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ORIGINAL ARTICLE

Stabilization of estrogen receptor α by USP37 contributes to the progression of breast cancer

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Abstract

Breast cancer is a major cause of cancer-related morbidity and mortality in women. Estrogen receptor-positive breast cancer accounts for roughly 70%-80% of breast tumors, and estrogen receptor alpha (ERα) has been considered as a key driver in promoting breast cancer progression. In the present study, we identified USP37 as a novel modulator in modulating ERα ubiquitination and stability. The expression of USP37 was upregulated in ERα-positive breast cancer and correlated with ERα protein level. High expression of USP37 was associated with unfavorable prognosis. USP37 depletion resulted in significantly decreased ERα protein level, ERα target genes expression as well as the estrogen response element activity in breast cancer cells. Further mechanistic study revealed the interaction between USP37 and ERα: USP37 regulated ERα signaling through modulating protein stability instead of gene expression, in which it stabilized ERα protein via inhibiting the K48-specific polyubiquitination process. Additionally, USP37 depletion led to growth inhibition and cell cycle arrest of ERα-positive breast cancer cells, which could be further rescued by ERα overexpression. Overall, our study proposed a novel post-translational mechanism of ERα in promoting breast cancer progression. Targeting USP37 may be proved to be a promising strategy for patients with ERα-positive breast cancer.

KEYWORDS
breast cancer, deubiquitylation, estrogen receptor α (ERα), stabilization, USP37

1 | INTRODUCTION

Breast cancer is the top leading cause of cancer-related death among women and the fourth leading cause of cancer-related disability–adjusted life years for both sexes combined globally.[1,2] The incidence has continued to rise over the past decades.[3,4] Due to its high heterogeneity, breast cancer could be classified into at least three subtypes: estrogen receptor (ER)-/progesterone receptor (PR)-positive, human epidermal growth factor receptor type-2 (HER2)-enriched, and triple-negative breast cancer. More than two thirds of all diagnosed breast cancers express estrogen receptor α (ERα) and thereby depend on estrogen for cellular

Abbreviations: ccRCC, clear cell renal cell carcinoma; DBD, DNA-binding domain; DUBs, deubiquitinating enzymes; E2, 17 beta estradiol hormone; EMT, epithelial-mesenchymal transition; ER, estrogen receptor; ERE, estrogen response elements; ERα, estrogen receptor α; HER2, human epidermal growth factor receptor type-2; JAMM, JAB1/MPN/MOV34 family; LBD, ligand-binding domain; MINDY, motif interacting with ubiquitin-containing novel DUB family; OS, overall survival; OUT, ovarian tumor proteases; PR, progesterone receptor; SMURF1, SMAD-specific E3 ubiquitin protein ligase 1; UCH, COOH-terminal hydrolases; UPS, ubiquitin proteasome system; USPs, ubiquitin-specific proteases.

growth and survival.[5] The treatment response and clinical outcome varies among different molecular subtypes of breast cancer. The ER-positive subtype, also known as luminal subtype, responds sensitively to endocrine therapy, which is associated with better prognosis, while triple-negative breast cancer is followed by a worse prognosis.[6]

Estrogen receptor α is a steroid hormone receptor and a typical estrogen ligand–dependent transcription factor driving tumorigenesis in hormone-dependent cancers. It is composed of three functional domains: transactivation domains AF-1 and AF-2, DNA-binding domain (DBD) and ligand-binding domain (LBD). After being activated by the binding of the 17 beta estradiol hormone (E2) through the LBD, ERα undergoes a conformational alteration to form homodimers and translocates from the cytosol to the nucleus. There, ERα recognizes the estrogen-responsive element (ERE) in the promoter regions of its targeted genes and recruits numerous coactivators to its DBD and LBD.[7–9]

ERα is one of the most important breast cancer diagnostic and prognostic biomarkers, playing a crucial role in regulating the growth of breast cancer.[10,11] Overexpression of ERα increases the levels of several oncogenic proteins, including cyclin D1 and c-Myc, which are essential for cell cycle progression by accelerating the G1/S phase transition of breast cancer cells.[12] Clinically, ERα-positive breast cancers are considered to have a better prognosis and sensitive response to endocrine therapy such as tamoxifen and aromatase inhibitor adminstration.[13] Tamoxifen is a widely used antiestrogen reagent in the treatment of patients with ERα-positive breast cancer, and has been demonstrated to contribute to significant improvements in clinical outcome.[14] Targeting ERα or ERα-mediated pathways precisely has been an effective strategy in ERα-positive breast cancer treatment as well as prevention.[15]

Recent studies have illustrated the significance of the post-translational modifications in regulating ERα protein activity or stability, such as phosphorylation, ubiquitination, and SUMOylation.[18] The ubiquitin proteasome system (UPS) is a key regulator for maintaining protein stability and cell function and is responsible for approximately 80%-90% of cellular proteolysis.[19] Several E3 ligases, including MDM2, CHIP, BARD1, SKP2, BRCA1, and E6AP, could increase polyubiquitin binding to lysine residues of ERα and induce proteasome-mediated degradation.[20–25] Contrarily, the RNF31, SHAPRIN, TRIM11, and RNF8, though belonging to E3 ligases as well, are reported to enhance ERα stability and promote breast cancer proliferation via inducing its monoubiquitination.[26–29] K63-linked ubiquitination on ERα protein mediated by TRIM56 also enhances ERα stability.[30]

Further, the ubiquitin process can be counteracted by the deubiquitinating enzymes (DUBs), which are involved in regulating various cell activities, including stress response, DNA repair, gene transcription, cell cycle, cell differentiation, and apoptosis. The DUBs involved in the human genome can be categorized into the following six families: ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTU), COOH-terminal hydrolases (UCH), the JAB1/MPN/MOV34 family (JAMM), Josephins, and motif interacting with ubiquitin-containing novel DUB family (MINDY).[31] Previous studies demonstrated that USP7 could remove the K48-ubiquitin chain from ERα, leading to inhibited proteasome-mediated ERα degradation.[32] OTUD7B and MINDY1 could promote breast cancer proliferation by stabilizing ERα in a deubiquitylation activity–dependent manner.[33,34] However, further research is still required to explore the exact mechanisms underlying ERα dysfunction in breast cancer.

In the study, we explored the oncogenic role of ubiquitin specific peptidase (USP37) in promoting Erα-positive breast cancer progression. USP37 was shown to interact with ERα protein and to enhance the stability of ERα via removing its K48-linked ubiquitin chain. As ERα signaling is essential for the proliferation and invasion of ERα-positive breast tumors, targeting USP37 could be a promising therapeutic strategy for the treatment of patients with ERα-positive breast cancer.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human ERα-positive breast cancer cell lines MCF-7 and T47D and human embryonic kidney cell line HEK293T were obtained from the American Type Culture Collection (ATCC). MCF-7 and HEK293T were cultured with Dulbecco's Modified Eagle's Medium (DMEM, 41965, Life Technologies) supplemented with 10% fetal bovine serum (FBS, 10270, Life Technologies). T47D cells were maintained with RPMI-1640 (Gibco) supplemented with 10% FBS. All cells were incubated at 37°C in a humidified environment with 5% CO_2.

2.2 | Plasmids, lentiviral construction, and RNA inference

The small interfering RNA (siRNA) library of DUBs, the Human ON-TARGETplus Deubiquitinating Enzyme siRNA library (set of four siRNAs), was obtained from Dharmacon™. This library targets members of the DUBs group which remove ubiquitin. The library covers 98 deubiquitinating enzymes.

The ERα and USP37 plasmids were obtained from Hanbio Biotechnology Co., Ltd. Lentiviral plasmids and siRNA used for knocking down the expression of USP37(5′-CAAAAGAGCUACCGAGUUA-3′, 5′- CCAAGGAUAUUUCAGCUAA-3′) were obtained from Ruibo Biotechnology Co., Ltd. The HA-K48 and HA-K63 Ubi plasmids were obtained from Addgene and applied into the experiment as described in a previous paper.[33] The pRL-TK control and the ERE-TK-luc reporter were gifted by Dr. Wu and described in a previous study.[33] For transient transfection of plasmids or siRNAs, respectively, cells at ~60% confluence were transfected with Lipofectamine 2000 (1662298, Invitrogen) for plasmid or Lipofectamine RNAiMAX (13778100, Invitrogen) for

siRNA. For stable cell line construction, cells were selected with 1 μg/ml puromycin for 1 week after being infected with shControl virus or shUSP37 virus.

