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TRIM3 facilitates estrogen signaling and modulates breast cancer cell progression

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Running title: TRIM3 stabilizes ER alpha in breast cancer

Key words: TRIM3, ER alpha, Breast cancer, Stabilize, Ubiquitin

Abstract

Background: Breast cancer ranks NO.1 in women cancer incidence worldwide, while 70% of breast cancers are estrogen receptor (ER) alpha positive. Compared with ER alpha negative breast cancer, which is more aggressive and shorter prognosis, ER alpha positive breast cancer could be well-controlled by endocrine therapy. Most of ER alpha positive breast cancer patients could benefit from selective ER alpha modulators, such as tamoxifen. However, approximately half of them will eventually develop endocrine resistance, making it an important clinical issue in breast cancer therapy. Thus, decoding the turnover of estrogen signaling, including the control of ER alpha expression and stability, are critical to the improvement of breast cancer therapeutics.

Methods: TRIM3 and ER alpha protein expression levels were measured by western blot, while the ER alpha target genes were measured by real-time PCR. MTT assay was used to measure cell viability. RNA sequencing was analyzed by Ingenuity

Pathway Analysis. Identification of ER alpha signaling was accomplished with luciferase assays, real-time RT-PCR and Western blotting. Protein stability assay and ubiquitin assay was used to detect ER alpha protein degradation. The ubiquitin-based Immuno-precipitation based assays were used to detect the specific ubiquitination manner happened on ER alpha.

Results: In our current study, we identified TRIM3 as an E3 ligase, which promotes ER alpha signaling and breast cancer progression. TRIM3 depletion inhibits breast cancer cell proliferation and invasion, while the unbiased RNA sequencing data indicates that TRIM3 is required for the activation of estrogen signaling in whole genomic scale. Molecular studies show that TRIM3 associates with ER alpha and promotes ER alpha mono-ubiquitination.

Conclusion: our study provides a novel post-translational mechanism in estrogen signaling. Modulation of TRIM3 expression or its function could be an interesting approach for breast cancer treatment.

Key words: TRIM3, ER alpha, Breast cancer, Ubiquitin, Stability

Introduction

Breast cancer is the most common women cancer malignancies and the second most common cause of cancer-related death worldwide [1]. The genomic based profiling analysis showed that breast cancer can be classified into four groups: Luminal A, Luminal B, HER2 type and basal-like type [2]. Among the groups, Luminal A and B types could accounts for approximately two thirds of the patients and could benefit from endocrine therapy [3]. The selective estrogen receptor modulators (SERMs) have become the standard treatment for ER alpha positive patients [4]. However, more than half of the patients will develop endocrine therapy resistance, which is a major challenge for breast cancer treatments [5]. Although some cases lose the ER alpha expression in breast cancer cells, most of the endocrine resistant breast cancers still maintain ER alpha expression [6]. Thus it is required and necessary for scientists to characterize the resistance mechanism together with the internal mechanism that controls ER alpha expression and stability.

Estrogen receptor alpha (ER) belongs to the superfamily of nuclear receptors, which was initially cloned from MCF-7 cells in 1985 [7]. ER alpha protein is composed of 595 amino acids by three functional domains: DNA binding domain, transcriptional activation domain and ligand binding domain [8]. When ER alpha is activated by 17 β -estradiol (E2), the ER alpha protein could trans-locate into the nuclear and subsequently bind to the promoter regions of ER alpha target genes, which facilitates certain downstream target gene expression, such as PS2 and GREB1 [9]. Besides,

the effect of ER alpha ligands, the ER alpha protein could also be regulated by several post-translational modifications. The modifications could be widely distributed over the ER alpha protein and affect ER alpha function in several ways. For example, the acetylation of ER alpha protein by P300 could enhance ER alpha activity [10], while the phosphorylation of ER alpha protein at Y537 sites by SRC kinase could change ER alpha conformation and increase the ligand independent activity [11]. Recent studies reveal that several atypical ubiquitination manners could also play important role in modulating ER alpha function. For example, our previous studies showed that RNF31/SHARPIN modulates ER alpha mono-ubiquitination and facilitates ER alpha stability in breast cancer cells [12, 13]. Based on the previous studies, we infer that the components of the ubiquitin-proteasome system, including several E3 ubiquitin ligases, might control the ER alpha ubiquitination, turnover and ER alpha target gene expression.

