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Knockdown of ZBTB7A inhibits cell proliferation of breast cancer through regulating the ubiquitination of estrogen receptor alpha

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ABSTRACT

Aims: ZBTB7A, a transcriptional repressor, accelerates the breast cancer progression. Over 70% of breast cancer samples are identified as ER-α positive. Due to the function of ZBTB7A in ER-α positive breast cancer incompletely known, we aimed to determine the role of ZBTB7A in ER-α positive cancer and explore the underlying mechanisms.

Main methods: In this study, the correlation between ZBTB7A and ER-α was confirmed by tissue microarray-based and TCGA database. Then, we explore if ZBTB7A maintains ER-α's level via targeting ER-α's expression or degradation. Finally, we examined the effect of ZBTB7A on the proliferation of breast cancer cells.

Key findings: We further confirmed that ZBTB7A shows a significant positive correlation with ER-α in clinical breast cancer samples by tissue microarray-based analysis. Mechanically, we identified that the inhibition of ZBTB7A could upregulate E3 ligase TRIM25 leading to enhancement of ER-α ubiquitination and proteasomal degradation, which could partly explain the correlation between ZBTB7A and ER-α. Besides, we uncovered that ZBTB7A could also transcriptionally increase the expression of ER-α via indirectly binding to the region +146 to +461 bp downstream of the transcription start site of *ESR1* (ERpro315) in breast cancer cells. Furthermore, ZBTB7A is found to stimulate the expression of ER-α's downstream genes, and promote the growth of estrogen receptor alpha (ER-α)-positive breast cancer cells.

Significance: Our data revealed the novel mechanisms through which ZBTB7A manipulates ER-α level and might provide a new avenue for endocrine therapy in breast cancer.

1. Introduction

Breast cancer is the most prevalent cancer and the leading cause of cancer death in females [1]. More than 70% of breast cancer expresses ER-α in clinical samples [2]. ER-α, encoded by *ESR1*, has been admitted as a nuclear hormone receptor [3]. It has been previously reported that most breast carcinomas can proliferate in response to estrogens through ER-α. ER-α participates in promoting proliferation, survival, and migration of breast cancer cells that contributing to tumor growth [4,5]. It is recognized as the most frequently applied therapeutic target in patient with ER-α positive breast cancer [6]. In this article, our results displayed that ZBTB7A is highly expressed in ER-α positive breast cancer cell lines, but not ER-α negative cell lines. We discovered the protein level of ZBTB7A is positively associated with ER-α, that implying that ZBTB7A is a potential therapeutic target in breast cancer.

ZBTB7A is a POK protein family member that has a crucial and pleiotropic function in cellular differentiation [7,8]. It is a transcription factor with proto-oncogenic or anti-oncogenic activity [9–11]. And the role of ZBTB7A has been assessed by a few studies in breast cancer [12]. We previously reported that ZBTB7A promotes breast cancer progression by upregulating survivin expression [13]. However, the function of ZBTB7A on cell proliferation is not demonstrated clearly. Here our findings supported that ZBTB7A regulates ER-α's level, which might explain its function on proliferation of breast cancer.

