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Deubiquitination and stabilization of estrogen receptor α by ubiquitin-specific protease 7 promotes breast tumorigenesis

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ABSTRACT

Breast cancer is the most common malignancy in women around the world. Estrogen receptor α (ERα) is expressed in approximately 70% of breast tumors, and considered as one of most effective targets in breast cancer therapy. It has been reported that the degradation of ERα protein is mediated by ubiquitin-proteasome system. However, little is known about the regulation of ERα deubiquitination, a critical constituent of its degradation control. The current study first reports that there is a positive correlation between ERα and ubiquitin specific protease 7 (USP7) protein levels in human breast tumor tissues. Subsequent studies showed that USP7 physically interacted with the ERα, thereby mediating the deubiquitination and stabilization of ERα. In addition, USP7 inhibition or silencing led to growth inhibition and apoptosis of ERα-positive breast cancer cells both in vitro and in vivo. Furthermore, overexpression of ERα rescued the USP7 silencing-induced cell cycle arrest and apoptosis, supporting that ERα status is essential to the function of USP7 in breast carcinogenesis. Overall, this study suggests that targeting USP7-ERα complex could be a potential strategy to treat ERα-positive breast cancer.

1. Introduction

Breast cancer has the highest incidence and mortality among women all over the world [1]. Breast cancer can be divided into three subtypes based on the molecular classifications or status of estrogen receptor (ER), HER2 and progestogen receptor (PR) [2,3]. ER is expressed in about 70% of breast cancer cases and is a well-known biomarker and well-established target for endocrine therapy [4]. Undoubtedly, ER is considered as one of the most successful molecular targets in therapeutic history of breast cancer [5,6]. ER alpha (ERα) plays a main role in the initiation and progression of breast cancer by increasing the level of oncogenic proteins, including cyclin D1 and c-myc which accelerate the G_1-S phase transition [7,8]. Therefore, studying the molecular regulatory mechanisms of ERα and exploring new therapeutics have become a long-term goal of breast cancer research.

ERα belongs to the nuclear receptor family and exerts transcriptional activity upon the binding of estrogen [9]. Altering the transcription of ESR1 via transcription factors, co-repressors and co-activators binding to ESR1 promoter could lead to dysregulation of ERα [10,11]. In addition to transcriptional regulation, some posttranslational modifications, such as ubiquitination, sumoylation, phosphorylation, neddylation and acetylation, are critical for the regulation of the protein stability or transcriptional activity of ERα [12–15]. Interestingly, the other posttranslational modifications can crosstalk to ubiquitination to regulate the ERα stability, indicating that ubiquitin proteasome system has a center role in regulation of ERα expression [16,17]. Several E3 ubiquitin ligases have been demonstrated to

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regulate ERα stability through inducing its proteasomal degradation in breast cancer, including BRCA1, RNF31, CHIP, and MDM2 [18–21]. Indeed, the ubiquitination and degradation of ERα protein might be reversed by its deubiquitination, which is opposed to ubiquitination. Deubiquitinases (DUBs), the critical effectors of deubiquitination, function to cleave mono- or poly-ubiquitin (Ub) chains from target proteins to modulate their degradation and regulate multiple cellular processes [22,23]. However, the DUB of ERα has not been reported.

Ubiquitin specific protease 7 (USP7) was originally documented as a herpes simplex virus type 1Vmw110-interacting protein and thus also known as herpesvirus-associated ubiquitin-specific protease (HAUSP). USP7 was showed to deubiquitinate and/or stabilize numerous proteins involved in multiple oncogenic pathways [24,25]. Some oncoproteins, such as androgen receptor, NOTCH1, histone demethylase PHF8, and mediator of DNA damage checkpoint 1 (MDC1) are cooperated with USP7 to drive the oncogenic progresses in several malignancies [26–29]. Collectively, these previous studies have suggested that USP7 is a tumor promoter and may serve as a therapeutic target for cancers. The current study is intended to investigate additional unknown roles of USP7 in breast cancer. We show that USP7 expression is positively correlated with ERα protein level in clinical breast tissues, but negatively correlated with the survival of patients with breast cancer. Further investigations revealed that USP7 is a DUB of ERα protein that promotes breast cancer growth and apoptosis escape in vitro and vivo.

2. Materials and methods

2.1. Cell lines and cell culture

The following cell lines were purchased from American Type Culture Collection (Manassas, VA, USA): MCF-7, MDA-MB231, MDA-MB468, HCC1937, MCF10A, HEK293T and T47D. HyClone DMEM with 10% fetal bovine serum (FBS) was applied to cell culture of MCF-7, MDA-MB231, MDA-MB468, HEK293T and T47D. HCC1937 was grown in RPMI 1640 with 10% FBS. MCF10A was grown in MEBM (Lonza). 37 °C and 5% CO_2 were kept in order to maintain all the cell cultures.

2.2. Reagents and antibodies

P5091 (S7132) and MG132 (S2619) were from Sellectchem (Houston, TX, USA). Cycloheximide (CHX) was from Sigma-Adrich (Sigma-Adrich, Louis, MO). Control siRNA (SC-37007) and USP7 siRNA (SC-41521) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). USP7-1 and −2 siRNA (S0222) were purchased from genechem (Shanghai, China). MTS (catalog no. G111) was obtained from Promega Corporation (Madison, WI, USA). Co-IP assay kit (14311D) was purchased from Life Technologies (Carlsbad, CA). Annexin V-FITC/PI apoptosis detection kits (KGA107) were from Keygen Company (Nanjing, China). The following antibodies were from Cell Signaling Technology (MA, USA): anti-PARP (9532), anti-ERα (8644) and anti-USP7 (4833) for western blot and Co-IP assay, anti-p21 (2947), anti-CDK4 (12790), anti-Cyclin D1 (2922), anti-Ubiquitin (3936), anti-K48-ub (12805), HA-tag (3724), and DYKDDDDK (FLAG) tag (14793). Anti-Bcl-2 (BS70205) and anti-GAPDH (MB001) were from Bioworld (Bioworld Technology, Inc., Louis Park, MN, USA). For western blot, the dilutions of the above antibodies were 1:1000; for confocal assay, the dilutions are as following: ERα (1:400); USP7 (1:50); FLAG (1:800); for Co-IP assay, the dilutions are as following: ERα (1:50); USP7 (1:50); FLAG (1:50); HA (1:50). Anti-ERα (ab32063) and anti-USP7 (ab108931) for immunohistochemical staining were from Abcam (USA) and the dilutions were 1:500. Lentivirus was from VigeneBio (Shandong, China) and plasmids were from Genechem (Shanghai, China).

2.3. Patients' selection

Two tissue arrays were obtained from Shanghai Outdo Biotech Company (Shanghai, China). The human breast cancer tissue arrays were from 140 patients and the adjacent tissue arrays were from 77 patients. All specimens were performed following the protocol and ethical standard of this company. Anti-ERα (ab32063) and anti-USP7 (ab108931) were used to immunolabel the paraffin embedded sections. ERα and USP7 expression in cancer tissue and adjacent tissue were evaluated.