TRIM3 (Tripartite Motif Containing 3) is one RING finger protein, which was initially founded to be a regulator for myosin function and facilitate the protein transportation in cells [14, 15]. Several studies showed decreased expression level of TRIM3 and potential role as a tumor suppressor in quite a few human cancers [16-19]. However, our data showed that TRIM3 specifically related to poor survival in endocrine therapy patients. Our data demonstrated TRIM3 as one candidate factor in modulating estrogen signaling in breast cancer cells. TRIM3 associates with ER alpha, facilitates ER alpha stability possibly by inducing ER alpha mono-ubiquitination and enhances ER alpha signaling activity. Our study uncovers a novel non-genomic regulation between TRIM3 and ER alpha signaling for breast cancer progression.

Materials and Methods

Cell culture

MCF-7, T47D and HEK293 cells were acquired from American Type Culture Collection (ATCC). MCF-7 and HEK293 were cultured with Dulbecco's Modified Eagle's Medium together with 4.5 g/L glucose and 4 mM L-glutamine (DMEM, 41965, Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS, 10270, Life Technologies). T47D cells are cultured with RPMI-1640 (42401, Life Technologies) supplemented with 2 mM L-glutamine (25030, Life Technologies) and 10% FBS. All cell lines are characterized by cell line authentication. The cell line authentication via Short Tandem Repeat (STR) is performed via PowerPlex 21 system. The STR data of MCF-7 and T47D cell lines are found consistent with STR data in ATCC.

Plasmids and siRNA

The Flag-TRIM-3 plasmid was acquired from Origene. The ER alpha full and deletion constructs were described in previous study [20]. The TRIM3 and ER alpha deletion variants were sub-cloned from the original plasmids. The HA-Ub-K48, HA-UbKo, HA-Ub-K63 and HA-Ub plasmids were used in previous study. The Estrogen-Response-Element (ERE)-TK reporter and renilla plasmids were used in previous study and are transfected with Lipofectamin 200 (1662298, Invitrogen). The Lipofectamin 2000 (1662298, Invitrogen) was used for the plasmids transfection. Small interfering RNAs were used for specific gene knocking-down. The TRIM3 siRNA sequences were: CAAACGAAAGGACAACCCAdTdT; UGGGUUGUCCUUUCGUUUGdTdT and GCAACAACCAGUGUAUUCAdTdT; UGAAUACACUGGUUGUUGCdTdT. The negative control siRNA sequences were: UUCUCCGAACGUGUCACGUTT; ACGUGACACGUUCGGAGAATT. The RNAiMAX reagent (13778150, invitrogen) was used for siRNA transfection.

RNA extraction and qPCR analysis

RNeasy plus mini kits were used to extract total RNA (Qiagen). Real-time PCR was performed as previously described [21]. 36B4 was used for internal control. The primer sequences were shown here. 36B4: F: GGCGACCTGGAAGTCCAACCT; R: CCATCAGCACCACAGCCTTC. GREB1 F: CGT GTG GTG ACT GGA GTA GC, R: ACC TCT TCA AAG CGT GTC GT; ER F: GCT ACG AAG TGG GAA TGA TGA AAG, R: TCT GGC GCT TGT GTT TCA AC; PS2 (TFF1) F: TGG GCT TCA TGA GCT CCT TC, R: TTC ATA GTG AGA GAT GGC CGG.

Quantification of cell viability

MCF-7 and T47D cells were transfected with siTRIM3 or siControl in 24-well plate. Twenty-Four hours after transfection, the cells number was countered and 4000 cells were seeded into 96-well plates. The relative cell viability was measured at indicated time points. Cell numbers were determined using the WST-1 cell proliferation reagent as previously described [21].

Wound healing assay

Fifty nM TRIM3 siRNA or siControl were transfected into MCF-7 or T47D cells. After twenty-four hours, cells were seeded into 12-well plates with 1%FBS. The cells were 100% confluence. The yellow pipette tips were applied for straight scratch. The wound distance was measured at indicated time points and normalized with starting time point. The wound healing recovery was expressed as: $[1 - (\text{Width of the wound at a given time} / \text{width of the wound at } t=0)] \times 100\%$.

Trans-well assay

Cell invasion capacity was measured using the modified two-chamber plates as before. For invasion assay MCF-7 and T47D cells were transfected with 50 uM TRIM3 siRNA or sControl. In order to stimulate invasion, the bottom wells were filled with complete medium, while the upper chambers were added with FBS-free medium. After twelve hours, cells were carefully removed and the cells that invaded through the membrane were fixed and stained with Crystal Violet Staining solution. The cell numbers were counted by using a microscope.

