpression of TRIM8 via lentivirus-based plasmid significantly inhibited MCF7 cell proliferation (**Supplementary Figure 2A, 2B**). The wound-healing assay indicated that the overexpression of TRIM8 reduced MCF7 cell migration capacity (**Supplementary Figure 2C**). TRIM8 knockdown promoted ER-positive BC proliferation, but had no effect on triple-negative BC cells (**Supplementary Figure 3**).

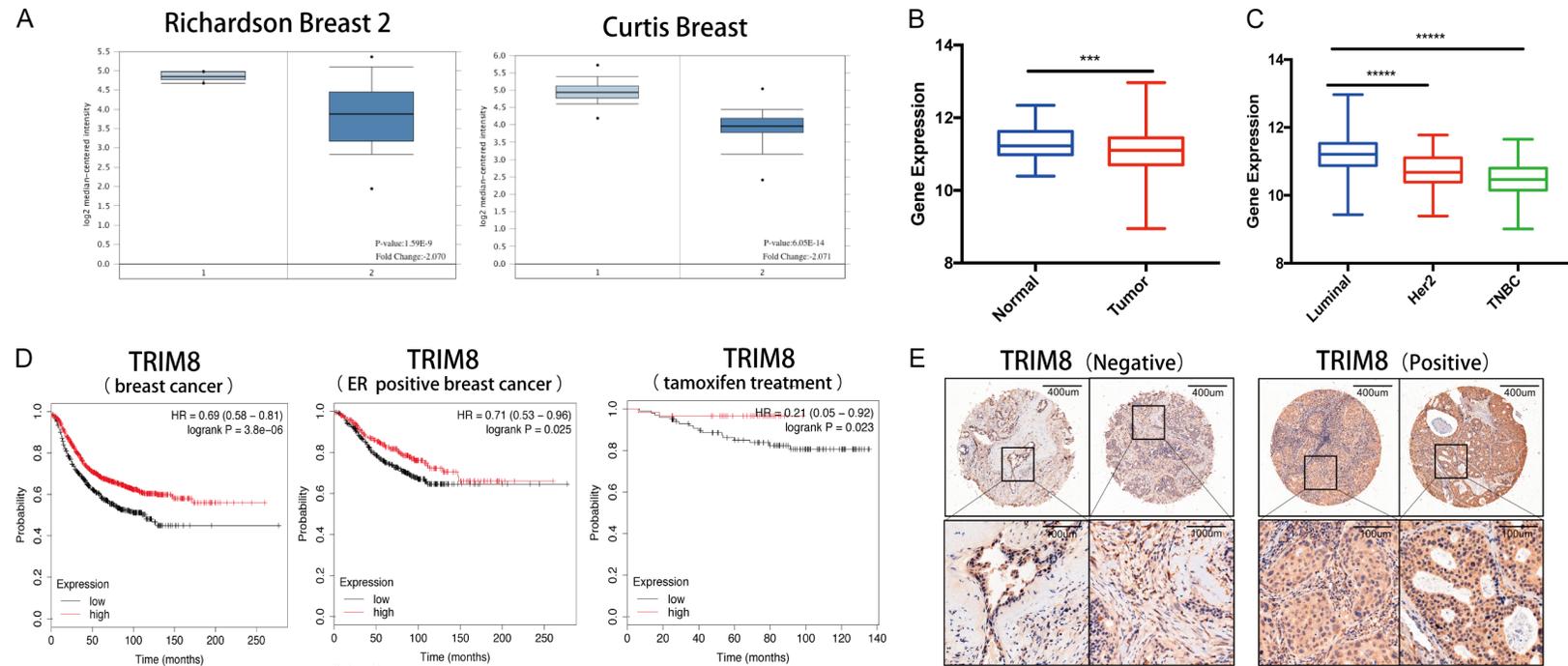
*TRIM8 knockdown promotes estrogen signaling activity*

Estrogen signaling plays a major role in ER $\alpha$ -positive BC. The RNA sequencing analysis was performed to investigate whether TRIM8 affects the ER $\alpha$  pathway. The result indicated that TRIM8 knockdown significantly increased ER $\alpha$  target gene expression in MCF7 cells (**Figure 3A**), and activated estrogen signaling at the same time (**Figure 3B**). TRIM8 knockdown also reduced the sensitivity of MCF7 and T47D cells to tamoxifen (**Figure 3C**). In comparison with the siControl group, TRIM8 depletion significantly upregulated ER $\alpha$  protein and target gene (GREB1, PS2, and CCND1) levels (**Figure 3D, 3E**). 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 4A, 4B**). 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 further depletion of ER $\alpha$  can restore 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

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**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) Downregulation of TRIM8 correlates with poor endocrine treatment outcomes from KM-plotter 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.

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**Table 1.** Statistics of clinical features and molecular characteristics

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

and cytoplasm (**Figure 5A**). Endogenous co-immunoprecipitation assay (COIP) in MCF7 cells indicated that TRIM8 can interact with ER $\alpha$  (**Figure 5B**). GST-pulldown analysis demonstrated that TRIM8 interacts with ER $\alpha$  directly (**Supplementary Figure 5**). A nuclear and cytoplasmic separation co-immunoprecipitation assay confirmed that the interaction of TRIM8 and ER $\alpha$  occurred in the cytoplasm (**Figure 5C**). In addition, E2 could induce the degradation of ER $\alpha$ , but had no effect on promoting the association between ER $\alpha$  and TRIM8 (**Supplementary Figure 6**). MG132 is a common proteasome inhibitor, which inhibits protein degradation by blocking the proteasome-dependent degradation pathway. TRIM8 overexpression downregulated ER $\alpha$  protein levels, and MG132 rescued this effect (**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-ER $\alpha$ -300-595 ( $\Delta$ AF2 domain) (**Figure 6A**); and for TRIM8: 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), and 6. GFP-TRIM8-182-551 ( $\Delta$ CC domain +  $\Delta$ RFP-Like domain) (**Figure 6B**). COIP results indicated that the AF1 domain of ER $\alpha$  was necessary for interactions with TRIM8 (**Figure 6C, 6D**). The TRIM8 RING domain retained the ability to interact with ER $\alpha$  (**Figure 6E**).

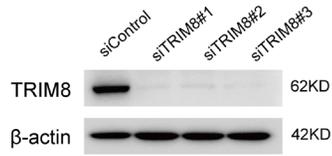
### *TRIM8 affects ER $\alpha$ protein stability through poly-ubiquitination*

As an E3 ubiquitin ligase, TRIM8 function is correspond with ubiquitin levels. TRIM8 overexpression enhanced the endogenous poly-ubiquitination of ER $\alpha$  protein in MCF7 cells (**Figure 7A**). E2 also positively regulated poly-ubiquitination of ER $\alpha$  (**Supplementary Figure 7**). The ubiquitin-based COIP analysis indicated that TRIM8 overexpression significantly increased ER $\alpha$  total ubiquitination level and K48-linked poly-ubiquitination, and decreased K63-linked poly-ubiquitination (**Figure 7B-D**). However, TRIM8 overexpression did not affect the mono-ubiquitination of ER $\alpha$  (**Supplementary Figure 8**). *In vitro* ubiquitination analysis revealed that TRIM8 can promote the poly-ubiquitination of ER $\alpha$  *in vitro* (**Supplementary Figure 9**). To determine the functional domain of TRIM8 that modulates ER $\alpha$  poly-ubiquitination, TRIM8 deletion constructs were transfected into HEK293 cells together with ER $\alpha$  and Ub/K48/K63 plasmids. Interestingly, TRIM8 promoted ER $\alpha$  K48-linked poly-ubiquitination, and inhibited K63-linked poly-ubiquitination only in the presence of the RING domain (**Figure 7E-G**).