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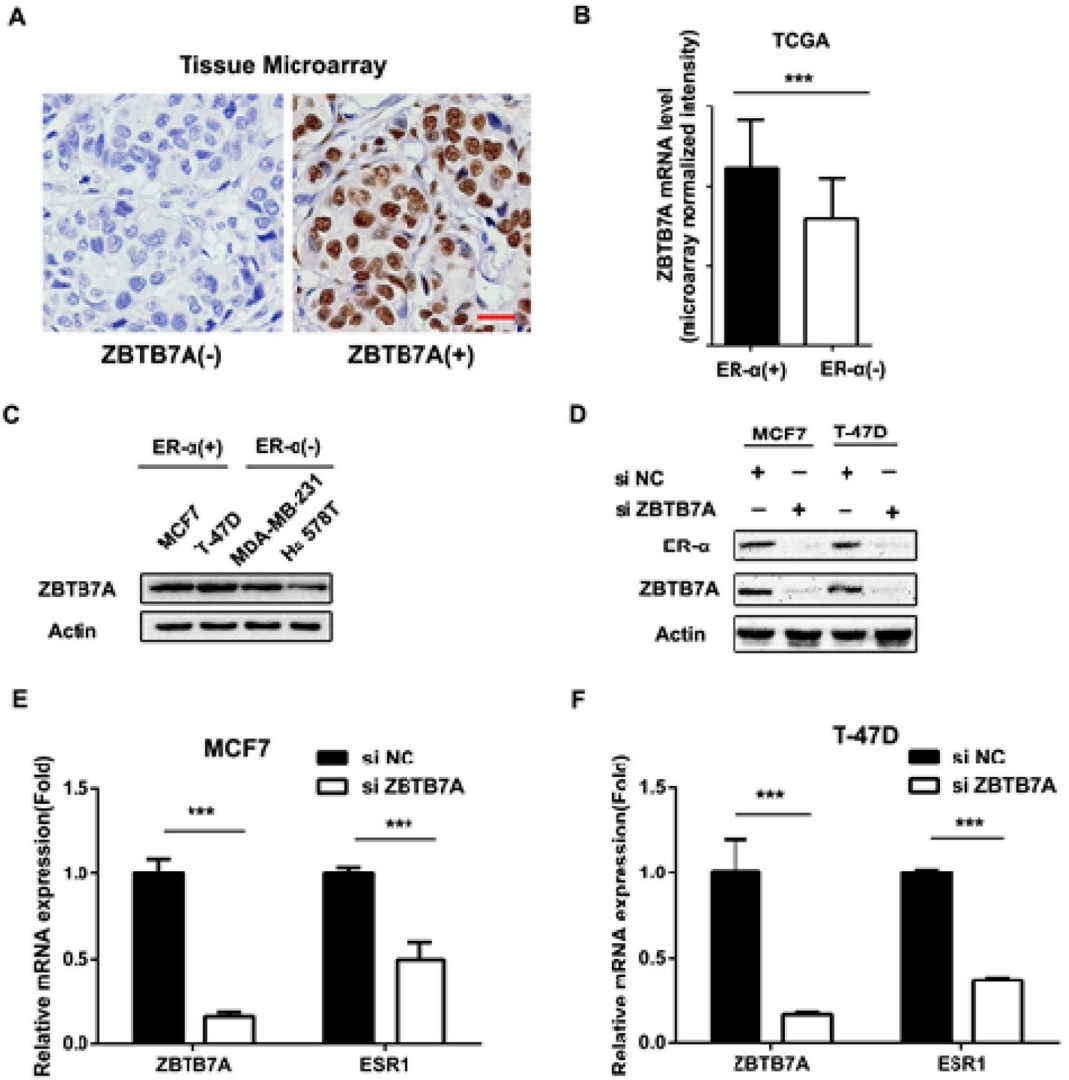


Fig. 1. The positive correlation of ZBTB7A expression with ER-α in breast cancer. (A) Representative images of ZBTB7A immunostaining in tissue microarrays (n = 50). Scale bar: 100 μm. (B) The mRNA level of ZBTB7A in ER-α positive or negative patient's samples from TCGA (n = 908). (C) Protein expression levels of ZBTB7A in ER-α positive (MCF7 or T-47D) or negative (MDA-MB-231 or Hs 578T) cancer cell lines. (D) The protein expression of ER-α in control or ZBTB7A knockdown cancer cells. Cells were treated with siRNA NC or ZBTB7A, and cell lysate was collected at 72 h for western blot. (E and F) ZBTB7A and ESR1 mRNA expression (normalized to the expression of β-actin) in breast cancer cells. MCF7 (E) or T-47D(F) cells were transfected with siRNA NC(Negative Control) or ZBTB7A, and qPCR was performed after 48 h.

Protein expression and degradation are key ways to maintain the steady-state level of ER-α protein [14]. In this study, it was demonstrated that ZBTB7A regulates ER-α's expression at the transcriptional level by indirectly binding with ERpro315. Meanwhile, we described the novelty mechanism of post-translational regulation that ZBTB7A attenuates the proteasome-mediated degradation of ER-α by upregulation of E3 ligase TRIM25. In conclusion, our study proved that ZBTB7A modulates expression and degradation of ER-α.