2.3 | RNA extraction and qPCR analysis

Total RNA was extracted using RNeasy plus mini kits (74134, Qiagen) according to the manufacturer's instructions. RNA reverse transcription to cDNA synthesis was carried out using a reverse transcription kit (Vazyme). RNA expression was determined by quantitative real-time PCR (qRT-PCR) with the CFX96TM real-time PCR detection system (Bio-Rad) normalized to GAPDH. All assays were performed in biological triplicates. Primers used in the study are listed as follows: GAPDH (forward: 5′- ACGGGAAGCTTGTCATCAAT-3′, reverse: 5′- TGGACTCCACGACGTACTCA-3′); GREB1 (forward: 5′- GGGATCTTGTGAGTAGCACTGT-3′; reverse: 5′- AATCGGTC CACCAATCCCAC-3′); PDZK1 (forward: 5′- GCCAGGCTCATTCAT CAAAGA-3′; reverse: 5′- CCTCTAGCCCAGCCAAGTCA-3′); PS2 (forward: 5′- GTCCCTCCAGAAGAGGAGTG-3′; reverse: 5′- AGC CGAGCTCTGGGACTAAT-3′).

2.4 | Cell proliferation analysis

The cell proliferation assay was performed with cell-counting kit-8 (CCK8) and ethynyl-deoxyuridine (EdU) staining assay as previously described.[33] Briefly, 50 nM siUSP37 or siControl were transfected into MCF-7 and T47D cells in six-well plates; 24 hours later, cell number was measured, and 3000 cells were seeded into 96-well plates. Then, 10 μl CCK8 solution was added into the culture medium (10 μl CCK8 to 100 μl medium), and relative cell viability was detected with a microplate reader at 490 nm wavelength per 24 hours. EdU cell proliferation assay kit (Ribobio) was used to perform the EdU labeling assay according to the manufacturer's protocol, and images were captured with Leica microsystem.

2.5 | Clone formation assay

MCF-7 and T47D cells were transfected with siUSP37 or siControl for 24 hours and were further plated at a density of 1000 cells per well into six-well plates for clone formation assay. After further cultivation for 2 weeks, cell clones were fixed by 4% formaldehyde for 15 minutes and stained with crystal violet for 30 minutes.

2.6 | Flow cytometry analysis

MCF-7 and T47D cells were transfected with siUSP37 or siControl for 48 hours and further collected and washed with cold PBS. Afterwards, cell cycle distribution was analyzed using a cell cycle analysis kit (Beyotime) and detected by flow cytometry analysis (Beckman).

2.7 | Animal experiments

MCF-7 cells stably expressing shControl or shUSP37 were selected with 1 μg/ml puromycin for 1 week. At the age of 4 weeks, female BALB/c nude mice (obtained from Vital River) were administered with slow-release 17β-estradiol pellets (0.72 mg/90-day release). After 1 week, 2×10^6 MCF-7 cells suspended in 10 μl serum-free medium were injected into the fourth mammary fat pad of mice. The tumor size was monitored regularly using vernier calipers every 5 days till the end point of the experiment. All animal procedures were approved by the Ethics Committee at Xiangya Hospital Central South University.

2.8 | Coimmunoprecipitation assay

For coimmunoprecipitation assay, the indicated cells were harvested and lysed with NP-40 lysis buffer. The cell lysates were incubated at 4°C overnight with the indicated antibodies, followed by incubation with protein A/G PLUS-Agarose beads (Santa Cruz) for 2 hours. The immune complexes were separated by immunoblotting.

2.9 | In vitro deubiquitination assay

His-ERα was coexpressed with HA-ubiquitin in HEK293T cells and purified using anti-His antibody under denaturing conditions. The purified ERα was further incubated with bacterially purified GST, GST-USP37 and GST-USP37C350A. Deubiquitination was analyzed by immunoblotting using anti-HA antibody.

2.10 | Immunoblotting analysis

Cell pellets were harvested and then lysed using RIPA extraction reagent (Meilun) supplemented with protease inhibitors (Beyotime). The BCA kit (Beyotime) was applied to detect the protein concentration. Afterward, proteins were separated on 10% SDS-PAGE by electrophoresis and electro-transferred to 0.45 μm PVDF membrane (Millipore). Antibodies used in this study were ERα (Invitrogen, MA5-13304), HA (Proteintech, 51064-2-AP), Myc (Proteintech, 60003-2-Ig), USP37 (Proteintech, 18465-1-AP), GST (Proteintech, 66001-2-Ig), and GAPDH (Proteintech, 60004-1-Ig) antibodies.

2.11 | Statistical analysis

The quantified data are presented as mean ± standard deviation (mean ± SD). Student's *t* test and one-way ANOVA were used to

evaluate the differences between two or more groups. Multiple comparisons with Bonferroni correction were performed. The difference was statistically significant with $p < 0.05$, and all tests were two-sided. All statistical data analyses were performed using Prism version 8.0 (GraphPad).

3 | RESULTS

3.1 | Depletion of USP37 inhibits ERα signaling pathway activity

First, we used the human DUBs siRNA library to identify DUBs associated with the stability of ERα in ERα-positive breast cancer cells. A pool of four nonoverlapping siRNA oligos targeting each DUB were transfected into MCF-7 cells. Immunoblotting results indicated that the expression of ERα dramatically decreased by knocking down USP37 (Figure 1A). We further silenced USP37 with two independent siRNAs in MCF-7 and T47D cells, respectively, and observed downregulation of ERα protein level upon the depletion of USP37. However, no obvious alteration in the mRNA level of ERα was observed after USP37 depletion (Figure 1B,C). We then used qPCR to examine Erα-targeted genes and found that the consumption of USP37 markedly suppressed the transcription levels of PDZK1, GREB1, and PS2 in both cell lines (Figure 1D,E). Moreover, we further detected ERα transcriptional activity after USP37 depletion with luciferase assay, which showed inhibited activity of the ERα-luciferase reporter gene in MCF-7 and T47D cell lines (Figure 1F,G). Taken together, it was suggested that USP37 acted as a potential regulator of the ERα signaling pathway.

3.2 | USP37 is associated with unfavorable clinical outcome and correlates with ERα protein levels in human breast cancer

In order to identify the gene expression of USP37 in human breast cancer samples, we applied the statistical mining application of the bc-GenExMiner database[35] (http://bcgenex.centregauducheau.fr/BC-GEM/GEM-Accueil.php?js=1). We observed that USP37 was upregulated in human breast cancer, especially in the ERα-positive subtype (Figure 2A–D). Through the analysis by the online tool KMplot with access to a public clinical breast cancer corhort[36] (http://kmplot.com/analysis/), we found that USP37 expression level was negatively associated with relapse-free survival, but not overall survival (OS) (Figure 2E,F). As USP37 was upregulated in ERα-positive breast cancer patients, we assessed the prognostic value of USP37 in ER-positive breast cancer. Consistently, high expression of USP37 indicated poor clinical outcomes for patients with ER-positive breast cancer (Figure 2G). Moreover, the expression of USP37 was also associated with poor response to endocrine treatment (Figure 2H). In order to figure out the relationships between USP37 and ERα protein levels, we applied tissue microarrays (TMA) and performed immunohistochemistry (IHC) analysis. A positive correlation between USP37 and ERα staining was observed. (Figure 3A). Further analysis from public breast cancer database also showed positive