Western blotting

Cells were harvested and lysed with RIPA buffer. Proteins were separated by electrophoresis on SDS-polyacrylamide gel electrophoresis (PAGE) and electro-transferred to PVDF membrane. The antibodies used in this study were listed here: Anti-TRIM3 (HAP043396, Sigma); Anti-ER alpha (D8H8, 8644, Cell signaling Technology); Anti-HA (MMS-101R, COVANCE); Anti-myc (9E10, ab32, Abcam); Anti-myc (Ab9106, Abcam); Anti-Actin (A5441, Sigma); Anti-Flag (20543-1-AP, Proteintech); Anti-GFP (Ab290, Abcam). Membranes were then washed with PBS for three times and incubated with secondary antibodies Peroxidase-Conjugated AffiniPure Goat Anti-Mouse IgG or Goat Anti-Rabbit IgG. Fluorescent signals were visualized with ECL system. (amersham imager 600, USA).

Luciferase assay

The luciferase activity of estrogen signaling activity was performed using the Dual-Luciferase Reporter kit (Promega, Germany). The ERE luciferase reporter was transfected together with the Renilla plasmid into the cells. Luciferase activity was measured after 24 h.

Co-immunoprecipitation assay

Immunoprecipitation was performed as described in previous study [20]. The MCF-7 total cell lysls were pre-cleared with rabbit IgG for 2 h and subsequently immunoprecipitated with ER alpha antibody (SC8005, Santa Cruz) over night, while rabbit IgG (Santa Cruz) was used as the negative control. The bounded protein was analyzed by Anti-TRIM3 (HAP043396, Sigma). For the overexpression experiment, HEK293 cells were transfected with 5ug GFP-TRIM3 (Full length or deletion domains) and ER alpha plasmid (Full length or deletion domains) in 10 cm dish. Cell lysates were pre-cleared with IgG and subsequently incubate with GFP (Ab290, Abcam) antibody, while rabbit IgG was used as the negative control. The bound proteins were analyzed by western blotting.

Poly-ubiquitination detection assay

To directly detect the enriched overall ubiquitinated, K63-ubiquitinated, mono-ubiquitinated and K48-ubiquitinated ER alpha from the cell extracts, HEK293 cells were transfected with 4 ug Ub, 4 ug K63 Ubi, 4 ug Ub-KO or 4 ug K48 Ubi plasmid, 2 ug ER alpha together with 0.5 ug Myc-TRIM3 or Myc-vector. After 48 h, total protein was extracted and pre-cleared with 20ul protein A (santa cruz, SC-2001) for 2 h. The supernatant was collected and immunoprecipitated by ER alpha antibody. Western blot with HA antibody was performed to detect specific ubiquitinated ER alpha.

Immunofluorescence assay

MCF-7 cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.2% Triton X-100 for 5 min, and blocked by 5% BSA in PBS for 1 h. A rabbit Anti-TRIM3 (HAP043396, Sigma) and mouse anti-ERα monoclonal antibodies (SC-56833) were used, followed by Alexa Flour 647 (Invitrogen) anti-rabbit antibody and FITC-conjugated anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA). As negative controls, the samples were incubated with the secondary antibodies without primary antibodies. Images were acquired under conditions fulfilling the Nyquist criterion using Nikon A+ laser scanning confocal system with a 60X oil NA1.4 objective and pinhole size of 1.0 Airy Unit. The acquired pictures were further processed and assembled using ImageJ.

Clinical breast tumor samples

One hundred and twenty three formalin-fixed paraffin-embedded breast cancer samples were collected from the Department of Pathology, Shandong Qilu Hospital. All the breast tumors samples were examined by TRIM3 status, ER alpha status, PR status, HER2 status by pathological specialists. The pathological grade plus lymph node metastasis status of each sample was also examined by pathological specialists. This study was reviewed and approved by the Ethical Board at the Qilu Hospital of Shandong University with written informed consent from all the patients.

Results:

TRIM3 is required for ER alpha positive breast cancer progression

We utilized two independent siRNAs to test TRIM3 effect on breast cancer cells. The knocking down efficiency of siRNAs were shown in Figure 1A. The WST-1 assay showed that TRIM3 knocking down decreased the speed of cell growth in MCF-7 and T47D cells (Fig. 1B-1C). We further investigated the role of TRIM3 in cell migration. The wound-healing assay showed that TRIM3 depletion inhibited the wound closure speed in both MCF-7 and T47D cells (Fig. 1D-1E). The trans-well assay indicated that TRIM3 depletion hampered the capability of cell migration in both MCF-7 and T47D cells (Fig.1F-1G). All these experiments might indicate the oncogenic role of

TRIM3 in ER positive breast cancer cells.