2. Results

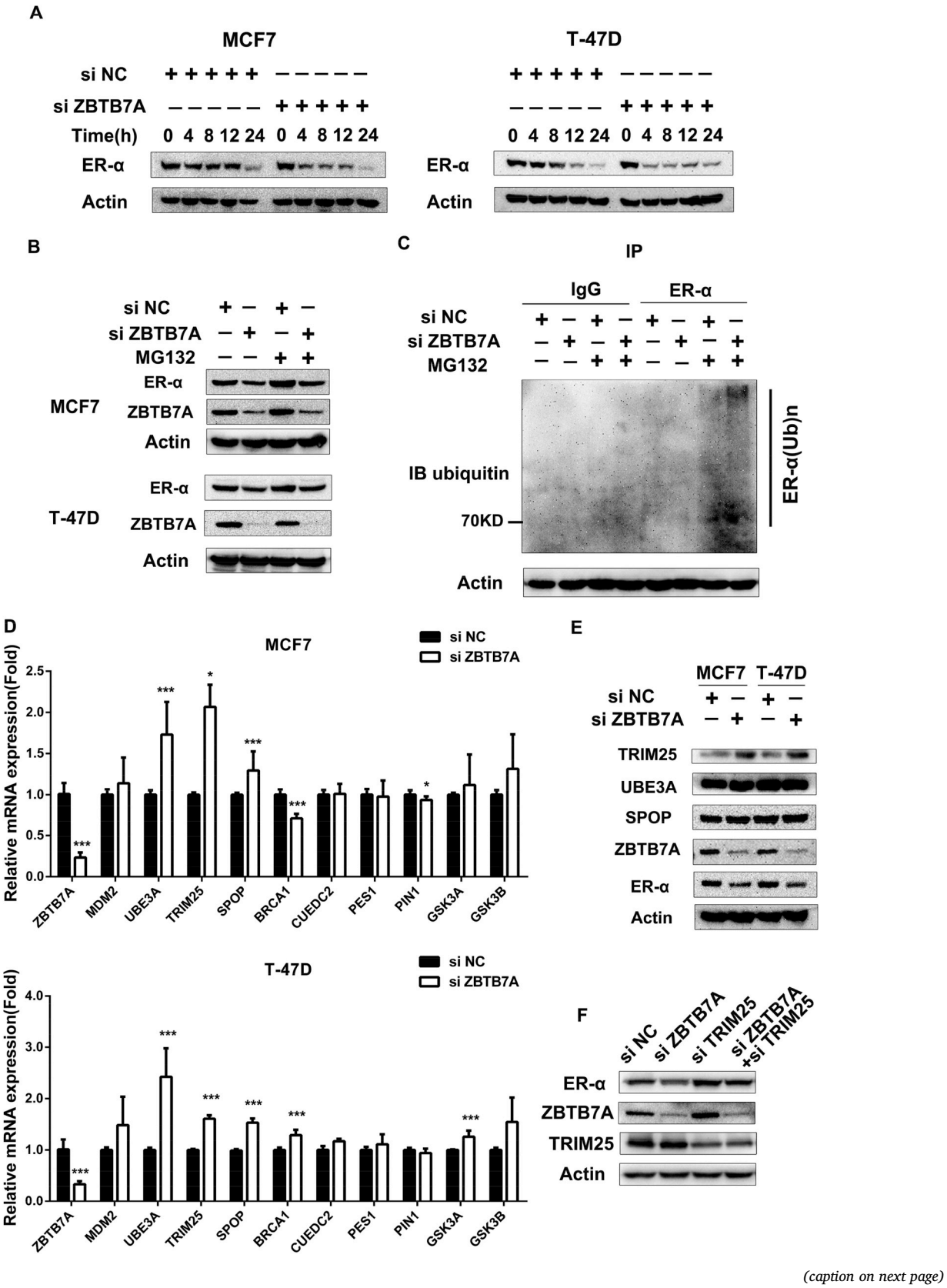
2.1. The positive correlation of ZBTB7A expression with ER-α in breast cancer

Here we found the expression of ZBTB7A is positively associated with ER-α in breast cancer tissue microarray (Fig. 1A, Table 1). Moreover, we collected available data from The Cancer Genome Atlas (TCGA) and analyzed the expression of ZBTB7A in ER-α negative or positive breast cancer tissue. The expression of ZBTB7A is higher in ER-α positive cancer tissue compared with negative cancer tissue (Fig. 1B).

Table 1
The expression of ZBTB7A and ER-α in breast cancer tissue and its lymph node metastasis.

Variables	n	ZBTB7A			*R*	*P* value
		–	+	+ +		
ER-α in breast, n (%)					0.515	< 0.001
–	32	14 (43.8)	16 (50.0)	2 (6.2)		
+	11	1 (9.1)	7 (63.6)	3 (27.3)		
+ +	3	0 (0)	1 (33.3)	2 (66.7)		
+ + +	4	0 (0)	2 (50.0)	2 (50.0)		
ER-α in lymph node, n (%)					0.419	0.002
–	36	13 (36.1)	21 (58.3)	2 (5.6)		
+	7	1 (14.3)	3 (42.9)	3 (42.9)		
+ +	4	1 (25.0)	0 (0)	3 (75.0)		
+ + +	3	0 (0)	2 (67.3)	1 (33.3)		

In addition, we tested the protein levels of ZBTB7A in ER-α positive (MCF7, T47D) and negative (MDA-MB-231, Hs 587T) breast cancer cell lines. As expected, ZBTB7A protein level is higher in ER-α positive



(caption on next page)

breast cancer cell lines compared to negative cell lines (Fig. 1C).

Then we utilized ZBTB7A siRNAs to confirm the correlation between ZBTB7A and ER-α. The obvious reduction of ER-α's mRNA level and protein was observed in ZBTB7A silencing group (Fig. 1D, E and 1F). Therefore, the ZBTB7A expression is positively correlated with ER-α in breast cancer, and these findings suggested ZBTB7A positively regulates the protein level of ER-α.