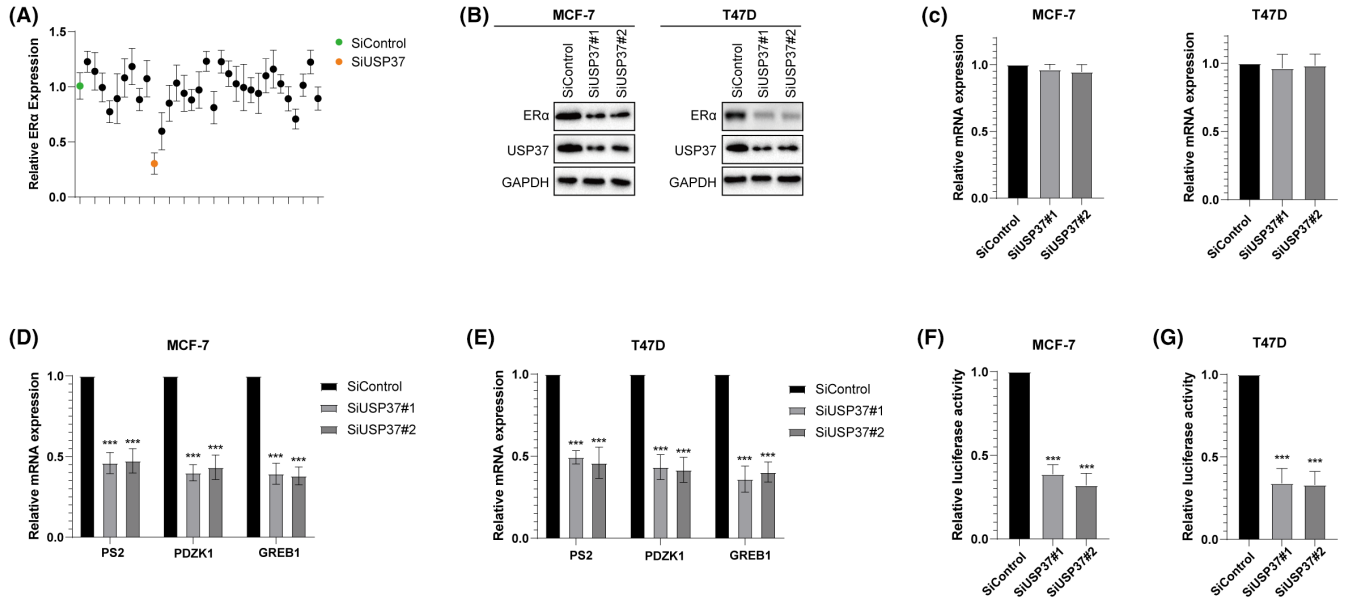

FIGURE 1 USP37 depletion decreases ERα signaling activity in breast cancer cells. A, The siRNAs specific to each deubiquitinating enzyme were transfected into MCF-7 cells. After 48 h, cells were lysed and the ERα protein level was analyzed by Western blot. Relative ERα protein level was normalized to GAPDH. B, USP37 depletion downregulated ERα protein level. C, USP37 depletion did not affect ERα mRNA level. D, E, USP37 depletion decreased ERα target genes. Total RNA was prepared, and the expression of the endogenous ERα target genes, PS2, GREB1, and PDZK1, were determined by qRT-PCR. F, G, USP37 depletion affected ERE-luciferase activity. Breast cancer cells were transfected with siUSP37 or siControl together with ERE luciferase reporter plasmid. Luciferase activity was measured 48 h after transfection. The experiment was independently repeated three times with three replicates. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

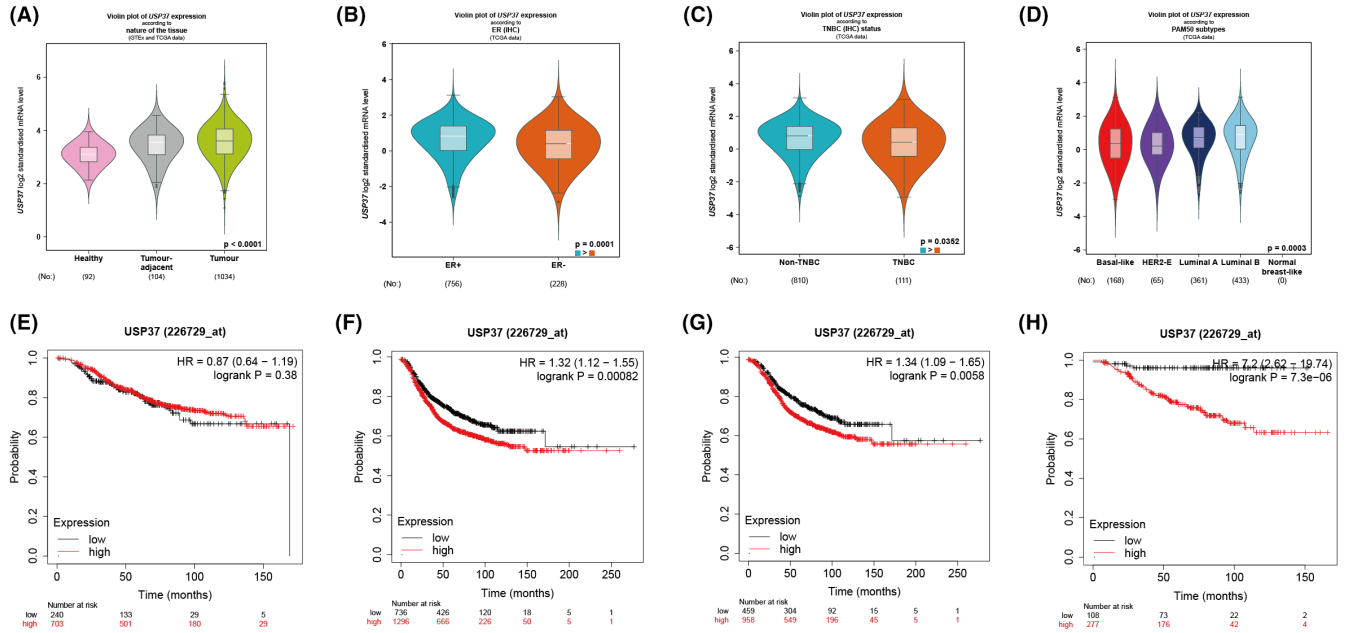

FIGURE 2 USP37 is overexpressed in breast cancer and correlates with poor prognosis. A-D, Expression of USP37 in breast cancer. All data are available at bc-GenExMiner v4.5(http://bcgenex.centregauducheau.fr/BC-GEM/GEM-Accueil.php?js=1). E, Kaplan-Meier curves of the overall survival between USP37-high and USP37-low breast cancer patients. F, Kaplan-Meier curves of the relapse-free survival between USP37-high and USP37-low breast cancer patients. G, Kaplan-Meier curves of the relapse-free survival between USP37-high and USP37-low ER-positive breast cancer patients. H, Kaplan-Meier curves of the relapse-free survival from patients receiving endocrine therapy.

correlation between the expression of USP37 and ERα (Figure 3B,C). Additionally, USP37 expression level was unraveled to be positively associated with lymph node status and tumor size in clinical patients (Figure 3C). These results suggested that USP37 may serve as a diagnostic and prognostic biomarker in patients with ER-positive breast cancer.

3.3 | USP37 interacts with ERα and enhances its stability

Immunostaining analysis demonstrated that in MCF-7 and T47D cells, ERα distributed mainly in the nucleus with less distribution in the cytosol, while USP37 was expressed both in cytosol and nucleus in our study. These two proteins presented clear colocalization mainly in the nucleus. (Figure 4A). Endogenous coimmunoprecipitation assay in MCF7 cells showed that USP37 could be coimmunoprecipitated with ERα under normal medium conditions (Figure 4B). To further interrogate the possibility that ERα might act as a substrate of USP37, we evaluated the possible deubiquitylation of ERα by USP37. We observed that deletion of USP37 remarkably reduced ERα expression. The decreased protein level of ERα could be reversed by the administration of the proteasome inhibitor MG132 (Figure 4C). As expected, ectopic expression of wild-type USP37, but not the catalytically inactive mutant C350A, could also rescue the ERα expression (Figure 4D). Further treatment with the protein synthesis inhibitor cycloheximide (CHX) has also proved that USP37 affected ERα stability. As shown in Figure 4E, the half-life of ERα was apparently decreased after knocking down USP37 in MCF-7 cells, while the half-life of ERα was prolonged once wild type USP37 was overexpressed in HEK293T cells. Besides, such prolonged half-life could not be detected with overexpression of USP37^{C350A} (Figure 4F).