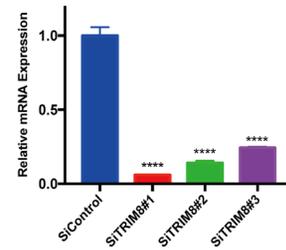
The above experiments indicated that the RING domain of TRIM8 plays an indispensable role in promoting ER $\alpha$  poly-ubiquitination. TRIM8 is mainly composed of 551 amino acid sequences, and amino acids 16-56 form the RING domain. Double mutation of Cys30 and Cys38 to Ser30 and Ser38 can inactivate RING domain function (**Figure 8A**), leading to loss of the ability to downregulate ER $\alpha$  protein level and reduce its stability (**Figure 8B, 8C**). Collectively, COIP results based on poly-ubiquitination sh-

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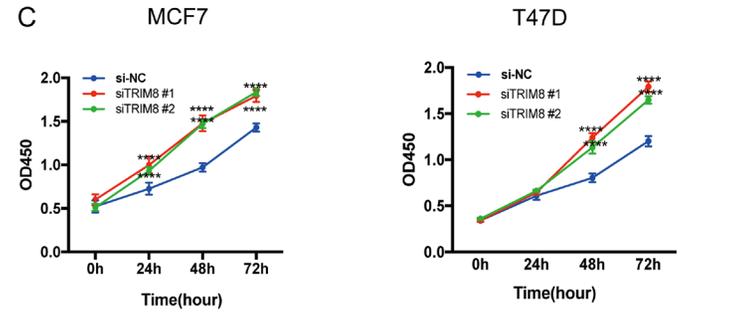
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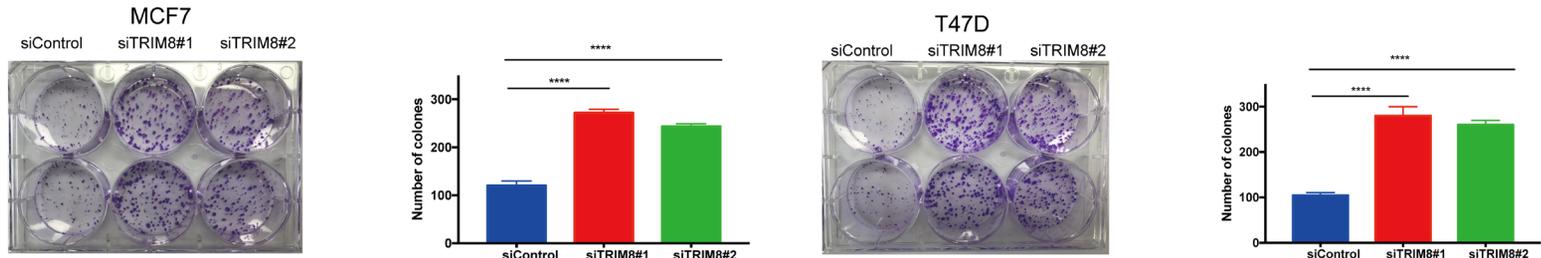
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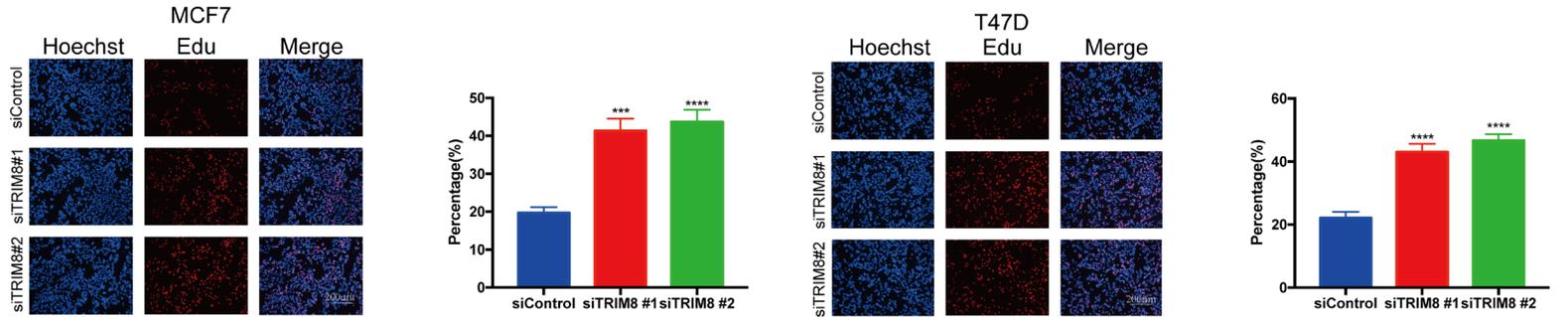
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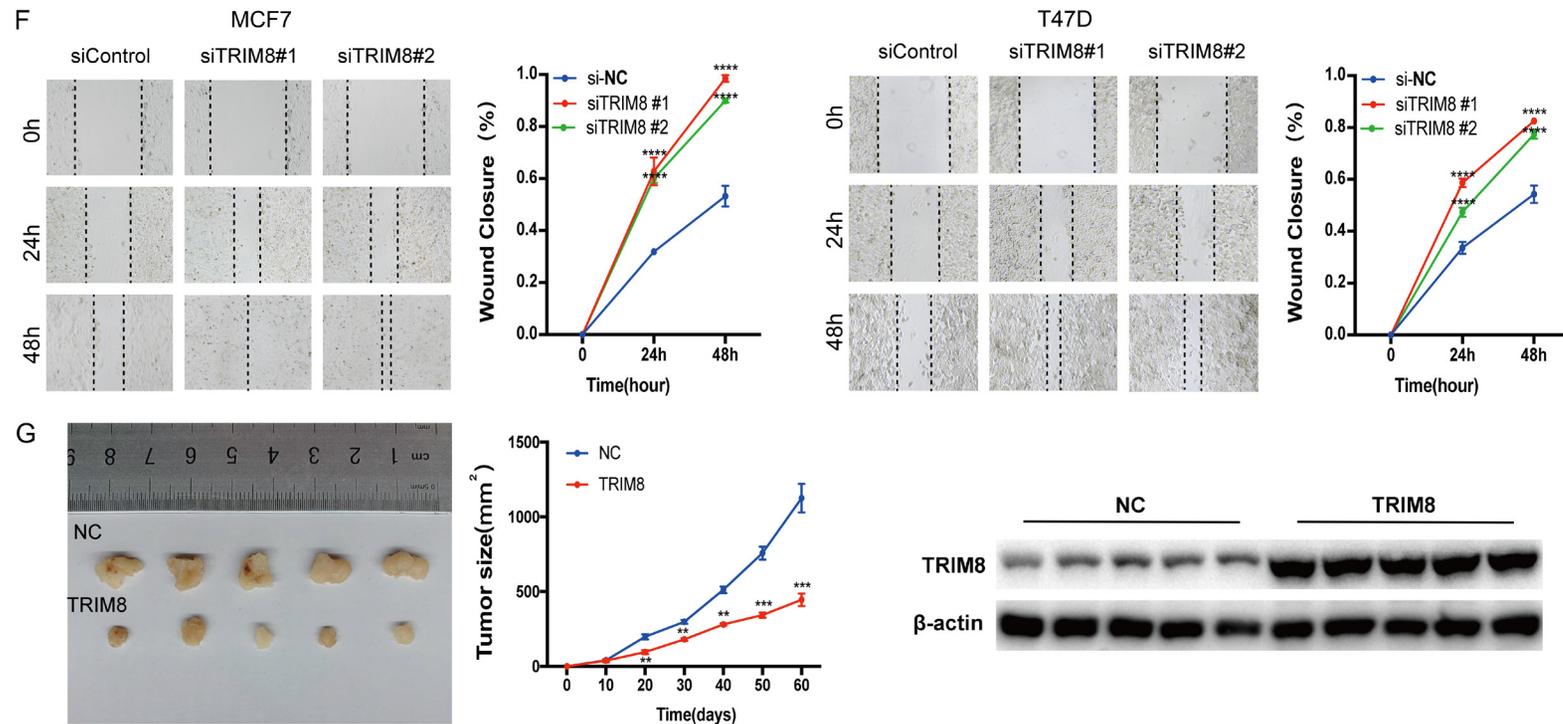
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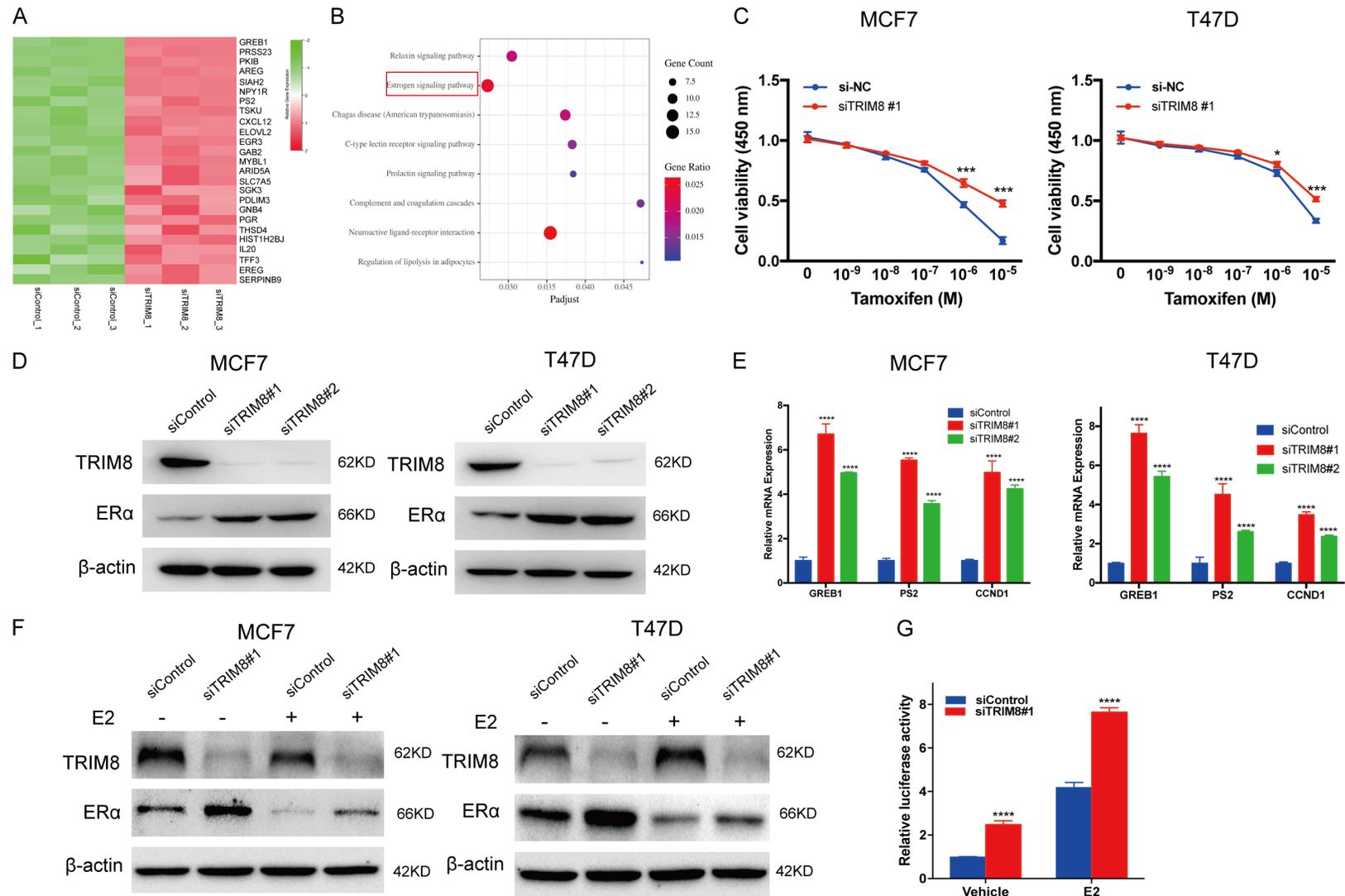