TRIM3 modulates estrogen signaling in breast cancer cells

We further investigated the role of TRIM3 in whole genomic scale. The RNA sequencing analysis showed that TRIM3 depletion inhibited several signaling, including ER alpha signaling (Fig. 2A). The heat map data showed that TRIM3 depletion affected a group of ER alpha signature genes, including PS2 and PR (Fig. 2B). Western blotting assay showed that TRIM3 depletion inhibited ER alpha protein level in MCF-7 cells (Fig. 2C). Further QPCR experiments showed that TRIM3 depletion reduced ER alpha classical target gene expression, including PS2 and GREB1. We further test TRIM3 effect on ER alpha signaling in both vehicle and E2-treated conditions. TRIM3 depletion could decrease ER alpha protein level and ER alpha target gene expression in vehicle and E2-treated conditions in MCF-7 cells (Fig. 2E-2F). In order to determine if TRIM3 knockdown could affect ER alpha transcriptional activity, we measure estrogen response element (ERE) luciferase activity in MCF-7 cells. The luciferase assay shows that TRIM3 depletion decreases ERE luciferase activity in MCF-7 cells (Fig. 2G).

TRIM3 correlates with ER alpha protein level and its target genes expression in breast cancer samples.

From the survival data analysis, we observed that ER alpha expression correlated with poor survival in endocrine therapy patients (Fig. 3A) (<https://kmplot.com/analysis/>). We further analyzed the expression relation between TRIM3 and ER alpha classical target genes. From TCGA database, we observed that TRIM3 correlated with TFF1/GREB1 expression in 1080 breast tumor samples (Fig. 3B-C) (<https://tcga-data.nci.nih.gov/tcga/>). In order to investigate the correlation between TRIM3 expression and breast cancer molecular biomarkers, 123 breast cancer tissues were collected for immunohistochemistry (IHC) analysis. We examined the protein expression levels including TRIM3, ER alpha, PR and HER2 (Fig. 3D). Besides, the pathological grade and lymph node metastasis data were also collected. The IHC analysis implicate that TRIM3 expression correlates with ER alpha protein levels ($P<0.001$) and PR protein levels ($P<0.001$). Besides, TRIM3 expression levels were also correlated with lower pathological grade ($P=0.04$) (Fig. 3E).

TRIM3 associates with ER alpha and modulates ER alpha stability

We further examined the localization of TRIM3 and ER alpha in breast cancer cells. The immuno-staining indicated that ER alpha was mainly located in the nuclear, while TRIM3 located both in the cytosol and nuclear (Fig. 4A). The endogenous

immuno-precipitation showed that TRIM3 could interact with ER alpha in MCF-7 cells (Fig.4B). Since TRIM3 could associate with ER alpha in breast cancer cells, we further investigated the biological effect of such interaction. Since ER alpha could regulate its own expression, making it difficult to distinguish direct effects of TRIM3 on ER alpha mRNA or protein levels in the cell line. We utilized HEK293 cells to investigate the mechanism. Co-transfection of ER alpha and TRIM3 in HEK293 cells showed that TRIM3 could increase ER alpha protein level (Fig. 4C), which effect could be minimized with the presence of the proteasome inhibitor MG132 (Fig. 4D). The protein half-life assay showed that TRIM3 could increase the protein stability of ER alpha (Fig. 4E-4F).

TRIM3 associates with ER alpha DNA binding domain via its Filamin/NHL domain

We further characterize the interaction domains between ER alpha and TRIM3. ER α is composed of three functional domains: AF1 domain, DNA binding domain and AF2 domain, while TRIM3 contains RING domain, B1/B2 domain, CC domain and Filamin/NHL domain. We made the ER α variants (AF1 domain, AF1+DBD domains, AF2 domain and AF2+DBD domains) and TRIM3 variants (Δ RING+ Δ B1/2 domains, Δ Filamin/NHL domain, Δ Filamin/NHL domain+CC domains) (Fig. 5A). Immuno-precipitation implicates that the DBD domain of ER alpha was required for its interaction with TRIM3 (Fig. 5B-5C), while the Filamin/NHL domain of TRIM3 was required for its interaction with ER alpha (Fig. 5D-5E). However, co-transfection of ER alpha with TRIM3 variants showed that only the full length of TRIM3 could exert its stabilization function on ER alpha protein (Fig.5F).