3.4 | USP37 deubiquitylates ERα

As USP37 is a member of DUBs belonging to the USP family, we speculated on the deubiquitination-based function of USP37 on ERα. Ubiquitin-based immunoprecipitation assay indicated that USP37 deletion remarkably promoted the level of polyubiquitinated ERα protein in MCF7 cells (Figure 5A). Conversely, it was observed that ectopic expression of the wild-type USP37 apparently reduced ERα ubiquitylation in cells, while the catalytically inactive mutant USP37 ^{C350A} had no such effect (Figure 5B). Moreover, we found that overexpression of USP37 decreased the ubiquitin chain of ERα in a dose-dependent manner (Figure 5C). As K48- and K63-linked ubiquitination are two common ubiquitination manners of ERα protein, we then performed a ubiquitin immunoblotting assay using HEK293T cells transfected with Myc-USP37/Flag-ERα/HA-K48 or HA-K63 plasmids for 48 hours. It was illustrated that USP37 efficiently eliminated the K48-linked ubiquitin chain from ERα, while the K63-linked ubiquitin chain on ERα was not affected by USP37 (Figure 5D,E). Further in vitro deubiquitination assay confirmed that the catalytical activity of USP37 indeed stabilized ERα via removing its ubiquitin chains (Figure S1F). These results demonstrated that

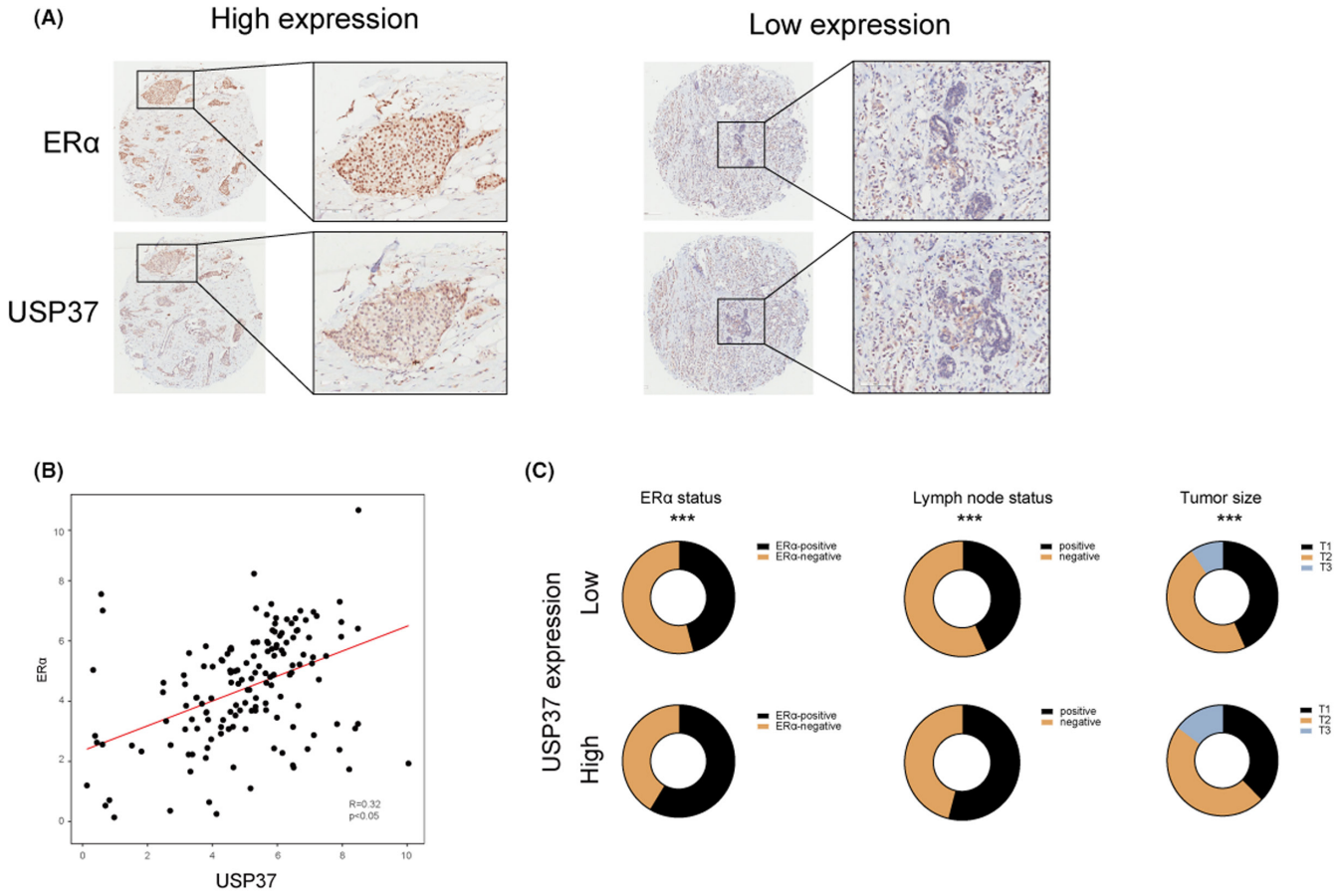

FIGURE 3 USP37 correlates with ERα protein levels and poor prognosis in human breast cancer samples. A, The representative immunohistochemistry (IHC) staining of ERα and USP37 in breast cancer specimens. Specific primary antibodies against ERα (Proteintech) and USP37 (China) were used. B, Positive correlation between ERα and USP37 was yielded among human breast cancer samples. C, The expression of USP37 was positively associated with ERα status, lymph node status, and tumor size in clinical breast cancer database.

USP37, serving as a potential DUB, de-polyubiquitylated and stabilized ERα via removing the K48-linked ubiquitination.

3.5 | USP37 promotes cell proliferation through ERα

With the intention to determine the potential influence of USP37 on ER-positive breast cancer, we knocked down USP37 in MCF7 and T47D cells. CCK8 assay revealed that USP37 ablation remarkably restrained the proliferation of MCF-7 and T47D cells. Flow cytometry analysis demonstrated that knocking down USP37 induced G1 phase arrest in MCF-7 and T47D, indicating that USP37 may block the G1-to-S transition of breast cancer cells (Figure 6A,B). Moreover, clone formation assay confirmed the pro-proliferative effect of USP37 (Figure 6C). The effects of USP37 depletion on ERα-negative cells were also examined in MDA-MB-231 cells. Though slight inhibition of cell proliferation was observed, the effects were not as drastic as in ERα-positive cell lines (Figure S1C–E). These results suggested that the significant biological effect of USP37 ablation in ERα-positive cells was mainly attributed to the potential relationship between USP37 and ERα. EdU is a thymidine analog which could be incorporated into the newly synthesized DNA strand when DNA is replicated, and detection of the EdU label could reflect cell proliferation. Consistently, Edu staining assay indicated that downregulation of USP37 inhibited DNA synthesis in MCF-7 and T47D cells (Figure 6D). To further illustrate the effect of USP37 in breast cancer tumorigenesis in vivo, xenograft mice models were constructed by injecting MCF-7 cells into the fourth mammary fat pad of female BALB/c nude mice. Our data demonstrated that depletion of USP37 by lentivirus-based shRNA dramatically decreased the growth of breast tumor in vivo (Figure 6F). ERα expression change upon USP37 by shRNA was confirmed (Figure S1A).