2.4. Immunohistochemical staining and intensity analysis

According to standard techniques we previously reported [30], xenografts were fixed and embedded in paraffin, followed by section. MaxVision kits (Maixin Biol) were incubated with tumor sections, and subjected to immunohistchemistry for ERα, Bax and Cyclin D1. 50 μl of MaxVisionTM reagent was added and the slide was stained with 0.05% diaminobenzidine and 0.03% H2O2 in 50 mM tris-HCl. DAB was applied to measure the primary antibodies. To distinguish and outline the targeted protein expressing cells within images, the Color Threshold, Make Binary and Analyze Particle functions within Image J were used.

2.5. Cell viability assay

The assay was performed as previously reported [31]. Cells were plated in 96-well plates in triplicate and cultured overnight. Cells were then exposed to the indicated drug or siRNA targeting USP7 for 24, 48, or 72 h, followed by MTS assay, according to the manufacture's instructions.

2.6. Edu staining

Cell-Light^{TM} EdU Apollo 567 In Vitro Kit (Cat number: C10310-1, RiboBio, Guangzhou, China) was applied for this assay. The indicated cells were plated onto a chamber slide and then exposed to the indicated treatment. After the indicated time, 50 μmol/L of Edu was added and incubated for 2 h. After that, fixed cells were incubated with 2 mg/ml of glycine, followed by 0.5% Triton X-100 for 10 min. Fluorochrome, catalytic agent, apollo reaction cocktail containing annexing agent buffer and apollo reaction buffer was used to incubate cells for half an hour. Cell nucleus was stained with DAPI in dark. Images were captured using an Olympus microscope.

2.7. Cell cycle and apoptosis assay

These two assays were performed as we previously described [32,33]. The indicated cells were treated with the indicated agents for 24 or 48 h. Cells were digested and collected. For cell cycle assay, 500 μl of PBS and 2 ml of 70% ethanol were added and resuspended overnight. The washed cells were incubated with 50 μg/ml of PI, 0.2% of Triton-X-100 and 100 μg/ml of RNase complex for 30 min in dark at 4 °C. Flow cytometry was then applied to the stained cells. For cell apoptosis analysis, the treated cells were washed with PBS thrice and centrifuged. Cells were resuspended for 30 min with a complex of 500 μl Annexin V-FITC binding buffer, 5 μl PI and 5 μl Annexin V-FITC. This was followed by flow cytometry.

2.8. Cell clonogenic assay

Clonogenic assay was performed as we reported previously [34]. Cells were plated into six-well plates in triplicate for overnight. Cells were then exposed to the mentioned inhibitor or siRNA targeting USP7 for the indicated time. Then cells were digested, collected and plated into six-well plates cultured with 10% FBS-DMEM for roughly 14 days. 4% paraformaldehyde was used to fix cells. Then cells were washed

with PBS for three times. And cells were then stained with crystal violet solution. The images were taken and presented.

2.9. Immunoblotting and Co-IP

This assay was performed as we previously reported [35]. Cell lysates obtained from the treated cells were resolved by SDS-PAGE, followed by transferring onto PVDF membranes. The membranes were then incubated with 5% non-fatted milk for 1 h and with the indicated antibodies overnight. This was followed by addition of the secondary antibodies and incubation for 1 h. Corresponding protein-antibodies complexes were analyzed by ECL detection reagents, followed by X-ray films. For immunoprecipitation, the dynabeads and antibodies mixtures were mixed overnight. This was followed by adding the extracted cell lysates to the combined mixtures for 1 h at 4 °C. The mixtures were washed with PBS-T thrice. Finally, appropriate SDS loading buffer was applied to dissolve the mixtures and subjected to western blot analysis.

2.10. Real-time polymerase chain reaction analyses

This assay was did as we previously described [36]. RNAs were extracted from cells exposed to the indicated treatment with RNAiso plus (TakaRa Biotechnology, Dalian, China), according to the manufacturer's instructions. The concentrations of RNAs were equal in each group and 1000 ng RNAs were used to synthesize first-strand cDNA by PrimeScript RT Master Mix kit (TaKaRa, Dalian, China). The mRNA levels of ERα and GAPDH were measured using Real-time quantitative PCR. According to the manufacture's instructions, SYBR Premix Ex Taq kit was then used. The primers for PCR in this study as follow: ERα: F: 5′ TCTTGGACAGGAACCAGGGA 3'; R: 5′ CAGAGACTTCAGGGTGCTGG 3; GAPDH: F: 5′TCCCATCACCATCTTCCA3'; R: 5′ CATCACGCCACAGTT TCC3'. .

2.11. ER-positive xenograft in mice

The mice (18–22 g, female) were from Guangzhou University of Chinese Medicine and animal protocols were approved by the Institutional Animal Care and Use Committee of Guangzhou Medical University. The nude BALB/c mice were housed in quarantine room for inspection. After two-three days, mice were housed in barrier facilities in animal facility. MCF-7 cells were plated into 6 cm dishes for overnight. The mixture containing lentivirus shRNA targeting USP7 and the medium containing polybrene (5 μg/ml, Santa Cruz, CA, USA) was added into cells for 48 h. For stably-transfected cells, puromycin was applied to select at the concentration of 3 μg/ml for two days. Surviving cells were collected and subjected to western blot for USP7 expression. First, 0.72 mg/90-day-release-17β-estradiol pellets were implanted subcutaneously into each mouse for one week. The mice were then subcutaneously implanted with MCF-7 cells or MCF-7 cells stably expressing USP7 deletion. After a month, mice were divided into two groups and administrated with the P5091 (20 mg/kg/d) or control for 15 days, followed by measuring the tumor volumes and body weight every other day.

2.12. Transfection assay

The transfection assay was performed as we described previously [37]. Cells were plated in dishes for 24 h. For siRNA transfection, the complex, including 500 μl RPMI opti-MEM, siRNAS/shRNA targeting USP7 and lipofectamine RNAiMax reagent was prepared to incubate with cells. After 6 h, fresh medium was replaced. Cells were collected after incubation for 48 h. For lentivirus USP7 shRNA transfection, the medium and polybrene (5 μg/ml, Santa Cruz, CA, USA) was added into cells for 48 h. For stably-transfected cells, puromycin was used to select at the concentration of 3 μg/ml for two days. For the transfection of plasmids, cells were transfected with plasmid or control vector mixed with lipofectamine 3000 reagent and then cells were incubated for 48 h for further analysis.

2.13. Luciferase reporter promoter assay

MCF-7 cells were cultured in 24-well plates for 24 h. Cells were transfected with 1000 ng luciferase reporter plasmid containing estrogen response elements (EREs) mixing RPMI opti-MEM and iMax for 24 h. Then cells were treated with USP7 inhibitor or siRNA for the indicated time. According to the manufacture's instructions, dual luciferase assays kit was used to measure the activity of luciferase. The relative luciferase was determined by firefly lucifersae to Renilla luciferase.

2.14. Confocal assay

The treated cells were fixed with paraformaldehyde, and then 0.5% Triton-X was used for 5 min. 5% BSA was applied to block the background, and then cells were incubated with the primary antibodies overnight. The indicated secondary antibodies were applied for 1 h. Cell nucleus was stained with DAPI, subjected to confocal microscope.