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**Figure 2.** TRIM8 inhibits ER-positive BC cell proliferation. (A, B) TRIM8 knockdown efficiency was determined by Western blot assay and qRT-PCR assays. (C-E) TRIM8 knockdown increases MCF7 and T47D cells proliferation. CCK8 assay of MCF7 and T47D 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 cell 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.

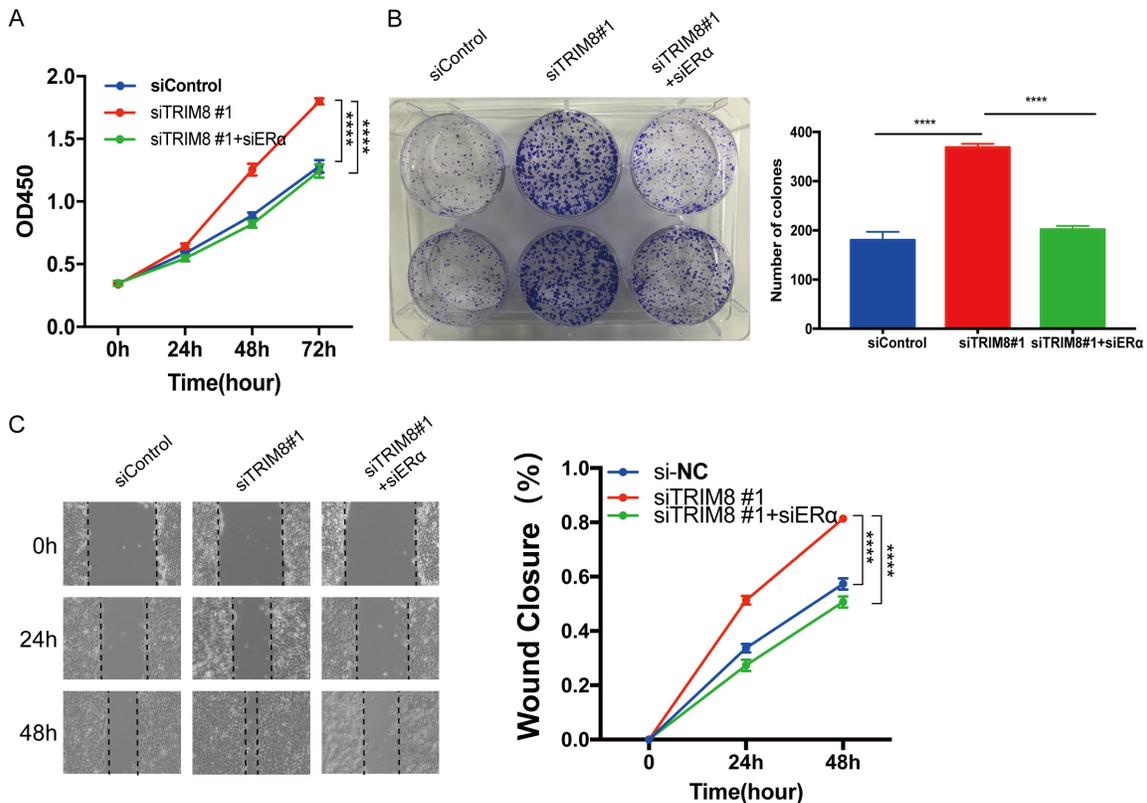
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**Figure 3.** TRIM8 depletion promotes estrogen signaling activity. (A) A heatmap indicating that TRIM8 knockdown increases ER $\alpha$  target genes in MCF7 cells. (B) TRIM8 depletion promotes estrogen signaling activity. The top signaling pathways 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 or control siRNA. Cells were treated with the indicated concentration of tamoxifen. Cell proliferation activity was measured via CCK8 assay. Three replicates were set for each group, and the experiment was repeated three times. (D-F) TRIM8 depletion improved ER $\alpha$  target genes and ER $\alpha$  protein levels. TRIM8 and ER $\alpha$  protein expression were determined by western

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blot. Actin was used as an internal control (D). ER $\alpha$  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. 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 6 h. 48 h 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.