To assess whether USP37 regulates breast cancer cell proliferation by stabilizing ERα, rescue assay by ectopic expression of Erα was carried out in MCF-7 cells transfected with siUSP37. CCK8 assay demonstrated that the extent of suppressed growth of USP37 was remarkably reversed by upregulated Erα expression in MCF-7 cells (Figure 7A). Similarly, the clone formation ability and cell cycle transition of MCF-7 cells were recovered by ectopic expression of ERα (Figure 7B,C). As expected, Edu staining assay also showed that ERα overexpression reversed the DNA synthesis in MCF-7 cells transfected with siUSP37 (Figure 7D). Collectively, these rescue experiments verified arguably that ERα overexpression restored the

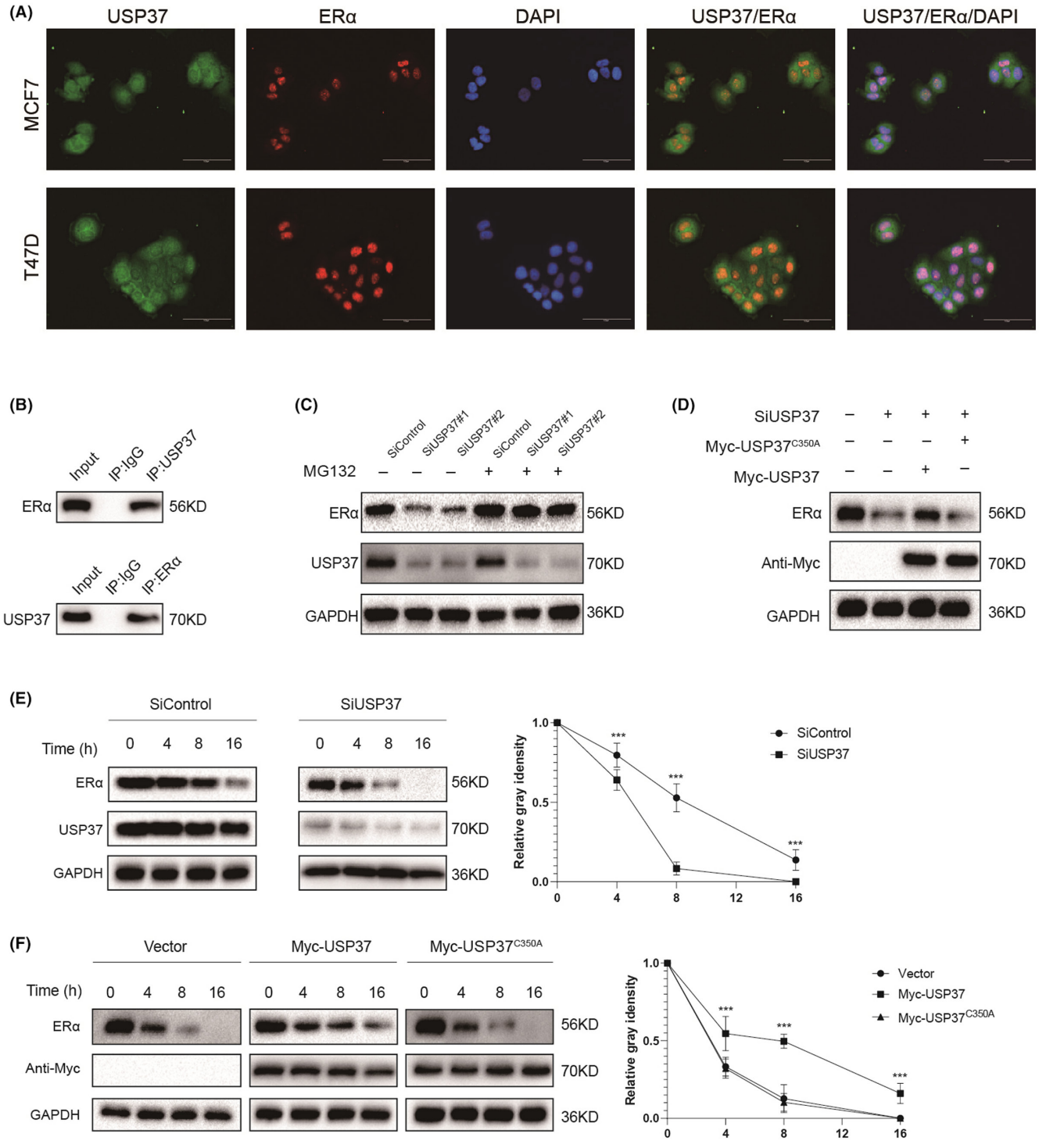

FIGURE 4 USP37 associates with ERα and increases ERα stability. A, An immunofluorescence assay demonstrated that USP37 and ERα at least partially colocalized in MCF7 and T47D cells. Scale bar: 100 μm. B, Coimmunoprecipitation (Co-IP) assay revealed an association between endogenous USP37 and ERα in MCF-7 cells. MCF-7 cells were harvested with RIPA lysis buffer. Co-IP was performed using antibody as indicated. C, In the presence of the proteasome inhibitor MG132, depletion of USP37 did not further decrease the ERα protein level. Breast cancer cells were transfected with siUSP37 or siControl. After 48 h, cells were treated with 10 μM MG132/vehicle for 6 h. Cell lysates were prepared for Western blot analysis. D, MCF-7 cells were transfected with USP37 (wild type or C350A) together with USP37 siRNA. The ERα levels were measured. E, USP37 depletion decreased ERα half-life in breast cancer cells. Breast cancer cells were transfected with siUSP37 or siControl. After 48 h, cells were treated with 100 μM cycloheximide/vehicle for indicated times. Cell lysates were prepared for Western blot analysis. F, USP37^{C350A} did not increase ERα half-life in HEK293 cells. HEK293 cells were transfected with HA-ERα plasmid and Myc-tag, Myc-USP37 or Myc-USP37^{C350A} plasmids. After 24 h, cells were treated with 100 μM cycloheximide/vehicle for indicated times. Cell lysates were prepared for Western blot analysis. The experiment was independently repeated three times with three replicates. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