2.15. Statistical analysis

The statistical significance between two groups was analyzed by student's t-tests. Survival curves were determined by the Kaplan-Meier way and ANOVA was used for differences among multiple groups. SPSS 20.0 was applied with all the analyses and p value of less than 0.05 was regraded significant. The present data were as mean ± SD from three independent experiments.

3. Results

3.1. USP7 expression is positively correlated with ERα and associated with poor prognosis in breast cancer

To identify the role of USP7 in breast cancer, we analyzed the mRNA levels of USP7 and ERα from published microarray datasets (TCGA). The results showed that USP7 mRNA level is upregulated in breast cancer and the upregulation was more remarkable in ERα-positive ($ERα^+$) breast cancer than that of in ERα-negative ($ERα^-$) breast cancer (Fig. 1A). Consistent with clinically statistical analysis [4], ERα mRNA level is upregulated in human breast cancer (Fig. 1B). In addition, by analyzing the TCGA database, we found that patients with lower USP7 expression had a higher overall survival (Fig. 1C). In light of statistical analysis from TCGA data sets, we further explored USP7 and ERα protein levels in human breast tumor and adjacent tissues by immunohistochemistry assay. We found that the expression of these two proteins were increased in human breast tumor tissues (Fig. 1D–F), and USP7 protein expression was positively correlated with that of ERα (Fig. 1G). Moreover, USP7 expression was an independent poor prognostic factor in survival analysis (Fig. 1H). Furthermore, the Kaplan-Meier curve of ERα protein showed that the prognosis of ERα positive breast cancer was better than that of in ERα negative breast cancer (Fig. 1I). Taken together, these results point to a tumor-promoting role of USP7 and the positive correlation between USP7 and ERα.

3.2. USP7 increases ERα protein expression and stability in breast cancer

Given the positive correlation between USP7 and ERα expression in human breast cancer tissues, we determined the protein expressions of USP7 and ERα in a human mammary epithelial cell line (MCF10A) and five breast cancer cell lines (T47D, MCF-7, MDA-MB-468, HCC1937, and MDA-MB-231). We observed higher protein level of USP7 in breast cancer cells than in MCF10A cells (Fig. 2A). Moreover, USP7 expression was the highest in ERα-positive breast cancer cells (T47D and MCF-7)

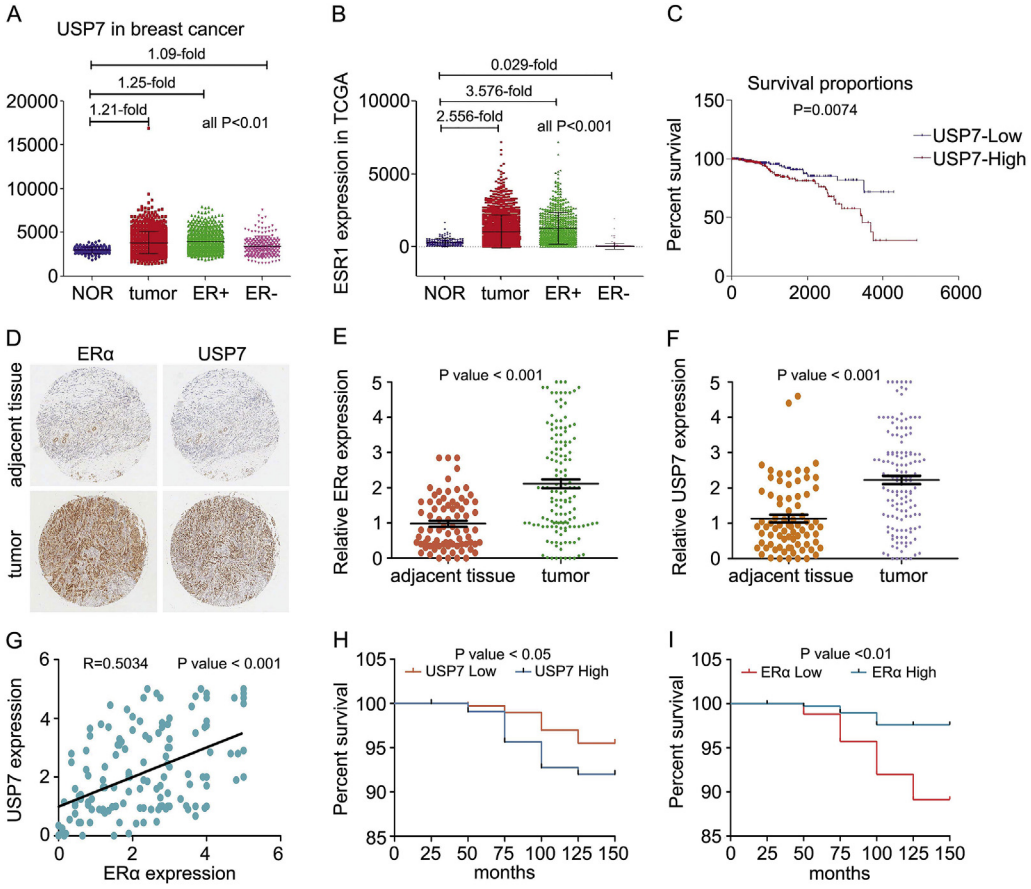

Fig. 1. USP7 expression is positively correlated with ERα and predicts poor prognosis in breast cancer. Expression of USP7 (A) and ERα (B) in human breast cancer clinical specimens from the TCGA mRNA expression array data (https://tcga-data.nci.nih.gov/), in comparison with normal tissues. (C) Kaplan-Meier curves from patients with breast cancer expressing low and high USP7 from the TCGA mRNA expression array data. (D) Representative images of USP7 and ERα expression in human breast cancer tissues (n = 140) and adjacent tissues (n = 77). Expression of ERα (E) and USP7 (F) in all human breast cancer and adjacent tissues including ERα positive and negative breast cancer patients. (G) The correlation of USP7 and ERα expression in human breast cancer (n = 140). (H) Kaplan-Meier curves of the survival between USP7-high and USP7-low breast cancer patients. (I) Kaplan-Meier curves of ERα in breast cancer patients.