Fig. 2. ZBTB7A inhibits ER-α's degradation through the proteasomal pathway. MCF7 or T47D cells were transfected with siRNA NC or ZBTB7A for 48 h. (A) The inhibition of ZBTB7A decreases the half-life of ER-α. Si NC and ZBTB7A treated cells were exposed to 100 μg/ml cycloheximide, a protein synthesis inhibitor. Cells were collected at the indicated times (0–24 h) after treatment and detected by western blot for ER-α and Actin. (B) Loss of ER-α in response to ZBTB7A knockdown is mediated by proteasomal degradation. Si NC and si ZBTB7A treated cells were treated with 20 μM MG132 for 4 h. And the whole-cell lysates were harvested and subjected to immunoblotting of ZBTB7A, ER-α and Actin. (C) ZBTB7A silencing increased ubiquitination level of ER-α compared to control group in 293T cells (after MG132 enrichment for ubiquitination). IB, Immunoblot; IP, immunoprecipitation. (D) Knockdown of ZBTB7A upregulates the mRNA expression of E3 ligases related to ER-α (UBE3A, TRIM25 and SPOP) in MCF7 and T-47D cells. Cells were collected for total mRNA extraction after transfection treatment. QPCR was performed to determine expression changes of indicated genes ($n \geq 4$, $^{*}P < 0.05$, $^{***}P < 0.001$). (E) Inhibition of ZBTB7A increases the protein level of TRIM25, but not UBE3A or SPOP. Cells were transfected with siRNA NC or ZBTB7A and harvested at 48 h for western blot. (F) Inhibition of ZBTB7A enhances the protein expression of ER-α. Cells were treated with siRNA ZBTB7A, TRIM25 or both. And cell lysate was harvested at 48 h for western blot.

2.2. ZBTB7A inhibits degradation of ER-α via proteasome pathway

We further explored the mechanism ZBTB7A modulates the level of ER-α. Interestingly, we discovered ZBTB7A promotes ER-α post-translational stability (Fig. 2A). Cycloheximide is a protein synthesis inhibitor [15]. Cycloheximide chase experiments were conducted to determine ER-α's stability in control and ZBTB7A-knockdown MCF7 cells. As shown in Fig. 2A, ZBTB7A knockdown reduced the half-life of ER-α from about more than 8 h to less than 4 h, suggesting that ZBTB7A plays an essential role in governing post-translational stability of ER-α. In addition, to determine whether ER-α's protein stability is regulated by ZBTB7A via the proteasome pathway, control and ZBTB7A-knockdown MCF7 cells were treated with the proteasome inhibitor MG132. The results indicated MG132 partly restores ER-α expression in cells with ZBTB7A knockdown (Fig. 2B). Moreover, Western blotting shows that ER-α is highly ubiquitinated upon ZBTB7A silencing, whereas control cells exhibited comparatively reduced ER-α ubiquitination (Fig. 2C). These data revealed ZBTB7A inhibits degradation of ER-α via proteasome pathway.

It is known that genes, such as MDM2 [16], ubiquitin protein ligase E3A (UBE3A) [17], tripartite motif containing 25 (TRIM25) [18], speckle type BTB/POZ protein (SPOP) [19] and BRCA1 [20], regulate ER-α proteasomal degradation. Here we suspected if ZBTB7A attenuates degradation of ER-α via the inhibition of E3 ligase or related genes. In order to identify the genes involving degradation of ER-α, we utilized siRNAs to silence ZBTB7A, and then their mRNA expression was detected. The results showed knockdown of ZBTB7A increased mRNA expression of UBE3A, TRIM25 and SPOP in both MCF7 and T47D cells. Subsequently, the protein levels of UBE3A, TRIM25 and SPOP were assessed by western blot after siRNA ZBTB7A treatment (Fig. 2D). Notably, the result displayed that the protein expression of TRIM25 is upregulated in ZBTB7A knockdown group, suggesting that TRIM25 participates in the process ZBTB7A regulates the ER-α's degradation (Fig. 2E). Consistently with these results, inhibition of TRIM25 increases the protein level of ER-α in ZBTB7A knockdown group (Fig. 2F).

2.3. ZBTB7A enhances transcriptional activity of ER-α via indirectly binding

As a transcription factor, ZBTB7A was speculated to promote expression of ER-α at transcriptional level. In order to determine the mechanism ZBTB7A regulates the expression of ER-α, luciferase reporter assay was performed. It is previous reported that Metastasis-associated protein 1(MTA1) regulates expression of ER-α, and MTA1 binds to the region +146 to +461 bp downstream of the transcription start site of *ESR1* [21](Fig. 3A). Therefore, the region the ERpro315 (+146 to +461) was cloned and used to drive the expression of luciferase reporter in MCF7 cells. Cotransfection of ZBTB7A siRNAs or plasmids together with ERpro315 in MCF7 decreases activity of ER-α promoter (Fig. 3B and C).

Furthermore, we investigated the interaction between the ERpro315 and ZBTB7A was by ChIP assay (Fig. 3D). Of note, as Fig. 3E shown that ZBTB7A is not recruited to the region ERpro315, confirming the indirect association of ZBTB7A with ERpro315.