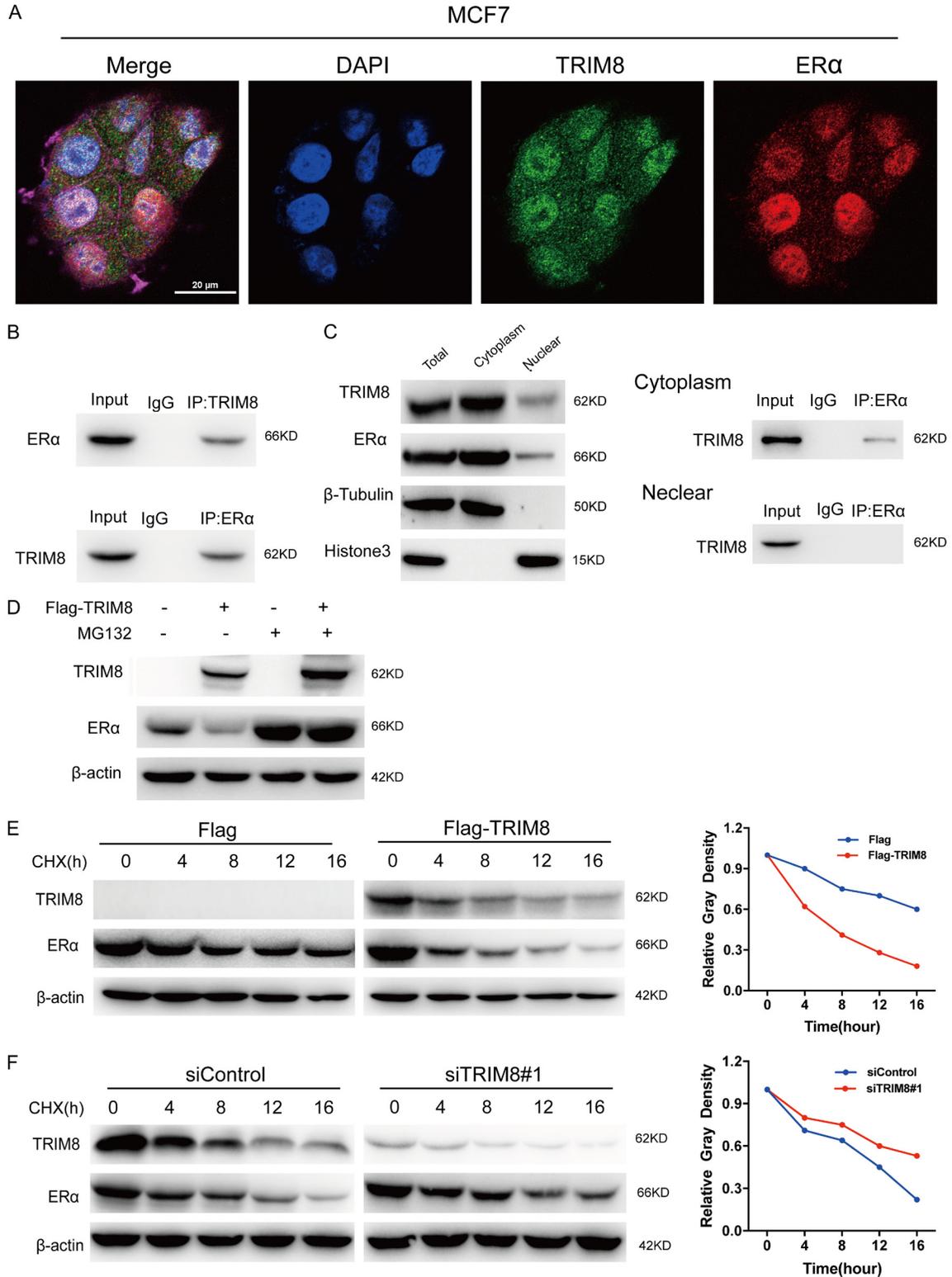
owed that mutant TRIM8 could no longer increase ER $\alpha$  poly-ubiquitination (Figure 8D-F).

### Discussion

BC is the most common cancer among women in the world, and 70% of cases are estrogen receptor  $\alpha$  (ER $\alpha$ ) positive [33]. Endocrine therapy is an important treatment strategy for ER $\alpha$ -positive patients [33]; however, endocrine resistance remains an important concern, and a host of confirmed and hypothetical mechanisms of ER $\alpha$  resistance have been proposed [11]. In addition to ER $\alpha$  gene mutation in the AF-2 domain and the low proportion of ER $\alpha$

gene amplification, endocrine resistance is mainly associated with two mechanisms [34, 35]. One is based on ER $\alpha$  could crosstalk with the EGFR/HER2, MAPK and NF $\kappa$ B signaling pathways, which could promote BC cell proliferation and lead to sustained signaling. In clinics, the crosstalk between ER $\alpha$  and EGFR/HER2 provides a good explanation for why ER-positive and HER2-positive patients do not respond well to tamoxifen [36, 37]. Another proposal is that the modification of ER $\alpha$  signaling via several mechanisms. Many post-translationally modified proteins have been reported to be involved in ER $\alpha$  modification, some of which can affect BC endocrine resistance. For

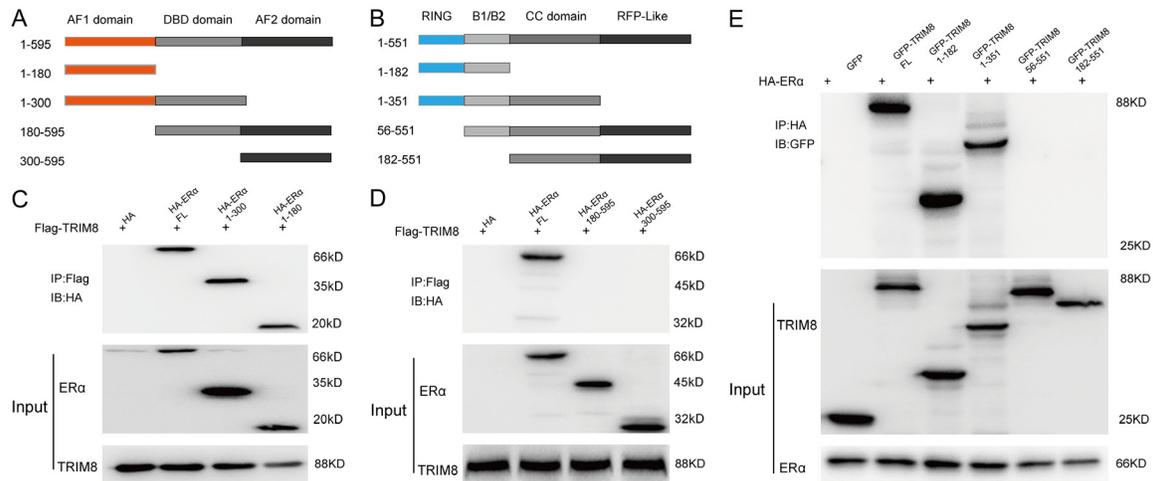
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**Figure 5.** TRIM8 interacts with ERα in the cytoplasm and affects its stability by promoting ERα protein degradation. A. Intracellular localization analysis of TRIM8 and ER alpha by immunofluorescence assay. The results demonstrated that ERα (red) and TRIM8 (green) were distributed both in the nucleus and cytoplasm. B. COIP results indicated that endogenous ERα and TRIM8 could interact with each other. The total cell lysates of MCF7 were precleared with IgG and agarose beads for 2 h and the immunoprecipitated with ERα or TRIM8 antibody overnight,

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while 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.