TRIM3 facilitates K63-linked ubiquitination and mono-ubiquitination of ER alpha.

Since TRIM3 is a putative E3 ubiquitin ligase, we further investigated the role of TRIM3 on ER alpha ubiquitination. The ubiquitination-based immuno-precipitation shows that TRIM3 could inhibit ER alpha overall poly-ubiquitination (Fig. 6A). Since K48-linked ubiquitination is the most common degradation manner, we examined the TRIM3 effect on K48-linked ubiquitination of ER alpha, which implicated that TRIM3 could inhibit K48-linked ubiquitination of ER α (Fig. 6B). Besides, since K63-linked ubiquitination or mono-ubiquitination of ER alpha could increase ER alpha stability and its signaling activity, we further analyzed effect of TRIM3 on ER alpha ubiquitination on these two forms. The data indicated that TRIM3 could significantly increase K63-linked poly-ubiquitination and mono-ubiquitination on ER alpha protein (Fig. 6C-6D).

Discussion

In our current study, we identified one RING family protein TRIM3 in modulating ER alpha signaling via its stability (Fig. 6E). Clinical data analysis indicated the positive correlation between TRIM3 and ER alpha protein level in IHC human sample. Besides, TRIM3 correlated with poor survival in endocrine therapy patients. Our study provided a non-genomic regulation on ER alpha stability control. Based on these study, we can propose that the modulating or inhibition of TRIM3 protein could an interesting strategy for ER alpha positive breast cancer treatments.

ER alpha gene was firstly discovered 30 years ago [7]. Based on the current understanding about breast cancer, ER alpha signaling is the main driver for two thirds of the breast cancer patients [22]. Although the gene amplification or mutation of ER alpha is not common in breast cancer, the increased expression could be observed in most of the breast tumors compared with normal breast tissue [23]. Thus, targeting ER alpha signaling is an excellent strategy for breast cancer, while the selective modulator of ER alpha, including tamoxifen, demonstrated a great success in inhibiting ER alpha signaling and breast cancer progression [22]. However, the occurrence of endocrine resistance is a headache problem for breast cancer therapy. Interestingly, most of the endocrine resistant breast tumors still maintain ER alpha expression, which lends to the possible involvement of ER alpha in drug resistance. Several confirmed and possible mechanisms indicate that the crosstalk between ER alpha and other signaling pathways or ER alpha modifications play important roles in medicating endocrine resistance. For example, ER alpha could trans-activate several growth factor pathways, including IGFR and AKT signaling, while the activation of these signaling cascades promotes the phosphorylation of ER alpha and facilitates ER alpha trans-activation [24]. Besides, recent studies reveal that the protein modifications of ER alpha could stabilize ER alpha or enhance ER alpha transcriptional activity in breast tumors [10, 25]. Thus, modulating of ER α expression and stability could be a promising strategy for breast cancer therapeutics and endocrine resistance.

There are about 500-1000 E3 ubiquitin ligases, which could be divided into four groups: HECT type, RING type, U-box type and PHD-finger type [26]. Among these, the RING family is the biggest and the function is not totally clear. Interestingly, recent studies showed that some RING family E3 ligases preferred to catalyze in atypical ubiquitin manner, which did not necessarily lead to degradation [27]. Several RING family members participate in ER α signaling activation and breast cancer progression via non-degradation related ubiquitinations. For example, RNF8 was shown to associate with ER alpha, promote ER alpha mono-ubiquitination and its signaling

transduction [28]. Besides, our previous work also identified a few E3 ubiquitin ligases in modulating ER alpha stability via non-genomic manner, including TRIM56 and RNF31 [29, 30].

TRIM3 belongs to the RING finger family proteins and is comprised of zinc-binding domain, RING domain, B1/2 domain and coiled region [19]. TRIM3 was firstly reported to interact with myosin and promoted the transportation of the target proteins [15]. TRIM3 decreased the expression in several human cancers, including gastric cancer, colon cancer and liver cancer [18, 19, 31, 32]. However, even TRIM3 was suggested as a tumor suppressor protein in several cancer types, we did not observe any dramatic expression change in breast cancer compared with normal breast tissue, which might indicated the uncertain role or dual roles of TRIM3 in breast cancer. Our molecular study revealed that TRIM3 could facilitate ER alpha signaling, promoting cell proliferation and invasion in breast cancer. Our finding indicates TRIM3 plays a oncogenic role in ER alpha positive breast cancer cells, which is opposite to previous studies [18, 19]. This interesting finding not only increases the understanding of ER alpha post-translational modifications but also implicates the multi-face of TRIM3 in different cancer background.