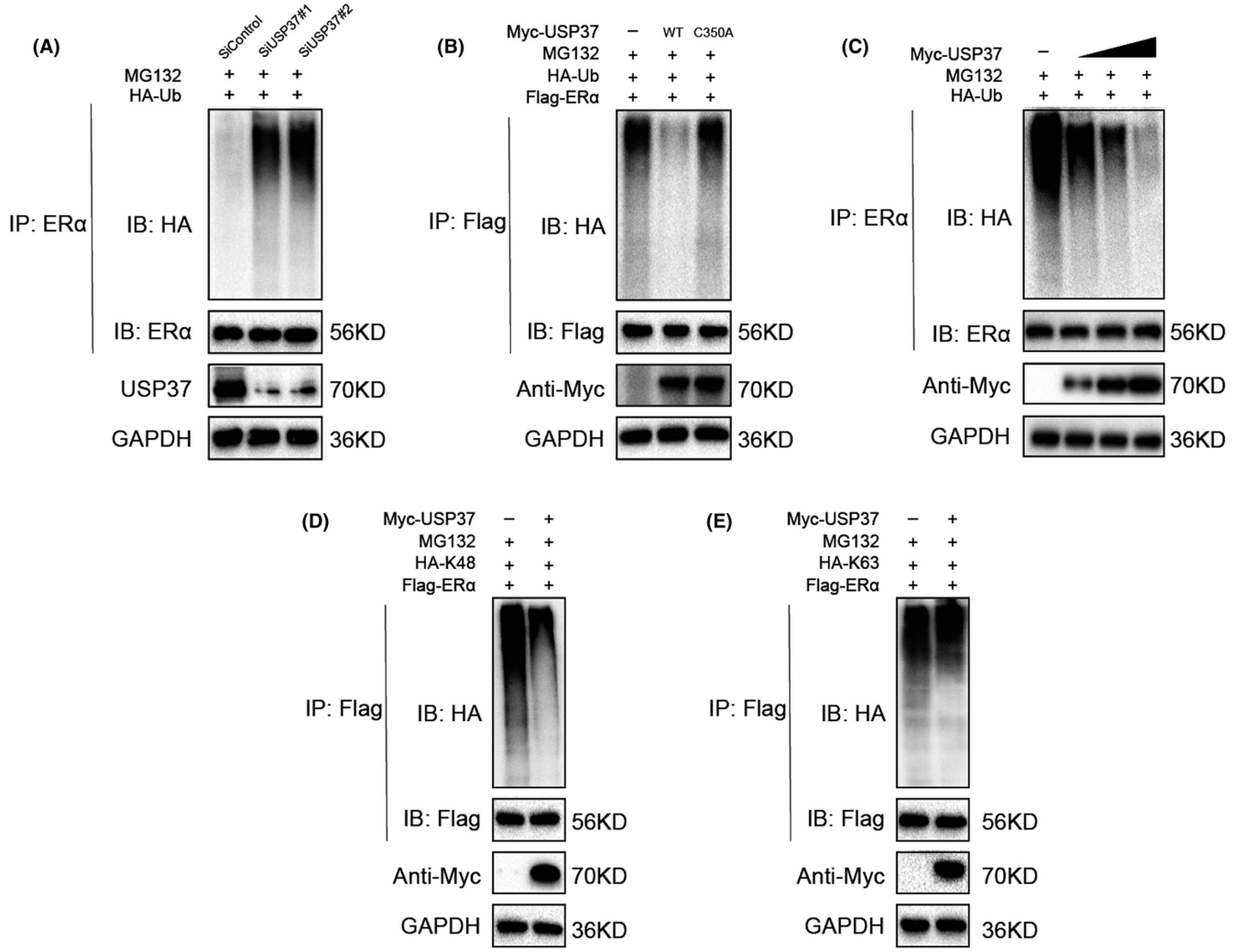

FIGURE 5 USP37 deubiquitylates ERα. A, MCF-7 cells transfected with the indicated siRNA were treated with MG132 for 6 h before collection. ERα was immunoprecipitated with anti-ERα and immunoblotted with anti-HA. B, Immunoblotting to detect the ubiquitination of ERα in HEK293 cells cotransfected with Flag-ERα, HA-ubiquitin, and Myc-USP37 (wild type or C350A). C, USP37 removed the ubiquitin chain of ERα in a dose-dependent manner. D, E, K48 or K63 Ub was cotransfected with Flag-YAP and Myc-USP37 into HEK293 cells. After treatment with 10 μM MG132 for 6 h, cell lysates were subjected to ubiquitination assay, and the ubiquitination level of ERα was detected by HA antibody.

promoted activity of USP37 on breast cancer cell proliferation, suggesting that USP37 promoted ERα-positive breast cancer cell proliferation via stabilizing ERα.

4 | DISCUSSION

Breast cancer has imposed a huge burden on global health due to its high morbidity and mortality. As is well known, the pathogenesis of breast cancer is extremely complicated given multiple endogenous and exogenous factors. ERα and ERα-mediated pathways have been recognized to play critical roles in regulating the pathogenesis of breast cancer. ERα has also been developed into a therapeutic target for breast cancer. ERα antagonists, such as tamoxifen and raloxifene, are currently used as first-line adjuvant treatment for premenopausal patients with ERα-positive breast tumors in order to inhibit the mitogenic stimulation of estrogens.[37] Integration of ERα antagonists into the treatment has resulted in robustly improved disease-free and OS for patients with ERα-positive breast cancers.[38]

However, due to inherent or acquired endocrine resistance, over 30% of patients receiving endocrine therapy relapse with resistant disease.[39] Therefore, endocrine resistance has become a rising concern in breast cancer therapy.[16,17] As the understanding of endocrine resistance mechanism evolves, various mechanisms have been proposed to explain the acquisition of endocrine resistance. ERα expression was maintained in most patients with endocrine resistance, while the subtle manipulations between ubiquitination and de-ubiquitination of ERα protein remain largely unclear in such a scenario. DUBs, which decrease the ubiquitination of proteins by dissociating ubiquitin from the substrates, are involved in the regulation of ERα stability.

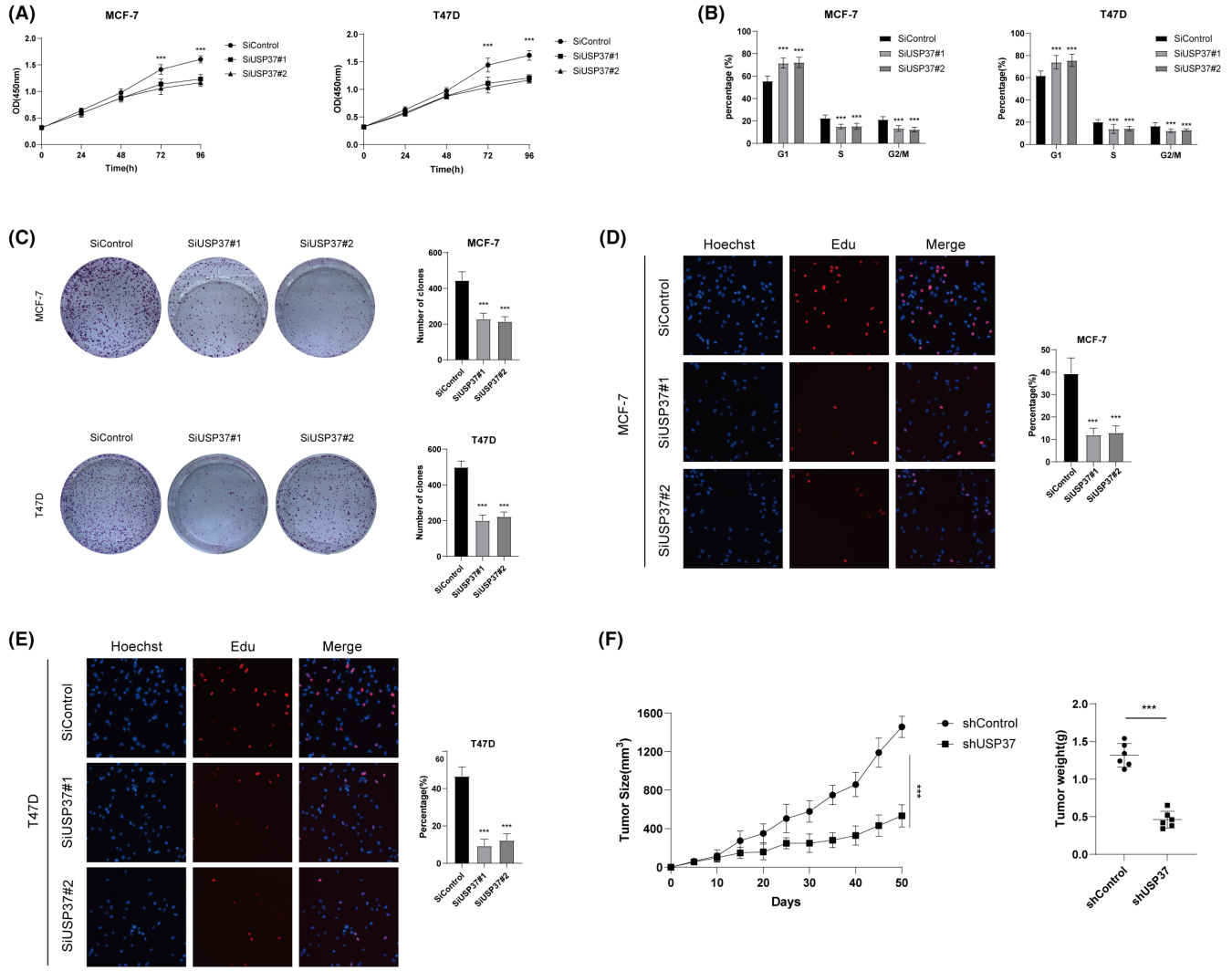

FIGURE 6 USP37 depletion inhibits ERα-positive breast cancer cell proliferation and migration. A, USP37 depletion inhibited cell proliferation in breast cancer cells. B, USP37 depletion induced G1 cell cycle arrest in breast cancer cells. C, USP37 depletion decreased clone formation capability of breast cancer cells. D, E, Representative images of EdU assay of breast cancer cells. F, MINDY1 depletion inhibits cell proliferation in breast cancer cells in vivo. The experiment was independently repeated three times with three replicates. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

USP37, a member of USPs, functions to remove the ubiquitin conjugates from substrates and protect them from ubiquitylation/degradation events. A growing number of studies demonstrated that USP37 could be a potential therapeutic target for cancer. In lung cancer, USP37 is reported as a Snail-specific deubiquitinase that promotes lung cancer cell migration.[40] In addition, USP37 regulates lung cancer cell proliferation and the Warburg effect via modulating the deubiquitination of c-Myc.[41] In clear cell renal cell carcinoma (ccRCC), USP37 interacts with HIF2α and enhances its deubiquitination. Thereby USP37 increases the protein stability of HIF2α in an enzymatically dependent manner, resulting in promoted cell proliferation, colony formation, and anchorage-independent growth in ccRCC.[42]

In breast cancer, USP37 is expressed at a high level in cancer tissues and cell lines. Based on the analysis of a breast cancer project in a TCGA program, high USP37 expression is found to be positively correlated with tumor growth and metastasis, while negatively associated with cell apoptosis. USP37 in breast cancer was reported to increase cell stemness, epithelial-mesenchymal transition (EMT), and invasion, while to decrease the sensitivity to cisplatin.[43]