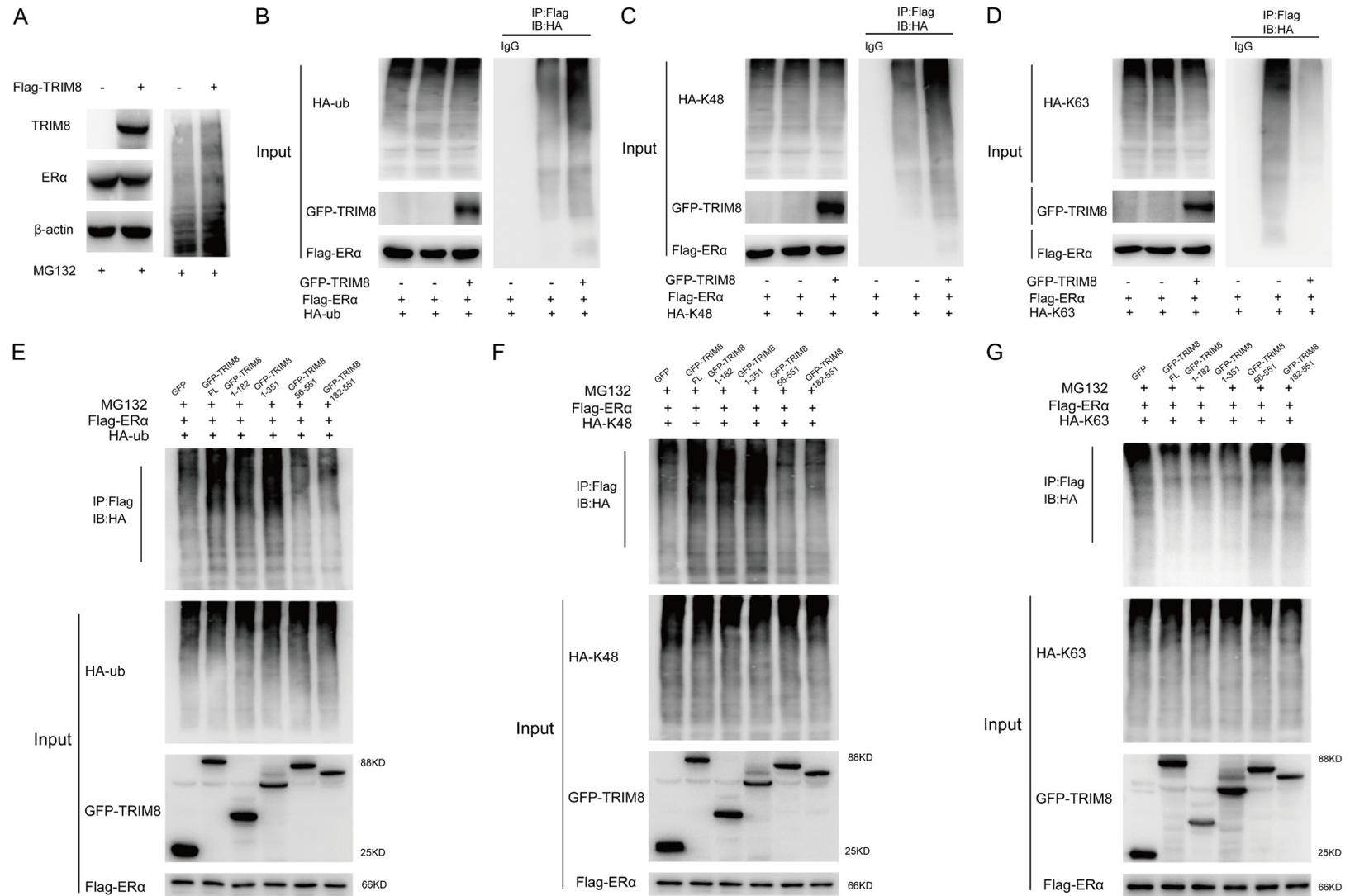


**Figure 6.** TRIM8 interacts with ER $\alpha$  AF1 Domain through Ring Domain. A. ER $\alpha$  domain structure and deletion mutants used in this study: 1. HA-vector, 2. HA-ER $\alpha$ -1-595 (Full Length), 3. HA-ER $\alpha$ -1-180 ( $\Delta$ AF1 domain), 4. HA-ER $\alpha$ -1-300 ( $\Delta$ AF1 domain +  $\Delta$ 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 lysates 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.

example, the phosphorylation of S118 and S167 sites in ER $\alpha$  indicates that the ER $\alpha$  signaling pathway is activated and may be used as a clinical indicator for BC endocrine therapy [38]. P300 promotes the acetylation of ER $\alpha$  at 302/303 site, which may be one reason for the reduced sensitivity of ER $\alpha$  to tamoxifen [39]. Moreover, the ubiquitination of ER $\alpha$  is a critical factor in endocrine resistance [20]. ER $\alpha$  protein ubiquitination does not necessary lead to its degradation, and some E3 ligases, such as RNF8, RNF31 and SHARPIN, promote the mono-ubiquitination of ER $\alpha$  to increase its stability [22-24]. A previous 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]. Since, it has been shown that