2.4. The inhibition of ZBTB7A attenuates the growth of breast cancer cells

Our previous report displayed that ZBTB7A promotes the breast cancer progression using tissue microarray combinated with clinicopathological data [13]. However, the cancer-promoting effect of ZBTB7A has not been verified on cell models. Here we used cell viability and colony formation assays to assess whether ZBTB7A affects cell proliferation. SiRNA ZBTB7A or control (si-NC) was transfected to MCF7 cells. It was observed that cell viability in ZBTB7A depletion group was reduced by 13% and 50%, respectively, at 72 h or 96 h (Fig. 4A). Meanwhile, the results of colony formation assay showed markedly reduced colony number of ZBTB7A-knockdown cells (Fig. 4B and C). Consistent with these data, the result of EdU incorporation indicated transient depletion of ZBTB7A in MCF7 by siRNAs results in a decrease of proliferation (Fig. 4D). Taken together, our data indicated that silencing of ZBTB7A hinders cell proliferation of breast cancer.

Progesterone receptor (PGR) [22], Cathepsin D (CTSD) [23], C-x-c motif chemokine ligand 12 (CXCL12) [24] and Cellular communication network factor 5 (CCN5) [25] are proliferation related genes that involved in estrogen receptor α signaling pathway. We observed ZBTB7A silencing inhibits the mRNA expression of PGR, CTSD, CXCL12 and CCN5 (Fig. 4E), Together these results implied ER-α signaling pathway might participate in process ZBTB7A stimulates cell proliferation.

3. Materials and methods

3.1. Cell culture

Breast cancer cell lines (MCF 7,T-47D) and human embryonic kidney cell line (293T) were obtained from American Type Culture Collection (Maryland, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin, at 37 °C, under a humidified atmosphere of 5% CO_2. Cells were confirmed to be without mycoplasma contamination.

3.2. Antibodies and reagents

Antibodies used in this study are listed as follows: ZBTB7A (A300-549A, Bethyl Laboratories, USA), ubiquitin(3966, Cell Signaling Technology, USA), ER-α(8644, Cell Signaling Technology), TRIM25(12573-1-AP, Proteintech), SPOP(16750-1-AP, Proteintech), UBE3A(ab126765, Abcam). Cycloheximide and MG132 were purchased from Sigma-Aldrich.

3.3. Cell viability assays

Cells were seeded in 96-well plates at 3000 cells per well in 0.1 ml medium. After treatment, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium (MTS, Promega) was added to the cells, and cell plates were incubated at 37 °C for 3 h. The optical absorbance was determined at 490 nm using a microplate reader (iMark, Bio-Rad).