(Fig. 2A). To assess the functional significance of correlation between ERα and USP7, we studied the role of USP7 on the ERα expression level. Western blotting analysis in two ERα$^+$ breast cancer cell lines (MCF-7 and T47D) showed that the level of ERα protein was notably decreased after treatment with the specific USP7 inhibitor (P5091) (Fig. 2B). Similarly, USP7 knockdown induced a dramatic reduction of ERα (Fig. 2C). To exclude off-target effects, two independent sets of siRNAs targeting different regions of USP7 and another independent knockdown tool, short hairpin RNA (shRNA) of USP7 were used to transfect MCF-7 and T47D cells, followed by determining effects on the level of ERα protein. All the results showed that USP7 inhibition leads to decreased ERα protein levels (Fig. 2C and D). Furthermore, Dual-luciferase reporter assay showed that the luciferase activity of ERα was dramatically reduced in USP7-depleted or -inhibited cells (Fig. 2E). We next explored whether USP7 was co-translocated with ERα. Results of confocal microscope showed that USP7 inhibitor significantly reduced the abundance of ERα in both the cytoplasm and nuclear (Fig. S1A). The same results were observed in both MCF-7 and T47D cell lines after USP7 knockdown (Figs. S1B and C). These results indicated that USP7 is not required for ERα translocation. Additionally, reverse transcription PCR (RT-PCR) results showed that USP7 inhibition or knockdown resulted in no significant decrease in mRNA level of ERα (Fig. 2F). To further study the potential role of USP7 in modulating the reduction in ERα protein level, the degradation of ERα was measured upon USP7 inhibition or knockdown and cycloheximide (CHX) treatment. Western blotting analysis revealed that a decreased half-life of ERα was clearly associated with USP7 deletion (Fig. 2G, H and J). Vice versa, overexpression of USP7 prolonged the half-life of ERα (Fig. 2I and J). We hypothesized that USP7-promoted ERα degradation was a result of proteasome-mediated protein degradation. Indeed, MG132, which blocks the 20S proteasome activity, effectively rescued the reduction in ERα protein level induced by USP7 deletion (Fig. 2K), supporting that USP7 is functionally essential for ERα protein stability.

3.3. USP7 interacts with ERα

It is well known that the degradation of substrate proteins are regulated by their deubiquitinases (DUBs) [38]. Given that the participation of USP7 in the ERα protein stability, we then investigated the potential interaction between USP7 and ERα proteins. Co-immunoprecipitation (Co-IP) with antibodies against ERα followed by immunoblot (IB) with antibodies against USP7 showed that endogenous USP7 notably interacted with ERα, and vice versa (Fig. 3A and B). Additionally, cellular extracts from HEK293T cells expressing FLAG-tagged ERα and HA-tagged USP7 were prepared and subjected to Co-IP and western blot assays. The results showed that exogenic FLAG-tagged ERα was able to bind to HA-tagged USP7, which further confirmed an efficient, direct interaction between ERα and USP7 proteins (Fig. 3C). Consistently, immunofluorescence staining followed by confocal assay showed that FLAG-tagged ERα appeared to be co-localized with endogenous USP7 in T47D cells (Fig. 3D). To gain a molecular insight into the interaction between ERα and USP7, and to map the USP7-binding region on ERα, a series of FLAG-tagged, truncated mutants of USP7 were used and transfected into HEK293T cells (Fig. 3E and F). Our Co-IP and western blot assays showed that the N-terminal domain (1–560 aa) of USP7 was required for its interaction with ERα (Fig. 3G).

3.4. USP7 deubiquitinates ERα

We hypothesized that USP7 regulates ERα through deubiquitination. To test it we next investigated whether the USP7-ERα interaction affects ERα ubiquitination. A Co-IP assay was performed to assess the level of poly-ubiquitinated ERα. We found that USP7 inhibition or knockdown dramatically increased expression of ubiquitinated ERα