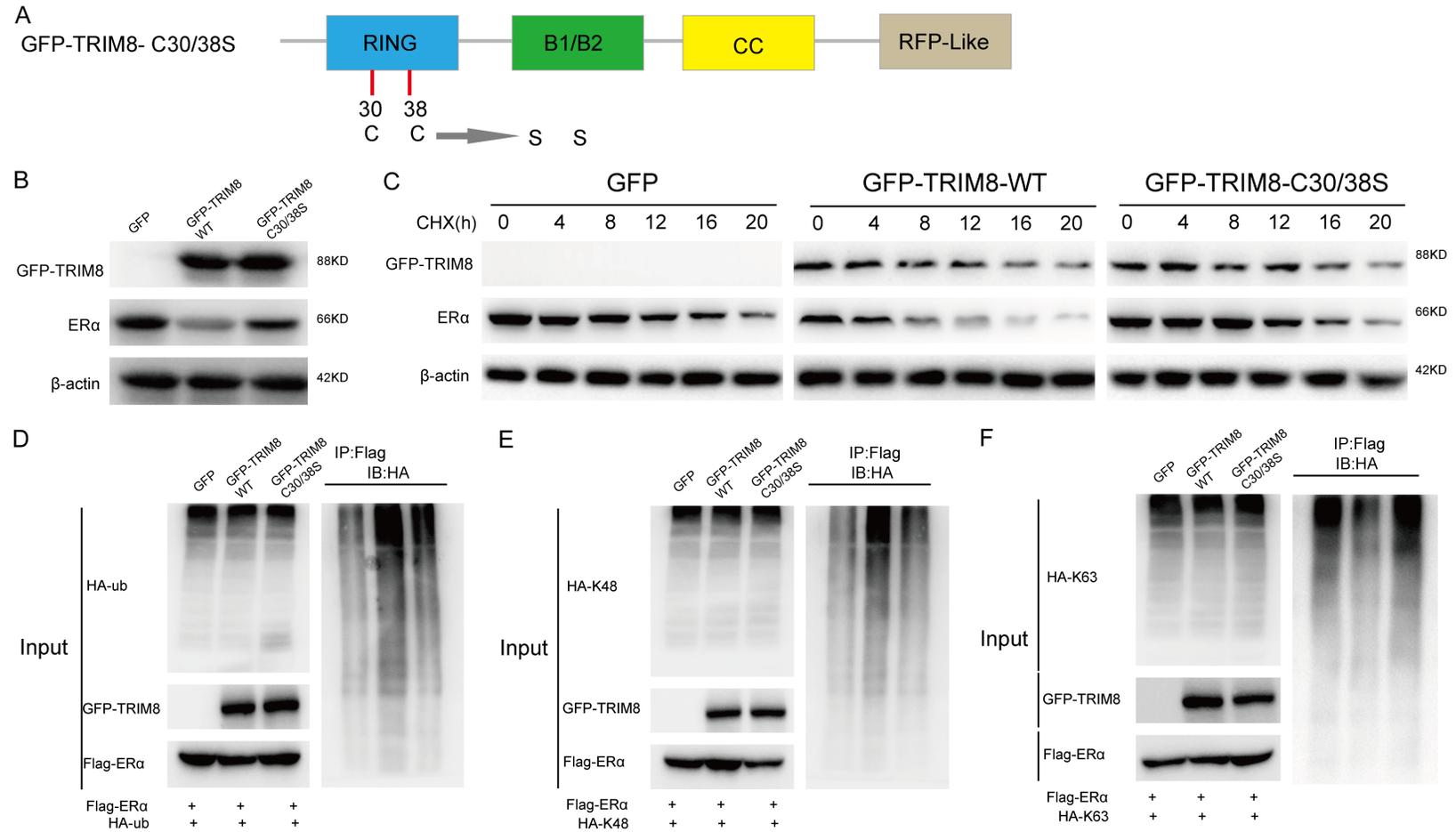
## TRIM8 inhibits BC proliferation



**Figure 7.** TRIM8 increases ER $\alpha$  K48-linked poly-ubiquitination via RING domain. **A.** TRIM8 enhances ER $\alpha$  endogenous poly-ubiquitination. MCF7 cells were transfected with Flag-tag or Flag-TRIM8 plasmid. After 48 hours, the cells were treated with 10  $\mu$ M MG132 for 8 hours. Western blot analysis was used to observe the ubiquitination of ER $\alpha$  protein. **B.** TRIM8 increased poly-ubiquitination of ER $\alpha$ . HEK293 cells were transfected with Flag-ER $\alpha$  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 ER $\alpha$  poly-ubiquitination. **C.** TRIM8 enhanced K48-linked ER $\alpha$  poly-ubiquitination. **D.** TRIM8 decreased K63-linked ER $\alpha$  poly-ubiquitination. **E.** TRIM8 increased ER $\alpha$  poly-ubiquitination via RING domain. HEK293

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cells were transfected with Flag-ER $\alpha$ , 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 ER $\alpha$  poly-ubiquitination. F. TRIM8 increased ER $\alpha$  K48-linked poly-ubiquitination via RING domain. G. TRIM8 decreased ER $\alpha$  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 downregulate ER $\alpha$  protein in MCF7 cells. (C) TRIM8-C30/38S mutant did not decrease the ER $\alpha$  half-life in HEK293 cells. (D-F) The TRIM8 mutant lost its ability to modulate ER $\alpha$  protein poly-ubiquitination. Total ubiquitination (D), K48-linked poly-ubiquitination (E), K63-linked poly-ubiquitination (F).

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 that promotes the regulation of TNF-induced NF- $\kappa$ B pathway by promoting PIAS3 nuclear translocation [48, 49]. However the mechanisms underlying the role of TRIM8 in BC have yet to be fully elucidated.

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 downregulate ER $\alpha$  protein and reduce its stability. COIP based on poly-ubiquitination revealed that mutant TRIM8 could not facilitate K48-linked ubiquitination and 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 K63-linked ubiquitination, thus promotes ER $\alpha$  degradation.

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 mechanisms of ER $\alpha$  signal regulation and decrease resistance to 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 BC cell proliferation *in vitro* and *in vivo*. Interestingly, there may be competition between K48 and K63 poly-ubiquitination. K48-linked poly-ubiquitination enhances ER $\alpha$  protein degradation, which further

inhibits K63-linked poly-ubiquitination. As an ER $\alpha$  regulator, targeting TRIM8 may provide a feasible treatment strategy for ER $\alpha$ -positive BC.

### *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 Ethics Committee. The Wuhan Animal Experiment Administration Committee reviewed and approved all animal experiments.

### **Acknowledgements**

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

### **Disclosure of conflict of interest**

None.

**Address correspondence to:** Gaosong Wu, Department of Thyroid and Breast Surgery, Zhongnan Hospital of Wuhan University, Wuhan, China. E-mail: wugaosongtj@163.com