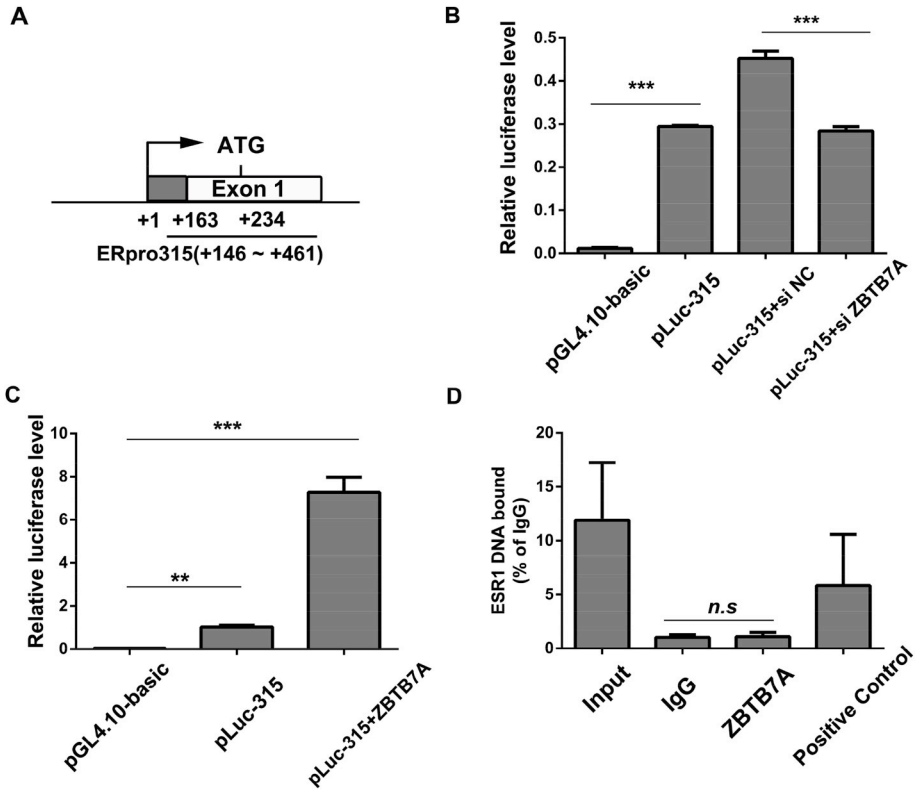


Fig. 3. ZBTB7A enhances transcriptional activity of ER-α via indirectly binding. (A) Schematic representation of human ER-α promoter (where +1 is the initiating ATG) +146~+461(ERpro315). (B and C) Regulation of ER-α promoter 315 activity by ZBTB7A in MCF7 cells. Wild-type(pGL4.10-basic) or mutant promoter(pLuc-315) was cotransfected with siRNA NC, siRNA ZBTB7A (B) or its overexpression plasmid (C), and luciferase activity was measured with pRL-TK as an internal control (n = 3, ***$P < 0.001$). (D) DNA binding of ZBTB7A on the ERpro315 region was accessed by ChIP analysis in MCF7 cells. DNA fragments that were immunoprecipitated by IgG (negative control), anti-ZBTB7A or anti-RNA Polymerase II (positive control) antibodies were amplified by qPCR using primers for ERpro315(n = 3, *n.s* means no significance).

3.4. RNA interference

Scramble(Negative Control, NC) and ZBTB7A siRNAs were obtained from GenePharma (Shanghai, China). ZBTB7A siRNAs are as follows: sense (CCCACAACUACGACCUGAAGAACCAdTdT) and antisense (UGG UUCUUCAGGUCGUAGUUGUGGGdTdT) siRNAs. SiRNAs were transfected using Lipofectamine RNAiMAX (Life Technologies) with OPTI-MEM (Thermo Fisher). After 6 h, cell medium was replaced with 10% foetal bovine serum in DMEM.

3.5. Colony formation assay

MCF 7 were cultured in 6-well plates at 1500 cells per well. Cells were transfected with Scramble or ZBTB7A siRNAs. Two weeks later, cells were fixed in 4% paraformaldehyde for 20 min, then washed with PBS and stained with Wright-Giemsa (BASO, China) for 10 min. Colonies with >50 cells or more were counted under an inverted microscope. The average number of colonies from three separate experiments was represented.

3.6. EdU incorporation assay

Cells were seeded in 96-well plates, and then transfected with siRNA ZBTB7A. According to the manufacturer's instructions, cell proliferation was assessed by Cell-Light EdU DNA cell proliferation kit (RiboBio, China) after 48 h.

3.7. Tumor tissue microarray

Tissue microarray were purchased from Alenabio Biotech Co., Ltd. Immunohistochemistry staining was carried on 5μm sections of the microarray to assess ZBTB7A expression. Immunohistochemistry stains on tissues without necrosis were also analyzed by two independent pathologists. According to the cell staining intensity, it was judged: non-staining(negative, -), light brown (weakly positive, +), brown (positive, ++) and dark brown (strongly positive, +++); The number of cells can be divided into: (+) refers to the number of positive cells below 25%, (++) refers to the number of positive cells between 25% and 49%, (+++) refers to the number of positive cells is above 50%. Finally, based on the above results, qualitative and semi-quantitative color intensity results were comprehensive evaluated. The images from at least with 5–10 high-powered fields.

3.8. Western blot

Cells were using RIPA lysis buffer (Beyotime, China) lysed on ice for 30 min, and sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed. After being electroblotted onto polyvinylidene fluoride membranes (Millipore, USA), target proteins were detected by with corresponding antibodies. The membranes were incubated with Immobilon Western HRP Substrate (Millipore), and the blots were visualized with FluorChem E system (ProteinSimple, USA).

3.9. Quantitative real-time PCR

Total RNA was extracted using Ultrapure RNA Kit (Cwbiotech, China), and reverse transcription was performed from 2 μg total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's recommendation. Quantitative PCR was performed with TB Green™ Premix Ex Taq™ II (TaKaRa, Japan) using an Applied Biosystems 7500 Real-Time PCR System (Life Technologies). The following amplification primers (Sangon, China) were used (5′ to 3′): ZBTB7A (sense, GAAGCCCTACGAGTGCAACATC; antisense, TGGTTCTTCAGGTCGTAGTTGTG), ER-α(sense, AGCCCGCT CATGATCAAACG; antisense, GCTCAGCATCCAACAAGGCA), TRIM25(sense, CTGCCTGCAGAAGTCCCAAG; antisense, CCTCGCCCA CAACACAATCA), UBE3A (sense, TCGCTATGGAAAATCCTGCAGAC; antisense, ACACCTCCCTCATCAACTCCT), SPOP(sense, TACACGGG - GAAGGCTCCAAA; antisense, TAAGCGCTCCAGGGCATACT), BRCA(sense, TCTGG -GCCACACGATTTGAC; antisense, CAACACGAGC TGACTCTGGG), PRGA (sense, TGGTGTCCTTACCTGTGGGA; antisense, CCAGCCTGACAGCACTTTCT), GREB1 (sense, GGACCAGCTTCAGTCA