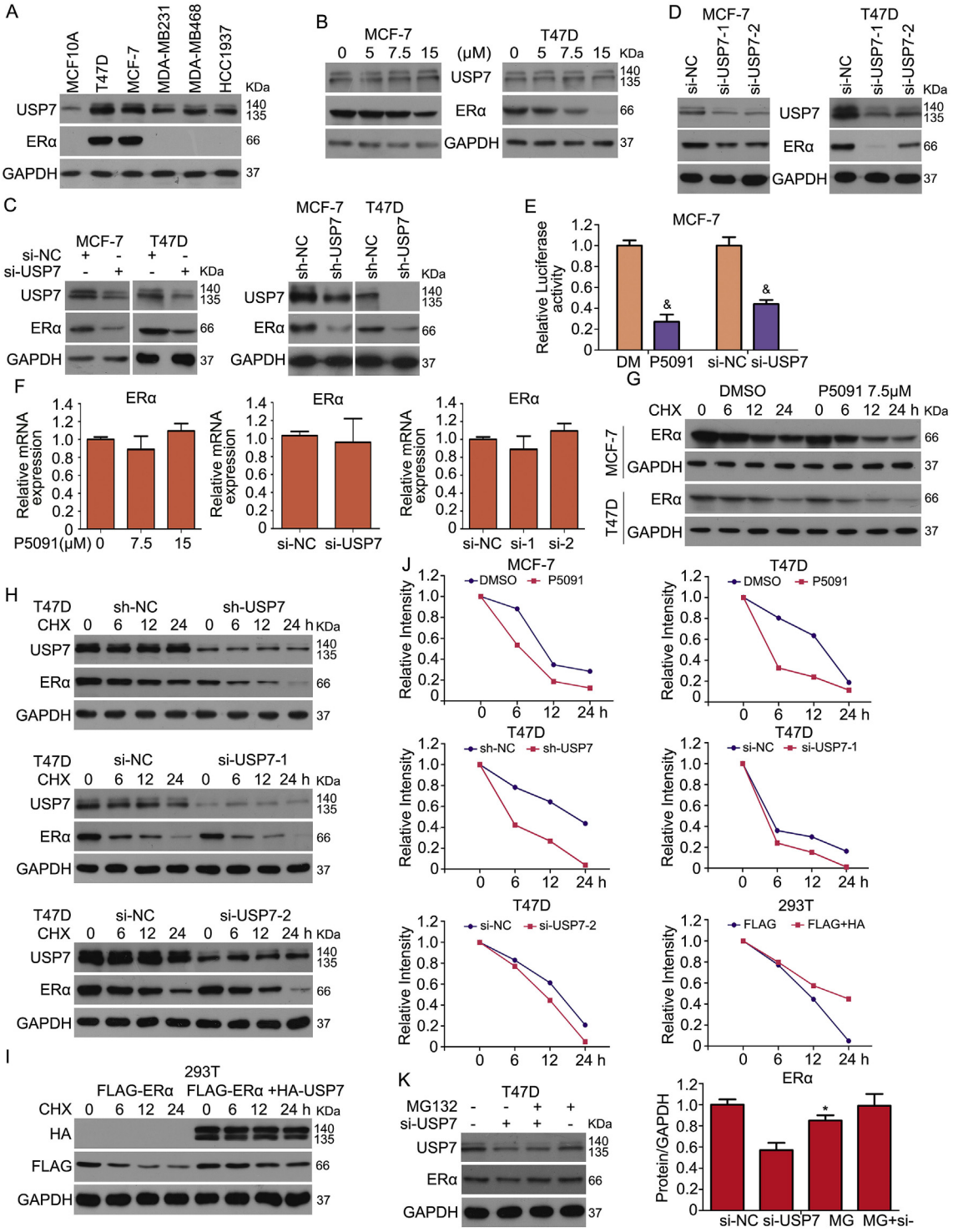

Fig. 2. USP7 increases ERα expression and stability in breast cancer. (A) Cells were collected and protein lysates were subjected to Western bolt analysis for USP7 and ERα expression. (B) Cells were exposed to USP7 inhibitor P5091 for 24 h and protein lysates were collected and detected by Western blotting for ERα and USP7 expression. (C and D) Breast cancer cells were transfected with Scramble siRNA or different sets of USP7 siRNAs (50 nM) for 48 h. Total proteins were prepared, followed by western blot to detect expression of ERα and USP7. (C) Protein lysates were extracted from the cells expressing stable control shRNA or USP7 shRNA. Western blotting analysis was performed to determine the expression of ERα and USP7. (E) MCF-7 cells were transfected with luciferase reporter plasmid containing estrogen receptor response elements (EREs) for 24 h. Then cells were treated with p5091 and USP7 siRNA. Protein lysates were collected, followed by dual-luciferase assay for luciferase activity. $^{\&}p < 0.001$ *versus* each vehicle control. (F) T47D cells were treated with P5091 (7.5 μM, 15 μM) or USP7 siRNAs (50 nM) targeting different sets. Total RNA were extracted and analyzed by RT-qPCR for ERα expression. $P > 0.05$ vs. each vehicle control. (G) MCF-7 and T47D cells were treat with USP7 inhibitor (7.5 μM) and CHX (50 μg/ml). The expression of ERα were analyzed by western blot. (H) Breast cancer cells were transfected with Scramble siRNA (shRNA) or USP7 siRNAs (shRNA) targeting different sets followed by western blotting analysis for detection of ERα and USP7 expression. (I) HEK293T cells were transfected with FLAG-tagged ERα and HA-tagged USP7 for 48 h. Then cellular extract were analyzed by western blotting. (J) The bands of ERα were quantified by densitometry with Image J. GAPDH was as a normalizer. (K) Protein lysate were collected from the treated cells with MG132 and USP7 siRNA. Western blotting analysis were performed for ERα and USP7 expression. $^{*}p < 0.05$ *versus* each vehicle control.

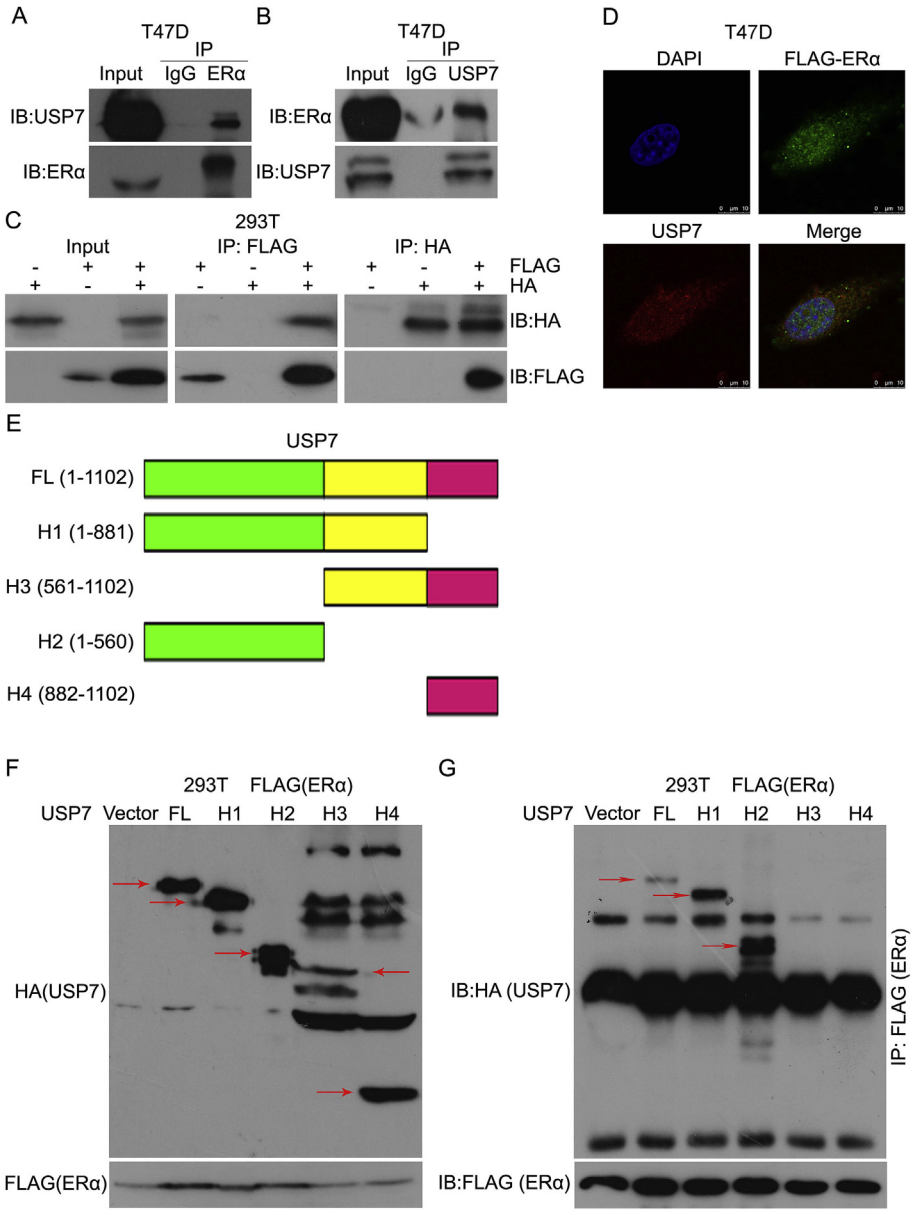

Fig. 3. USP7 interacts with ERα. (A and B) Cellular extracts from T47D cells were subjected to co-immunoprecipitation assay. Western blotting assay were used to determine the interaction between USP7 and ERα. (C) HEK293T cells were transfected with FLAG-tagged ERα and HA-tagged USP7 for 48 h. Co-immunoprecipitation assay was performed to measure levels of FLAG and HA. (D) T47D cells were transfected and then stained with anti-USP7 and anti-FLAG. Scale bar, 10 μm. (E) Schematic representation of HA-tagged full-length USP7 (FL) and its various deletion mutants (H1–H4). (F) HEK293T cells were transfected HA-tagged USP7 (FL) or its deletion mutants, followed by western blotting analysis. (G) Immunoprecipitates with FLAG beads were subjected to immunoblot for HA and FLAG.

(Fig. 4A–C). To further determine whether the decreased ERα expression triggered by USP7 deletion is due to ERα degradation, we assessed the levels of K48- and K63-poly-ubiquitinated ERα. Western blotting analysis showed that K48-poly-ubiquition of ERα was increased, whereas no obvious change in K63-poly ubiquition of ERα was detected (Fig. 4C). To confirm that, we generated HEK293T cells expressing MYC-tagged full-length USP7 (USP7/WT) and MYC-tagged catalytically inactive mutant of USP7 (USP7/C223S). The abundance of poly-ubiquitinated ERα was increased in cells expressing USP7/C223S compared to that in cells expressing USP7/WT (Fig. 4D), suggesting that the enzymatic activity of USP7 is indispensable for USP7-dependent ERα deubiquitination.