In conclusion, we identified an interesting E3 ligase TRIM3 in facilitating ER alpha signaling in breast cancer cells. TRIM3 could promote breast cancer cell invasion and proliferation via stabilizing ER alpha protein. As a novel modulator of ER alpha signaling, disturbing TRIM3 activity or affecting TRIM3 expression could be a plausible way to treat luminal types of breast cancer.

Public available clinical data analysis

Analysis of TRIM3 with clinical prognosis was carried out through KMPLLOT database (<https://kmplot.com>). Analysis of TRIM3 correlation with ER alpha target gene (TFF1 and GREB1) was carried out by TCGA database with 1080 breast cancer samples. The analysis was carried out with “ggcorrplot” package in the statistical environment R-3.6.1 version. The “ggcorrplot” package version is “ggcorrplot 0.1.3”. The detailed information of “ggcorrplot” can be found in the following link: <https://github.com/kassambara/ggcorrplot>.

RNA sequence analysis

The global gene expression analysis (siControl and siTRIM3) was based on RNA sequencing platform from BGI (Beijing Genomic Institute). The RNA sequence data are deposited in the Gene Expression Omnibus (GEO) database (Assessing number: GSE143610). Analysis was performed for differentially expressed genes ($P < 0.01$

and fold change > 2) by Ingenuity Pathway Analysis (IPA).

Statistics

Student's t-test, Pearson correlation coefficient, and Cox regression analysis were used for comparisons. A P-value of < 0.05 was considered to be significant.

Abbreviations:

TRIM3: Tripartite Motif Containing 3

AF1: Transcriptional activation domain 1

DBD: DNA binding domain

AF2: Transcriptional activation domain 2

ER alpha: Estrogen receptor alpha

HER2: Human epidermal growth factor receptor 2

PR: Progesterone receptor

TBNC: Triple negative breast cancer

RBR: RING-between-RING domain

ZF: Zinc finger domain

EMT: Epithelial-mesenchymal transition

ATCC: American Type Culture Collection

Declarations

Ethics Approval and Consent to participate: This study was reviewed and approved by the Ethical Board at Xinxiang Medical University. This usage of clinical samples was reviewed and approved by the Ethical Board at the Qilu Hospital of Shandong University with written informed consents from all the patients.

Consent for publication: All authors read the manuscript and agree for publication.

Availability of supporting data: The RNA sequence data are deposited in the Gene Expression Omnibus (GEO) database (Assessing number: GSE143610)

Competing Interests: The authors declare no conflict of interest.

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Authors' contributions: BB.W, XJ.T L.W and P.S performed most of the bench work. X.L, HJ.Y, YQ.C, X.Y, ZB.L, C.Y, Y.X, CM.Z, ZG.N and Q.C participated in western blot, real time PCR work. YL.D, ZP.Z, QS.H and T.Z supervised the process of the study and performed the manuscript writing.

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Figure legends:

Figure 1: TRIM3 is required for ER alpha positive breast cancer progression.

A: TRIM3 knocking down efficiency in MCF-7 cells. MCF-7 cells were transfected with TRIM3 siRNA. After 48 h, TRIM3 mRNA levels were determined by QPCR.

B: TRIM3 depletion inhibited MCF-7 cells proliferation. MCF-7 cells were transfected with siControl or siTRIM3. After 24 hours, the WST assay was used to determine the cellular metabolic activity at indicated time points after infection. Experiments were done in triplicates. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for cell growth comparison.

C: TRIM3 depletion inhibited T47D cells proliferation. T47D cells were transfected with siControl or siTRIM3. After 24 hours, the WST assay was used to determine the cellular metabolic activity at indicated time points after infection. Experiments were done in triplicates. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for cell growth comparison.

D and E: Wound-healing assay indicated that TRIM3 depletion inhibited breast cell migration capacity. MCF-7 or T47D cells were transfected with siControl or siTRIM3. After 24 hours, cells were transfected with siTRIM3 or siControl. Quantification of wound closure at the indicated time points. Data are presented as $\pm$ SD. **, $P < 0.01$, ***, $P < 0.001$ (student's t-test).