In the current study, we identified USP37 as a novel deubiquitinase responsible for the stabilization of ERα by means of the siRNA library of human DUBs. Among the DUBs, USP37 exerted a prominent influence on ERα as well as ERα signaling activity. With further analysis of a public database from bc-GenExMiner, we observed that USP37 was upregulated in ERα-positive breast cancer. Though some other research studies had demonstrated that USP37 could regulate cell growth in both ERα-positive and -negative breast cancer cells, it could be also implied from their work that ERα-positive cells responded much more sensitively than ERα-negative cells.[43] Our data also confirmed that cell colony formation and migration were dramatically affected upon silencing USP37 in ERα-positive cells, while such effects remained subtle in ERα-negative cells. We also show

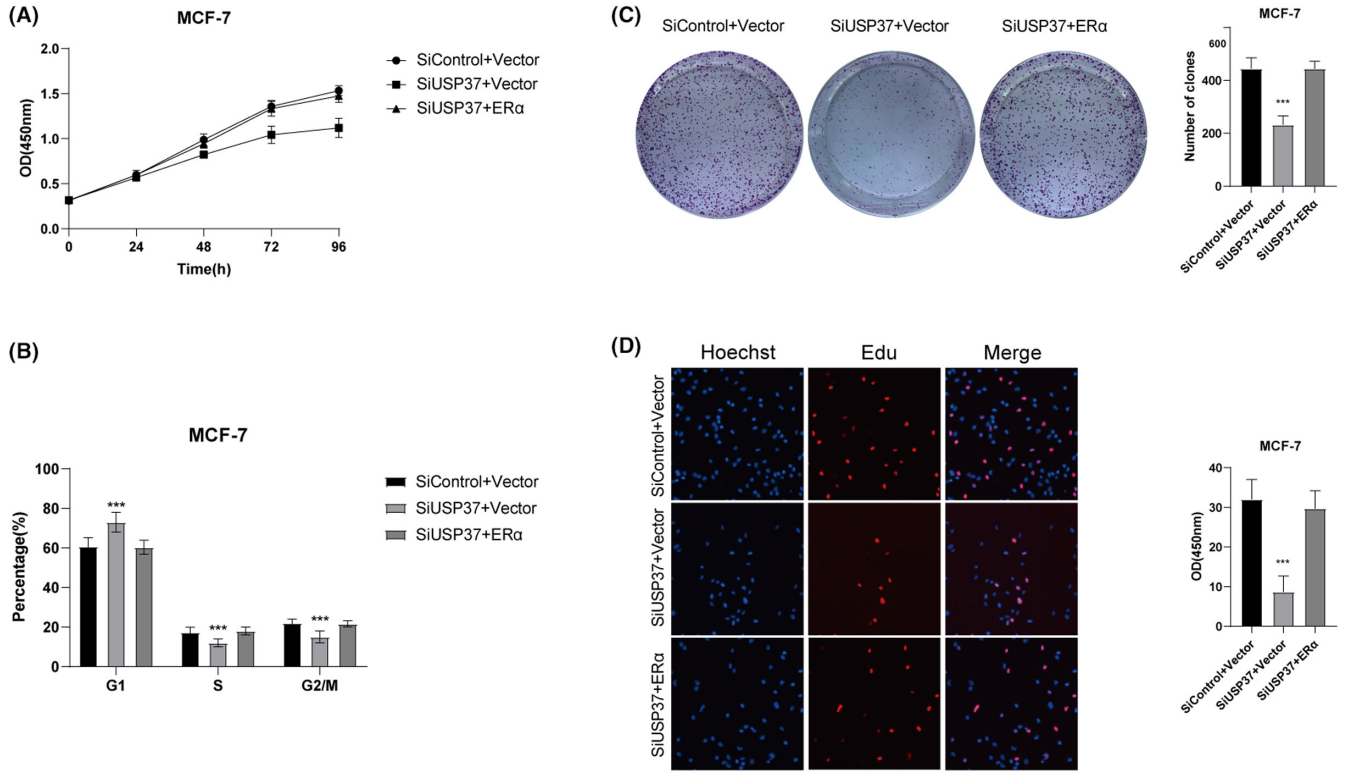

FIGURE 7 Increased ERα expression reverses the effect of USP37 depletion. A, Cell proliferation assay of MCF-7. B, Cell cycle analysis of MCF-7. C, Clone formation assay of MCF-7. D, Representative images of EdU assay of breast cancer cells. The experiment was independently repeated three times with three replicates. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

that the protein level of ERα was positively correlated with USP37 in human breast cancer samples. Their colocalization in human breast cancer tissues also confirm the potential relationship between USP37 and ERα.

Our study investigated the underlying mechanism of USP37 in regulating ERα and demonstrated that the protein stability of Erα increased upon ectopic expression of wild-type USP37, but not the catalytically inactive mutant C350A. Further, we confirmed that USP37 depletion–induced ERα degradation was dependent on the UPS, and endogenous USP37 could coimmunoprecipitate with endogenous ERα under normal conditions. Previous studies have revealed that K48-linked ubiquitination of ERα induced its degradation, while K63-linked ubiquitination of ERα was associated with nonproteolytic modification and contrarily stabilized ERα protein. In the current study, we found that USP37 removed the K48-linked ubiquitin conjugates from ERα, thus enhancing ERα stability. Moreover, we demonstrated that the catalytically inactive mutant of USP37 (C350A) lost the capability to deubiquitylate Erα, while such effect could be rescued by the introduction of exogenous wild-type ERα, indicating that USP37-induced ERα stability relied on its catalytical activity.

In previous studies on breast cancer, USP37 was suggested to regulate breast cancer malignancy via stabilizing the hedgehog pathway component Gli-1, yet the evidence for the direct regulating mechanism behind was not strong enough.[43] Besides, USP37 was also demonstrated to deubiquitinate and stabilize BLM, a human RecQ helicase, further sustaining the DNA damage response (DDR) and resulting in chemotherapy resistance.[44]

Our study has presented a novel regulating mechanism of USP37, especially in the ERα-positive subtype of breast cancer. Mechanistically, USP37 interacts with ERα and removes the K48-linked ubiquitin through its catalytical activity, leading to the stabilization of ERα. Collectively, our study explored the oncogenic role of USP37 in supporting breast cancer progression. These findings demonstrate that USP37 is an essential regulator of estrogen signaling and that the USP37-ERα axis is a potential target for breast cancer treatment.

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DISCLOSURE

The authors declare no conflict of interest.

ETHICAL APPROVAL

The research was carried out according to the World Medical Association Declaration of Helsinki and was approved by the Ethics Committee at Xiangya Hospital Central South University.

ANIMAL STUDIES

Animal studies involved in the current research were carried out according to the World Medical Association Declaration of Helsinki and approved by the Ethics Committee at Xiangya Hospital Central South University.