3.5. USP7 loss inhibits the growth of breast cancer cells

Downregulating ERα expression is an effective strategy to treat ERα-positive breast cancer [5,6]. After obtaining the results of the inhibition of ERα level by USP7 inhibition or knockdown, we next assessed the effect of USP7 in proliferation of ERα$^+$ breast cancer cells. We showed that USP7 inhibitor, P5091, dramatically suppressed cell viability of ERα-positive breast cancer cells in a concentration-dependent manner (Fig. 5A, Fig. S2A). In addition, siRNA targeting USP7 exerted an inhibitory effect on cell viability (Fig. 5B, Fig. S2B). To explore the long-term effect of USP7 deletion on MCF-7 and T47D cells, we did cell colony formation assay, and the results revealed that USP7 inhibition by either inhibitor or shRNA targeting USP7 decreased the colony formation (Fig. 5C, Fig. S2C). Furthermore, Edu, a thymidine analog which binds to replicated chromosomal DNA when the cell is in S phase, was applied in MCF-7 and T47D cells treated with USP7 inhibitor or siRNA. The cells labeled with Edu were reduced after USP7 inhibition (Figs. S2D–F). These findings supported a role of USP7 in promoting growth of ERα$^+$ breast cancer cells.

3.6. USP7 inhibition suppresses ERα$^+$ breast cancer growth in MCF-7 xenografts

After finding the inhibitory effect of USP7 deletion on cell growth in vitro, we next determined whether USP7 inhibition suppresses ERα$^+$ breast cancer in vivo. MCF-7 cells were implanted into nude mice to establish in vivo xenografts. We found that USP7 inhibitor P5091

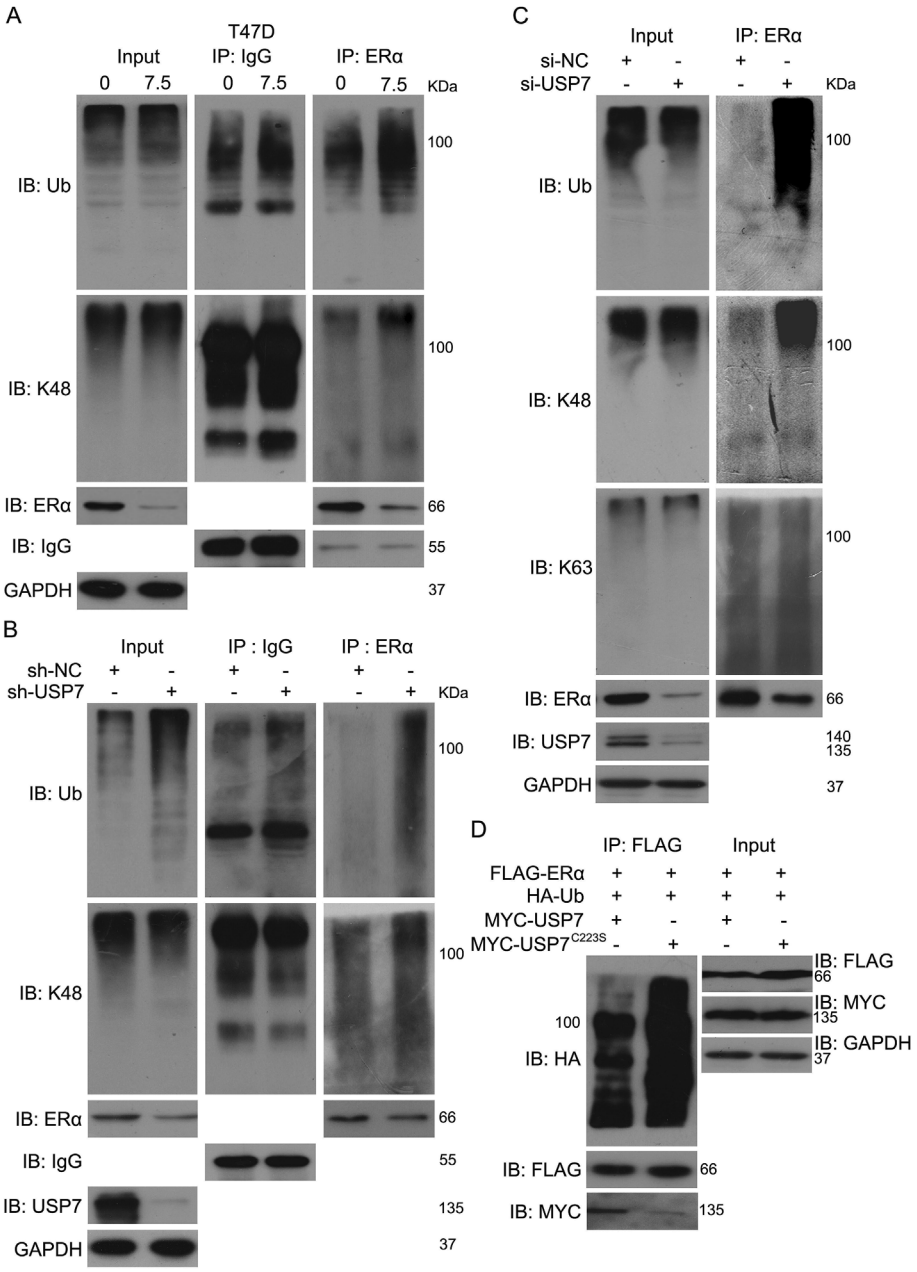

Fig. 4. USP7 deubiquitinates ERα. (A) T47D cells were treated with P5091 (7.5 μM) and MG132 (10 μM) for additional 6 h, immunoprecipitated with ERα and IgG beads and then immunoblotted with ubiquitin (Ub), K48, ERα and IgG. (B) Protein lysates were extracted from T47D cells expressing USP7 shRNA. Co-immunoprecipitation assay was performed using ERα and IgG and then immunoblotted with ubiquitin, K48, ERα, USP7 and IgG. (C) Cells were transfected with USP7 siRNA for 48 h and MG132 for 6 h. Cells were harvested for immunoprecipitation and immunoblotting. (D) HEK293T cells were transfected MYC-USP7, MYC-USP7^{c223s}, HA-Ub and FLAG-ERα, immunoprecipitated with FLAG and then immunoblotted with HA.

resulted in xenograft shrinkage (Fig. 5D). Consistently, the tumor volumes and weight were significantly declined in the P5091-treated group (Fig. 5E, Fig. S2H), while the body weight has no obvious loss during the treatment (Fig. S2G). In addition, levels of pro-apoptosis protein Bax were increased while that of Cycin D1 and ERα were decreased (Fig. 5F, Fig. S2K). To evaluate the inhibitory mechanism of USP7 inhibitor, TUNEL assay was performed to determine levels of apoptosis. The results showed that the TUNEL-positive cells were increased by P5091 treatment, indicating that USP7 inhibition induced tumor apoptosis (Fig. 5G). Furthermore, we implanted subcutaneously USP7 shRNA stably expressing MCF-7 cells, and obtained similar results. As shown in Fig. 5H, I and Figs. S2I and J, we observed the decreased tumor volumes and weight in USP7 shRNA group while no change in body weight. Immunochemical staining and TUNEL analysis showed that USP7 silence triggered tumor apoptosis (Fig. 5J and K, Fig. S2K). Collectively, we conclude that USP7 inhibition suppresses tumor growth derived from MCF-7 cells.