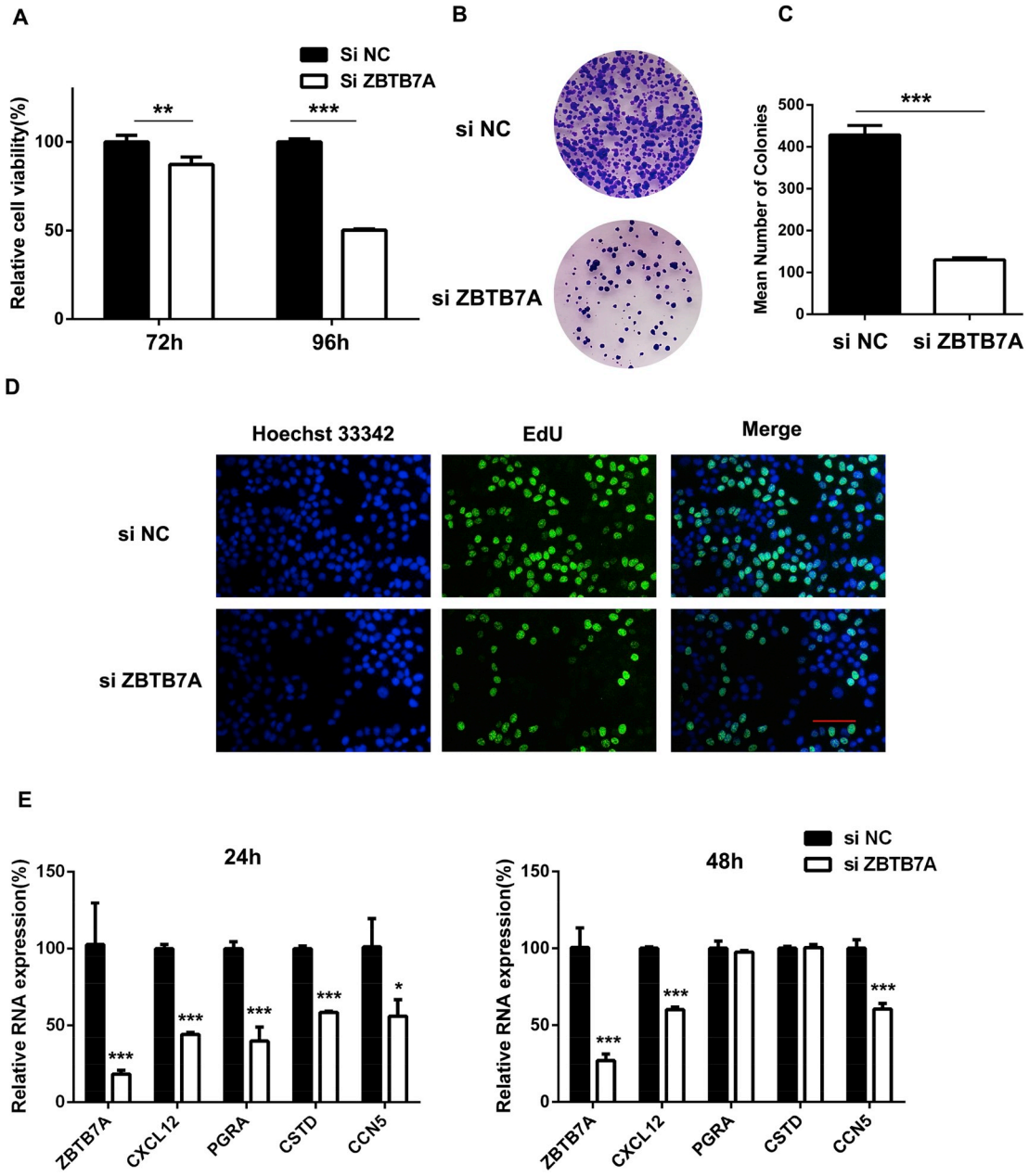


Fig. 4. The inhibition of ZBTB7A attenuates the growth of breast cancer cells. MCF7 cells were transfected with siRNA NC or ZBTB7A. (A) Cell viability of MCF7 cells after siRNAs treatment 72 or 96 h ($n = 5$, $^{**}P < 0.01$, $^{***}P < 0.001$). (B) Representative images of colony formation assay in si NC and si ZBTB7A groups after 14 days. (C) Number of colonies in MCF7 tumor cells with siRNAs control and ZBTB7A ($n = 3$, $^{***}P < 0.001$). (D) EdU incorporation images of MCF7 cells after transfection with siRNA NC or ZBTB7A. The ability of proliferation was evaluated through EdU incorporation assay. Scale bar: 50 μm. (E) QPCR anlaysis of ER-α related genes mRNA expression after treated with siRNA NC or ZBTB7A for 24 h or 48 h ($n \geq 3$, $^{***}P < 0.001$, $^{*}P < 0.05$).