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REFERENCES

1. Kocarnik JM, Compton K, Dean FE, et al. Cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life years for 29 cancer groups from 2010 to 2019: a systematic analysis for the global burden of disease study 2019. *JAMA Oncol*. 2022;8(3):420-444.
2. Zhang S, Huang P, Dai H, et al. TIMELESS regulates sphingolipid metabolism and tumor cell growth through Sp1/ACER2/S1P axis in ER-positive breast cancer. *Cell Death Dis*. 2020;11(10):892.
3. Azubuike SO, Muirhead C, Hayes L, McNally R. Rising global burden of breast cancer: the case of sub-Saharan Africa (with emphasis on Nigeria) and implications for regional development: a review. *World J Surg Oncol*. 2018;16(1):63.
4. Rivera-Franco MM, Leon-Rodriguez E. Delays in breast cancer detection and treatment in developing countries. *Breast Cancer (Auckl)*. 2018;12:1178223417752677.
5. Flores JR, Salcedo AMC, Fernández LM. Micellar electrokinetic chromatographic study of the separation of an aromatase inhibitor and a tryciclic antidepressant in the breast cancer treatment. *Anal Chem Insights*. 2008;3:91-101.
6. Waks AG, Winer EP. Breast cancer treatment: a review. *JAMA*. 2019;321(3):288-300.
7. Greene GL, Press MF. Structure and dynamics of the estrogen receptor. *J Steroid Biochem*. 1986;24(1):1-7.
8. Ng HW, Perkins R, Tong W, Hong H. Versatility or promiscuity: the estrogen receptors, control of ligand selectivity and an update on subtype selective ligands. *Int J Environ Res Public Health*. 2014;11(9):8709-8742.
9. Kumar R, Zakharov MN, Khan SH, et al. The dynamic structure of the estrogen receptor. *J Amino Acids*. 2011;2011:812540.
10. Miyoshi Y, Murase K, Saito M, Imamura M, Oh K. Mechanisms of estrogen receptor-α upregulation in breast cancers. *Med Mol Morphol*. 2010;43(4):193-196.
11. Tecalco-Cruz AC, Ramírez-Jarquín JO. Mechanisms that increase stability of estrogen receptor alpha in breast cancer. *Clin Breast Cancer*. 2017;17(1):1-10.
12. Wong SC, Chan JK, Lee KC, Hsiao WL. Differential expression of p16/p21/p27 and cyclin D1/D3, and their relationships to cell proliferation, apoptosis, and tumour progression in invasive ductal carcinoma of the breast. *J Pathol*. 2001;194(1):35-42.
13. Chen X-S, Wang K-S, Guo W, et al. UCH-L1-mediated downregulation of estrogen receptor α contributes to insensitivity to endocrine therapy for breast cancer. *Theranostics*. 2020;10(4):1833-1848.
14. Gnant M, Thomssen C, Harbeck N. St. Gallen/Vienna 2015: a brief summary of the consensus discussion. *Breast Care (Basel)*. 2015;10(2):124-130.
15. Bafna D, Ban F, Rennie PS, Singh K, Cherkasov A. Computer-aided ligand discovery for estrogen receptor alpha. *Int J Mol Sci*. 2020;21(12):4193.
16. D'Souza A, Spicer D, Lu J. Overcoming endocrine resistance in metastatic hormone receptor-positive breast cancer. *J Hematol Oncol*. 2018;11(1):80.
17. Li X, Lu J, Zhang L, Luo Y, Zhao Z, Li M. Clinical implications of monitoring ESR1 mutations by circulating tumor DNA in estrogen receptor positive metastatic breast cancer: a pilot study. *Transl Oncol*. 2020;13(2):321-328.
18. Traboulsi T, El Ezzy M, Dumeaux V, Audemard E, Mader S. Role of SUMOylation in differential ERα transcriptional repression by tamoxifen and fulvestrant in breast cancer cells. *Oncogene*. 2019;38(7):1019-1037.
19. Kwon YT, Ciechanover A. The ubiquitin code in the ubiquitin-proteasome system and autophagy. *Trends Biochem Sci*. 2017;42(11):873-886.
20. Bhatt S, Xiao Z, Meng Z, Katzenellenbogen BS. Phosphorylation by p38 mitogen-activated protein kinase promotes estrogen receptor α turnover and functional activity via the SCF(Skp2) proteasomal complex. *Mol Cell Biol*. 2012;32(10):1928-1943.
21. Eakin CM, Maccoss MJ, Finney GL, Klevit RE. Estrogen receptor alpha is a putative substrate for the BRCA1 ubiquitin ligase. *Proc Natl Acad Sci USA*. 2007;104(14):5794-5799.
22. Fan M, Park A, Nephew KP. CHIP (carboxyl terminus of Hsc70-interacting protein) promotes basal and geldanamycin-induced degradation of estrogen receptor-alpha. *Mol Endocrinol*. 2005;19(12):2901-2914.
23. Hashizume R, Fukuda M, Maeda I, et al. The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. *J Biol Chem*. 2001;276(18):14537-14540.
24. Saji S, Okumura N, Eguchi H, et al. MDM2 enhances the function of estrogen receptor alpha in human breast cancer cells. *Biochem Biophys Res Commun*. 2001;281(1):259-265.
25. Sun J, Zhou W, Kaliappan K, Nawaz Z, Slingerland JM. ERα phosphorylation at Y537 by Src triggers E6-AP-ERα binding, ERα ubiquitylation, promoter occupancy, and target gene expression. *Mol Endocrinol*. 2012;26(9):1567-1577.
26. Tang J, Luo Y, Tian Z, et al. TRIM11 promotes breast cancer cell proliferation by stabilizing estrogen receptor α. *Neoplasia*. 2020;22(9):343-351.
27. Wang S, Luo H, Wang C, et al. RNF8 identified as a co-activator of estrogen receptor α promotes cell growth in breast cancer. *Biochim Biophys Acta Mol Basis Dis*. 2017;1863(6):1615-1628.
28. Zhu J, Zhao C, Kharman-Biz A, et al. The atypical ubiquitin ligase RNF31 stabilizes estrogen receptor α and modulates estrogen-stimulated breast cancer cell proliferation. *Oncogene*. 2014;33(34):4340-4351.
29. Zhuang T, Yu S, Zhang L, et al. SHARPIN stabilizes estrogen receptor α and promotes breast cancer cell proliferation. *Oncotarget*. 2017;8(44):77137-77151.
30. Xue M, Zhang K, Mu K, et al. Regulation of estrogen signaling and breast cancer proliferation by an ubiquitin ligase TRIM56. *Oncogenesis*. 2019;8(5):30.
31. Abdul Rehman SA, Kristariyanto YA, Choi S-Y, et al. MINDY-1 is a member of an evolutionarily conserved and structurally distinct new family of deubiquitinating enzymes. *Mol Cell*. 2016;63(1):146-155.
32. Xia X, Liao Y, Huang C, et al. Deubiquitination and stabilization of estrogen receptor α by ubiquitin-specific protease 7 promotes breast tumorigenesis. *Cancer Lett*. 2019;465:118-128.
33. Tang J, Wu Z, Tian Z, Chen W, Wu G. OTUD7B stabilizes estrogen receptor α and promotes breast cancer cell proliferation. *Cell Death Dis*. 2021;12(6):534.
34. James C, Zhao TY, Rahim A, et al. MINDY1 is a downstream target of the polyamines and promotes embryonic stem cell self-renewal. *Stem Cells*. 2018;36(8):1170-1178.
35. Jézéquel P, Campone M, Gouraud W, et al. bc-GenExMiner: an easy-to-use online platform for gene prognostic analyses in breast cancer. *Breast Cancer Res Treat*. 2012;131(3):765-775.
36. Győrffy B, Lanczky A, Eklund AC, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer

prognosis using microarray data of 1,809 patients. *Breast Cancer Res Treat*. 2010;123(3):725-731.
37. Lappano R, Santolla MF, Pupo M, et al. MIBE acts as antagonist ligand of both estrogen receptor α and GPER in breast cancer cells. *Breast Cancer Res*. 2012;14(1):R12.
38. Molloy ME, Lewinska M, Williamson AK, et al. ZBTB7A governs estrogen receptor alpha expression in breast cancer. *J Mol Cell Biol*. 2018;10(4):273-284.
39. Portman N, Milioli HH, Alexandrou S, et al. MDM2 inhibition in combination with endocrine therapy and CDK4/6 inhibition for the treatment of ER-positive breast cancer. *Breast Cancer Res*. 2020;22(1):87.
40. Cai J, Li M, Wang X, et al. USP37 promotes lung cancer cell migration by stabilizing snail protein via deubiquitination. *Front Genet*. 2019;10:1324.
41. Pan J, Deng Q, Jiang C, et al. USP37 directly deubiquitinates and stabilizes c-Myc in lung cancer. *Oncogene*. 2015;34(30):3957-3967.
42. Hong K, Hu L, Liu X, et al. USP37 promotes deubiquitination of HIF2α in kidney cancer. *Proc Natl Acad Sci USA*. 2020;117(23):13023-13032.
43. Qin T, Li B, Feng X, et al. Abnormally elevated USP37 expression in breast cancer stem cells regulates stemness, epithelial-mesenchymal transition and cisplatin sensitivity. *J Exp Clin Cancer Res*. 2018;37(1):287.
44. Wu C, Chang Y, Chen J, et al. USP37 regulates DNA damage response through stabilizing and deubiquitinating BLM. *Nucleic Acids Res*. 2021;49(19):11224-11240.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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