3.7. Cell cycle arrest and apoptosis are triggered by inhibition of USP7

To explore the underlying mechanism by which USP7 mediates proliferation of ERα$^+$ breast cancer cells, we performed cell cycle analysis. USP7 inhibition or silence increased the population in G_0/G_1 phases, indicating that USP7 regulates G_1 to S transition in ERα$^+$ breast cancer cells (Fig. 6A–C). As well known, p21, Cyclin D1 and CDK4 are all essential players for G_0/G_1 to S phase transition. We hypothesize that these three proteins could be regulated by USP7. To test this idea, we performed western blotting analysis and found that indeed the expression of Cyclin D1 and CDK4 proteins was decreased and the expression of p21 protein was upregulated in MCF-7 and T47D cells after USP7 inhibition (Fig. 6D–F). Next we determined whether apoptosis occurrence is required for growth inhibition induced by USP7 deletion. To do so, we applied flow cytometry analysis, and found that USP7 inhibition increased the number of cell death. To study the molecular mechanism by which the inhibition of USP7 induces cellular apoptosis, we determined levels of several classical pro-apoptotic and anti-

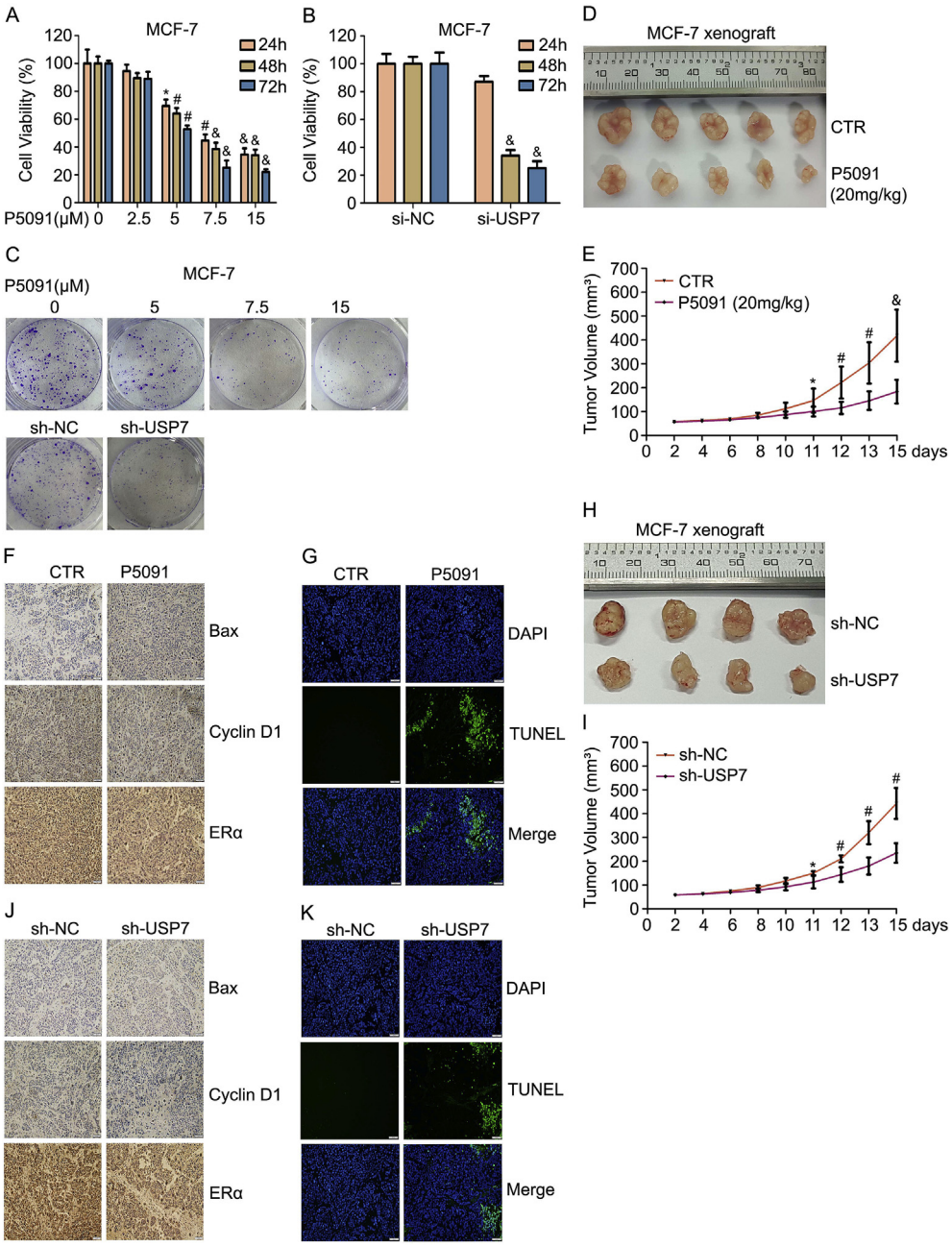

Fig. 5. USP7 inhibition suppresses ERα$^+$ breast cancer growth in MCF-7 xenografts. Cells were treated with P5091 in different doses for the indicated times (A) or USP7 siRNA (B). 20 μl MTS was added for 2 or 3 h. Cell viability was measured. $^*p < 0.05$, $^\#p < 0.01$, $^\&p < 0.001$ *versus* each vehicle control. (C) Cells were collected from the indicated cells with P5091 or USP7 shRNA treatment. Colony formation assay was performed and represent images are shown. Tumor images of mice with subcutaneous injection of MCF-7 cells (D) or MCF-7 cells transduced with USP7 shRNA (H) were shown. (E and I) Growth curves of tumor were shown. $^*p < 0.05$, $^\#p < 0.01$, $^\&p < 0.001$ *versus* each vehicle control. (F and J) Immunohistochemical assay were performed for ERα, Bax, Cyclin D1 expression. Scale bar, 50 μm. (G and K) Tumor was sectioned, followed by TUNEL staining. The represented images from three independent experiments were shown. Scale bar, 20 μm.

apoptotic proteins, including PARP and Bcl-2. The results of western blotting showed that PARP cleavage and downregulation of Bcl-2 were induced by USP7 inhibition (Fig. 6G–I).

3.8. USP7-promoted breast carcinogenesis depends on ERα status

Given that USP7 regulates the growth of ERα$^+$ breast cancer cells and stabilizes ERα protein expression, we then asked whether USP7-promoted ERα stabilization is crucial for increasing the cellular malignant phenotype. To test this idea, we transduced full-length plasmid of human ERα into MCF-7 cells and assessed the effect of ERα overexpression on cell growth inhibition induced by silencing USP7. As shown in Fig. 7A and B, ERα overexpression potently blocked USP7 knockdown-induced cell death. Importantly, PARP cleavage and Bcl-2 downregulation induced by USP7 inhibition were attenuated upon ERα overexpression (Fig. 7C). To further investigate this finding, we evaluated the role of ERα on cell cycle progression in USP7-silenced MCF-7 cells. Overexpression of ERα was able to rescue USP7 silencing-induced G_1 arrest (Fig. 7D and E). Notably, increased p21, which blocks the G_1-S phase transition, was precluded by overexpressing ERα upon USP7 knockdown (Fig. 7C). These results indicate that USP7 promotes cell growth in part through ERα stabilization (Fig. 7F).