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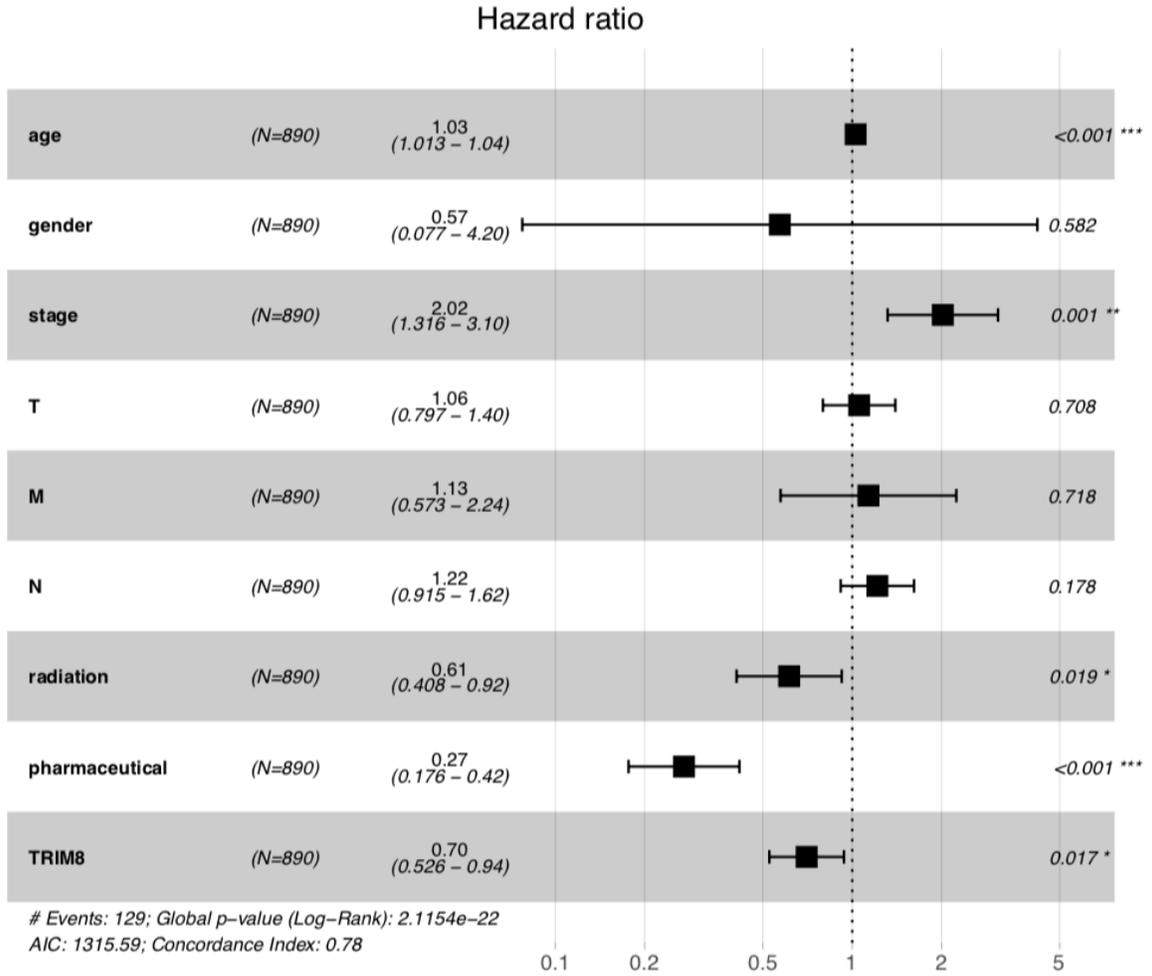
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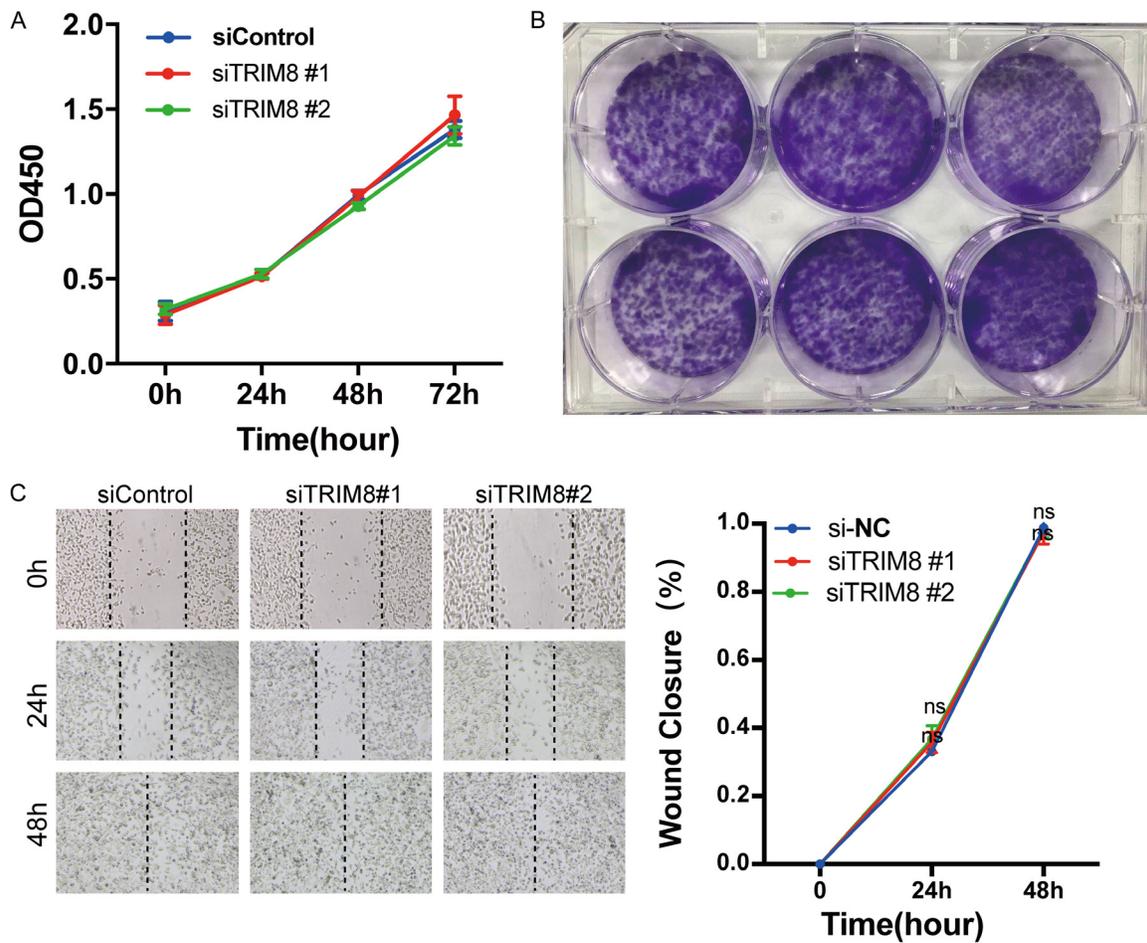
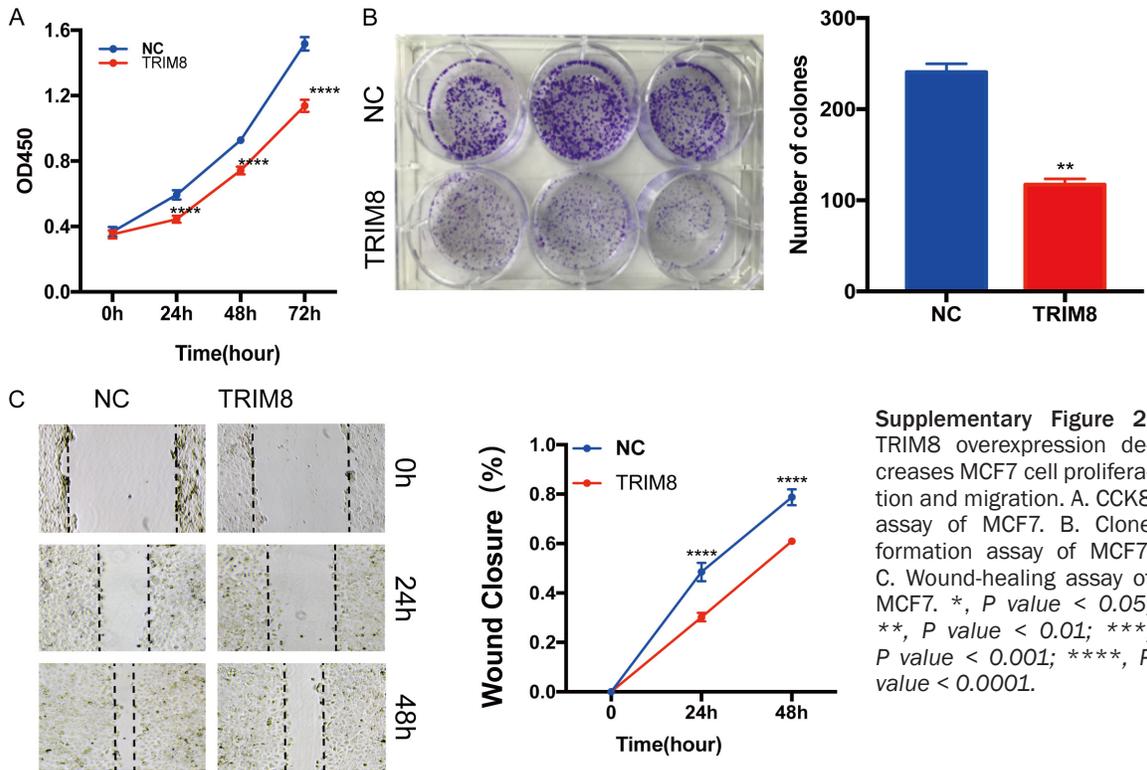
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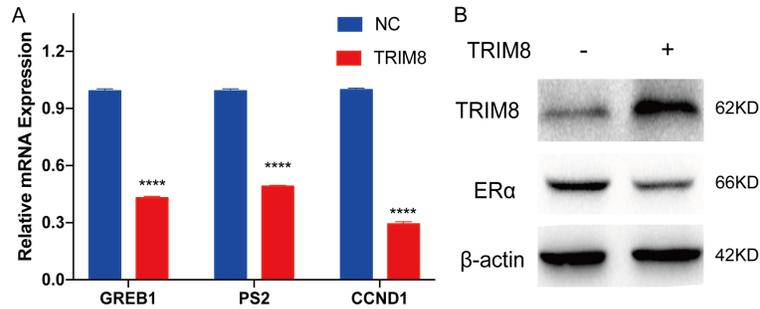
Supplementary Figure 1. Multivariate COX regression analysis of ER-positive BC clinical data in TCGA database.