F and G: TRIM3 depletion inhibited breast cancer cell migration capacity. MCF-7 or T47D cells were transfected with siControl or siTRIM3. After 24 hours, cells were transfected with siTRIM3 or siControl. After another 24 hours, cancer cells were seeded into the chamber for trans-well assay. The cell number was counted and Data are presented as $\pm$ SD. **, $P < 0.01$, ***, $P < 0.001$ (student's t-test).

Figure 2: TRIM3 modulates estrogen signaling in breast cancer cells.

A: Top 10 signaling pathways significantly decreased by TRIM3 depletion in MCF7 cells. The pathway-enrichment analysis was used by the threshold $P < 0.001$ and fold change > 2 to derive regulated genes. TRIM3 was depleted by siRNA (mix of siTRIM3 #1 and siTRIM3 #2) or treated with siControl. After 48 hours, the whole mRNA was extracted for RNA sequence analysis. The siControl and siTRIM3 were done in triplicates.

B: The heat-map graph showed the ER alpha regulating genes, which is significantly inhibited by TRIM3 depletion in MCF-7 cells.

C: TRIM3 consumption decreased ER alpha protein levels via two independent siRNAs in MCF-7 cells. MCF-7 cells were transfected with siControl or siTRIM3. After 48 hours, cells were harvested for western blot analysis. TRIM3 and ER alpha protein levels were determined by Western blot. Actin was used as internal control.

D: TRIM3 consumption decreased ER alpha target gene expression via two independent siRNAs in MCF-7 cells. MCF-7 cells were transfected with siControl or

siTRIM3. After 48 hours, total RNA was extracted for gene expression analysis. *P<0.05; ** P<0.01; ***P<0.001 for target gene expression comparison.

E: TRIM3 depletion decreased ER alpha protein levels in both vehicle and E2-treated conditions in MCF-7 cells. MCF-7 cells were transfected with siTRIM3 or siControl. After 48 h, cells were treated with either ethanol or 10nM estradiol for 6 h. TRIM3 and ER alpha protein levels were determined by Western blot analysis. Actin was used as internal control.

F: TRIM3 depletion decreased ER alpha target genes in both vehicle and E2-treated conditions in MCF-7 cells. MCF-7 cells were transfected with siTRIM3 or siControl. After 48 h, cells were treated with either ethanol or 10nM estradiol for 6 h. Total RNA was prepared and the expression of the endogenous ER alpha target genes, PS2, GREB1, and PKIB were determined by qPCR. Shown are the results from three experiments. *P<0.05; ** P<0.01; ***P<0.001 for target gene expression comparison.

G: TRIM3 depletion affected ERE-luciferase activity in MCF-7 cells. MCF-7 cells were transfected with siTRIM3 or siControl together with ERE luciferase reporter plasmid. Cells were treated with 10 nM estradiol or vehicle. Luciferase activity was measured 48 h after transfection. Shown are the results from three experiments. *P<0.05; ** P<0.01; ***P<0.001 for luciferase activity comparison.

Figure 3: TRIM3 correlates with ER alpha protein level and its target genes expression in breast cancer samples.

A: TRIM3 correlated with poor endocrine therapy outcome in public available database (<https://kmplot.com>).

B and C: Public available data shows that TRIM3 was reversely correlated with ER alpha target genes: GREB1 and TFF1 (<https://www.cbioportal.org>).

D: Example tumor cases showing that TRIM3 reversely correlated with ER alpha and PR protein in IHC

E: Statistical analysis of TRIM3 correlation with ER alpha in 123 human breast tumor samples.

Figure 4: TRIM3 associates with ER alpha and modulates ER alpha stability.

A: Intracellular localization analysis of ER alpha and TRIM3 by immunofluorescence assay. MCF-7 cells were cultured in normal medium before fixation. Intracellular localization of ER alpha (red) and TRIM3 (green) were shown. Nuclei (blue) were stained with 4',6-diamidino-2-phenylindole (DAPI).

B: Co-IP assay revealed association between endogenous ER alpha and TRIM3 protein in MCF-7 cells. MCF-7 cells were harvested with RIPA lysis buffer. CO-IP was performed using antibody as indicated.

C: TRIM3 could stabilize ER alpha protein. HEK293 cells were transfected with 1 ug

ER alpha together with different amounts of TRIM3 plasmids. After 24 h, cell lysates were prepared for Western blot analysis. The results are representative for three independent experiments.