CCTT; antisense, CCAAGGGCTACCATTTGGGT), CSTD(sense, CTGGAC ATCGCTTGCTGGAT; antisense, TGCCTCTCCACTTTGACACC), CXCL12(sense, GTGCCCTTCAGATTGTAGCCC; antisense, GCCCTTCCC TAACACTGG -TT), GAPDH (sense, AGCCTCAAGATCATC-AGCAAT GCC; antisense TGTGGTCATGAGTCCTTCCACGAT). The ΔCt value of genes were normalized to GAPDH. The $2^{-\Delta\Delta Ct}$ method was applied to determine the relative gene expression levels.

3.10. Dual-luciferase reporter assays

Cells were planted in 12-well plates and transfected with ER-α vector containing luciferase or with empty control pGL 4.1. A luciferase assay kit (Promega) was applied to assess the reporter activity according to the manufacturer's instructions. Luciferase activity was normalized by using Renilla luciferase as internal control.

3.11. Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) was performed using EZ-ChIP™ chromatin immunoprecipitation kit (Millipore) according to the manufacturer's recommendation. Briefly, cross-linked chromatin was sonicated to 200–1000 bp fragments. The chromatin was immunoprecipitated using an anti-ZBTB7A antibody (A300-549A, Bethyl Laboratories). Normal human IgG (Cell Signaling Technology) was used as a negative control. ChIP samples were analyzed by quantitative real-time PCR using SYBR Green. The primers specific for ER-α promoter were 5′- GCTGTGCTCTTTTT CCAGGT-3′(forward), 5′-GTCTGACCGTA GACCTGCGCGTTG-3′(reverse).

3.12. Transfection and ubiquitination assay

293T cell transfection was conducted using Lipofectamine 2000 (Life Technologies). Plasmids encoding ubiquitin were transfected in 293T cells with and without knockdown of ZBTB7A. Cells were harvested and lysed with RIPA buffer after 48 h. Precipitated ER-α protein was isolated using SDS-PAGE, transferred to PVDF membranes and blotted with an anti-ubiquitin antibody (3936, Cell Signaling Technology).

3.13. Statistical analysis

The data displayed correspond to the mean ± standard deviation (SD) and all statistical analyses were performed using GraphPad Prism 6.0 software. The statistical significance of differences between the two groups was determined by a two-tailed Student's t-test, and one-way ANOVA was used for comparison between multiple groups. When data could not satisfy the conditions of ANOVA, Kruskal–Wallis test was used. Pearson correlation coefficient was used to calculate statistical dependence. $P < 0.05$ was considered to statistically significant. All experiments were repeated at least three times.

4. Discussion

Studies have shown that ZBTB7A regulates the expression of proto-oncogene or tumor suppressor genes at the transcriptional level [11,13,26]. It was proved that ZBTB7A binds to ER-α promoter sequence, which is located at −1381, −2079, −2729, and −3253 bps from the transcription start site [27]. Interestingly, different from the previous report, we found ZBTB7A transcriptionally regulates ER-α by indirectly binding to its promoter region +146 to +461 bp from the transcription start site of *ESR1*. Even more important, we provided the evidence for the first time another novel mechanism that ZBTB7A maintains ER-α protein level at post-translational level by inhibiting proteasome-mediated degradation.

Fulvestrant, a selective oestrogen receptor down-regulator, is an effective approach for ER-α positive breast cancer treatment in the clinic [28]. Mechanistic studies have shown that fulvestrant induces proteasomal degradation of ER-α protein, shutting down the estrogen signaling to causes proliferation arrest and apoptosis of breast cancer cells [29,30]. In this article, we further confirmed ZBTB7A promotes breast cells proliferation and expolred its underlying mechanisms ZBTB7A regulates expression and ubiquitinational degradation of ER-α. Our research highlights an apparently promising avenue developing ZBTB7A as an anticancer target.

We screened for genes that directly regulates proteasomal degradation of ER-α protein by ZBTB7A silencing. The results displayed that TRIM25's mRNA and protein levels are upregulated in ZBTB7A knockdown group. The present research confirmed the E3 ligase TRIM25 interacts with and ubiquitylates ER-α, consequently promotes the degradation of ER-α [18]. Moreover, our data display inhibition of TRIM25 rescued the protein expression of ER-α that was downregulated by ZBTB7A. Therefore, our findings provide evidences that TRIM25 mediates the ubiquitylation of ER-α, which may be responsible for the degradation by ZBTB7A.

Author contributions

X.X., Y.Y.S., L.Y.Y., X.Y.N. and G.L. designed and performed most of the experiments, X.X. and X.G.C. analyzed data, X.X., J.H. and X.Y.Z. wrote the manuscript, W.B.Z, J.Z. and J.H.L. provided ideas and critical comments. X.D.P. and X.Y.Z. conceived and directed the project.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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