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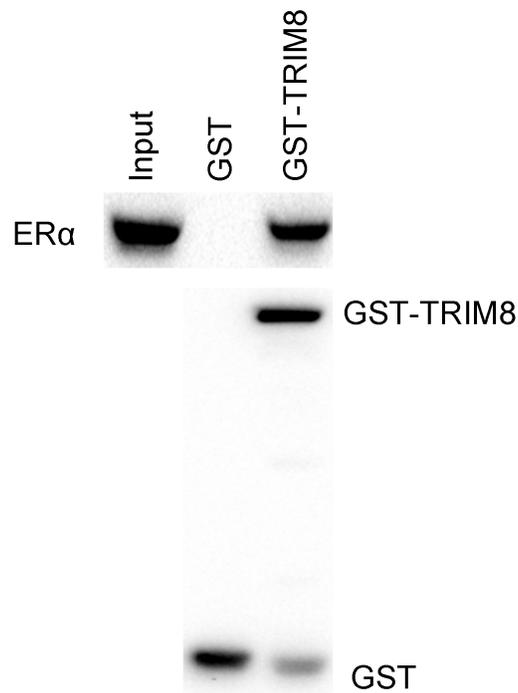


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**Supplementary Figure 3.** TRIM8 depletion had no effect on triple-negative breast cancer cell proliferation. A. CCK8 assay of MDA-MB-231. B. Clone formation assay of MDA-MB-231. C. Wound-healing assay of MDA-MB-231. \*, *P* value < 0.05; \*\*, *P* value < 0.01; \*\*\*, *P* value < 0.001; \*\*\*\*, *P* value < 0.0001.

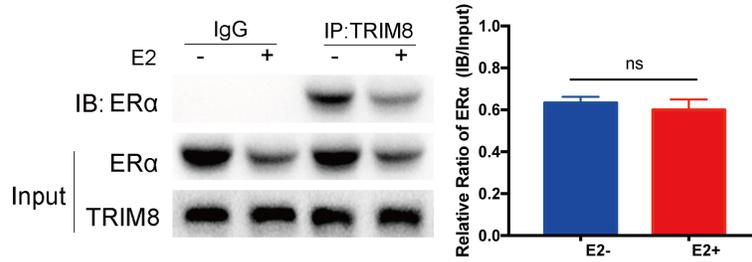


**Supplementary Figure 4.** TRIM8 overexpression decreases ERα protein and target gene levels in MCF7 cells. A. TRIM8 and ERα protein expression were determined by Western blot. Actin was used as an internal control. B. ERα target genes expression (GREB1, PS2, and CCND1) were determined by qRT-PCR. Three replicates were set for each group, and the experiment was repeated three times. 36B4 was used as an internal control. \*, *P* value < 0.05; \*\*, *P* value < 0.01; \*\*\*, *P* value < 0.001; \*\*\*\*, *P* value < 0.0001.

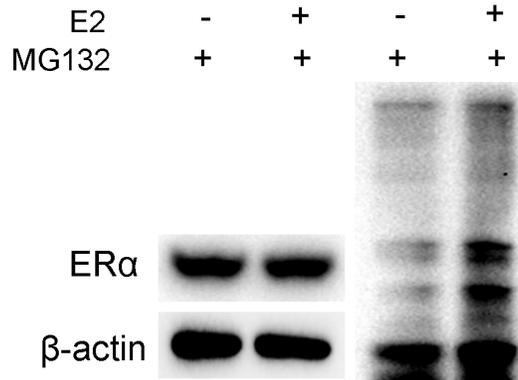


**Supplementary Figure 5.** GST-pulldown assay indicates TRIM8 interacts with ERα directly. The purified GST or GST-TRIM8 was incubated with ERα recombinant protein at 4 °C for 2 h. The mixture was washed three times with GST-pulldown buffer and interacted protein was detected by Western blot.

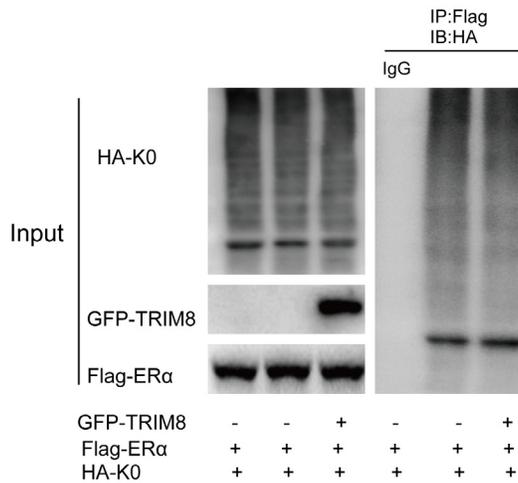
## TRIM8 inhibits BC proliferation



**Supplementary Figure 6.** E2 has no effect on promoting the association between ER $\alpha$  and TRIM8. MCF7 cells were treated with ethanol or 10 nM estradiol for 6 h. The total cell lysates of MCF7 were precleared with rabbit IgG and agarose beads for 2 h and the immunoprecipitated with Anti-TRIM8 antibody overnight, while rabbit IgG was used as the negative control. The bounded protein was analyzed by Anti-ER $\alpha$  antibody.



**Supplementary Figure 7.** E2 enhanced ER $\alpha$  endogenous poly-ubiquitination. MCF7 cells were transfected with Flag-tag or Flag-TRIM8 plasmid. After 48 hours, cells were treated with 10  $\mu$ M MG132 for 8h plus ethanol or 10 nM estradiol for 6 h. The endogenous ubiquitination of ER $\alpha$  was observed by Western blotting.



**Supplementary Figure 8.** TRIM8 overexpression did not affect the monoubiquitination of ER $\alpha$ . HEK293 cells were transfected with Flag-ER $\alpha$  plasmid, HA-KO plasmid, and GFPTRIM8 plasmid. Anti-Flag antibody was used for COIP, and Anti-HA antibody was used for Western blot analysis to detect mono-ubiquitination of ER $\alpha$ .

TRIM8 inhibits BC proliferation

E1/E2/ub	+	+	+
ER $\alpha$	+	+	+
GST	-	+	-
GST-TRIM8	-	-	+

WB: anti-  
Ubiquitin



ER $\alpha$



**Supplementary Figure 9.** TRIM8 overexpression promotes the poly-ubiquitination of ER $\alpha$  *in vitro*. *In vitro* ubiquitylation of ER $\alpha$ . 10  $\times$  E3 Ligase Reaction Buffer, E1, E2, and purified GST-TRIM8 and purified ER $\alpha$  were incubated at 37 °C for 1 h. Anti-ubiquitin antibodies were used to detect poly-ubiquitination of ER $\alpha$ .