D: In the presence of the proteasome inhibitor MG132, the stabilization effect of TRIM3 on ER alpha did not further increase ER alpha protein levels. HEK293 cells were transfected with TRIM3 and ER alpha plasmids. After 24 h, cells were treated with 10 uM MG132/vehicle for 6 h. Cell lysates were prepared for Western blot analysis. The results are representative for three independent experiments.

E: TRIM3 increases ER alpha half-life in HEK293 cells. HEK293 cells were transfected with HA-ER α plasmid and Myc-tag or Myc-TRIM3 plasmids. After 24 h, cells were treated with 100 μ M cycloheximide/vehicle for indicated times. Cell lysates were prepared for Western blot analysis. The results are representative for three independent experiments. The ER alpha relative density was measured by Image J software.

Figure 5: TRIM3 associates with ER alpha DNA binding domain via its Filamin/NHL domain.

A: ER alpha domain structure and deletion mutants were used in the study and TRIM3 full length and deletion mutants were used in the study.

B and C: ER alpha interacted with TRIM3 through its DBD domain. HEK293 cells were transfected with 2 μ g Myc-TRIM3 together with HA-ER alpha full length or mutants (Δ AF1, Δ AF1+ Δ DBD, Δ AF2 and Δ AF2+ Δ DBD). After 24 h, cells were harvested with NP-40 lysis buffer. CO-IP was performed using Myc antibody. The possible interacted ER alpha domains were detected by HA antibody.

D and E: The Filamin/NHL domain was required for TRIM3 to interaction with ER alpha. HEK293 cells were transfected with 2 μ g HA-ER alpha together with GFP-TRIM3 full length or mutants (Δ N-RING domain, Δ RING+ Δ CC domain, Δ Filamin/NHL domain, Δ Filamin/NHL+ Δ CC domain). After 24 h, cells were harvested with NP-40 lysis buffer. CO-IP was performed using HA antibody. The possible interacted TRIM3 domains were detected by GFP antibody.

F: The intact TRIM3 protein was required for its stabilization effect on ER alpha. HEK293 cells were transfected with 2 μ g Flag- ER alpha and 0.5 μ g GFP-TRIM3 full length or mutants (Δ N-RING domain, Δ RING+ Δ CC domain, Δ Filamin/NHL domain, Δ Filamin/NHL+ Δ CC domain). The ER alpha protein levels were detected via western blotting analysis.

Figure 6: TRIM3 facilitates K63-linked ubiquitination and mono-ubiquitination of ER alpha.

A: TRIM3 decreased poly-ubiquitination of ER alpha. HEK293 cells were transfected

703 with 2 µg ER alpha plasmid, 0.5 µg HA Ub plasmid and 0.5 µg GFP-tag or
704 GFP-TRIM3 plasmids. The cell extracts were immunoprecipitated with HA antibody.
705 The poly-ubiquitinated ER alpha was detected via western blotting analysis.

706 **B:** TRIM3 decreased K48-linked poly-ubiquitination of ER alpha. HEK293 cells were
707 transfected with 2 µg ER alpha plasmid, 0.5 µg HA-K48 Ubi plasmid and 0.5 µg
708 GFP-tag or GFP-TRIM3 plasmids. The cell extracts were immunoprecipitated with HA
709 antibody. The K48 specific poly-ubiquitinated ER alpha was detected via western
710 blotting analysis.

711 **C:** TRIM3 promoted K63-linked poly-ubiquitination of ER alpha. HEK293 cells were
712 transfected with 2 µg ER alpha plasmid, 0.5 µg HA-K63 Ubi plasmid and 0.5 µg
713 GFP-tag or GFP-TRIM3 plasmids. The cell extracts were immunoprecipitated with HA
714 antibody. The K63 specific poly-ubiquitinated ER alpha was detected via western
715 blotting analysis.

716 **D:** TRIM3 promoted mono-ubiquitination of ER alpha. HEK293 cells were transfected
717 with 2 µg ER alpha plasmid, 0.5 µg HA-Ub-KO plasmid and 0.5 µg GFP-tag or
718 GFP-TRIM3 plasmids. The cell extracts were immunoprecipitated with HA antibody.
719 The mono-ubiquitinated ER alpha was detected via western blotting analysis.

720 **E:** The hypothesis model of TRIM3 regulating ER alpha signaling in breast cancer:
721 TRIM3 interacted with ER alpha protein, stabilized ERα and facilitated its signaling
722 activation and breast cancer progression.

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6