4. Discussion

Breast cancer is the biggest threat to women's health worldwide. Targeting ERα is the common scheme for endocrine therapy in patients with breast cancer because of its sensitivity and effectiveness. ERα has been demonstrated to be one of the most successful molecular targets [5]. The studies exploring the regulatory mechanism of ERα are limited. In this study, we report that USP7 is a novel ERα co-regulator. ERα is bound, and stabilized by USP7. In particular, the USP7-ERα interaction is essential in breast carcinogenesis.

We first found that the mRNA level of USP7 is upregulated in breast

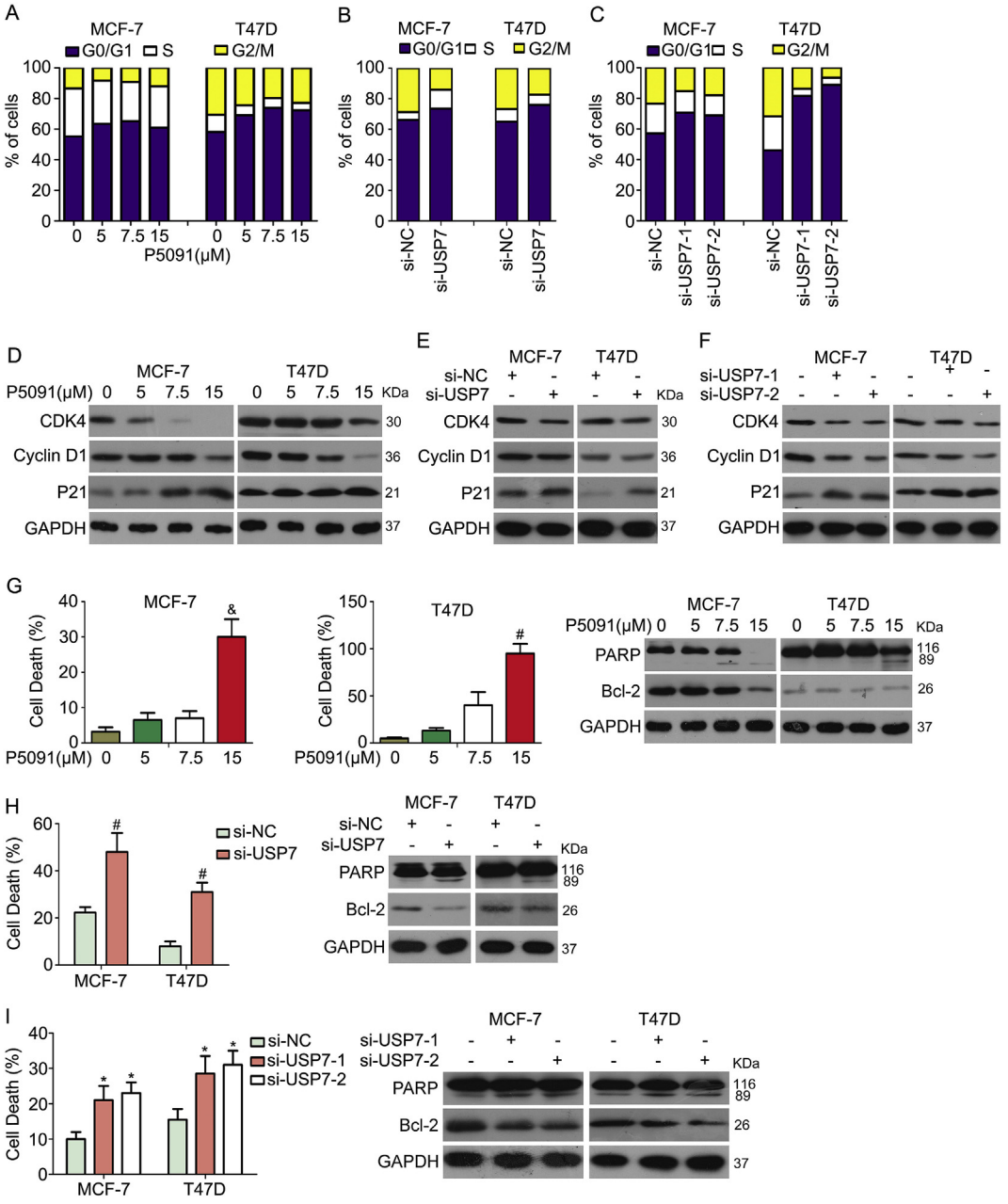

Fig. 6. Cell cycle arrest and apoptosis are triggered by inhibition of USP7. (A–C) Cells were treated with USP7 inhibitor or siRNAs. FACS was performed to test cell distribution. The percentage of cells were calculated. (D–F) Protein lysates were prepared to western blotting for CDK4, p21 and Cyclin D1. GAPDH was served as a loading control. (G–I) Cells were treated with P5091 or USP7 siRNAs treatment. Apoptotic cells were detected with Annexin V- FITC/PI staining followed by flow cytometry analysis. Cell death populations were shown. *p < 0.05, #p < 0.01, &p < 0.001 *versus* each vehicle control. Western blotting analysis was used for PARP and Bcl-2 expression.

cancer based on the analysis from TCGA datasets. Significantly, USP7 levels were higher in ER-positive disease and are associated to poorer prognosis. This findings is consistent with the continued risk of relapse in patients with ER-positive tumor after initial diagnosis [39]. In tissue microarray staining from 140 human breast cancer and 77 adjacent tissue, expression levels of USP7 and ERα were higher in human breast tumor and there was an intimate correlation between these two proteins. Moreover, the survival rate from 2005 to 2015 indicates that USP7 expression is associated with poor prognosis. ER-positive tumors were identified to have a better prognostic than that of ER-negative in the first 5–10 years, however, ER-positive patients show a poor outcome after 5–10 years in a meta-analysis of 10,000 breast cancer patients [40]. In our study, USP7 showed no prognostic value in the first 80 months in breast cancer patients, but after 80 months, USP7 expression was associated with poor prognosis. The explanation for the results of the first 80 months may be that the good prognostic value of ER is superior to the poor prognostic value of USP7. After 80 months, USP7 is superior and indicates a poor prognostic value which maycontribute to the resistance and recurrence in patients with breast cancer.

We further explored the molecular mechanism regulating ERα expression. USP7 inactivation downregulated ERα expression via promoting its degradation. Previous reports have demonstrated that ERα can be degraded by ubiquitin-proteasome system (UPS) [41]. We asked whether UPS is required to ERα degradation induced by USP7 inhibition. We found that MG132 blocked partially the decreased ERα

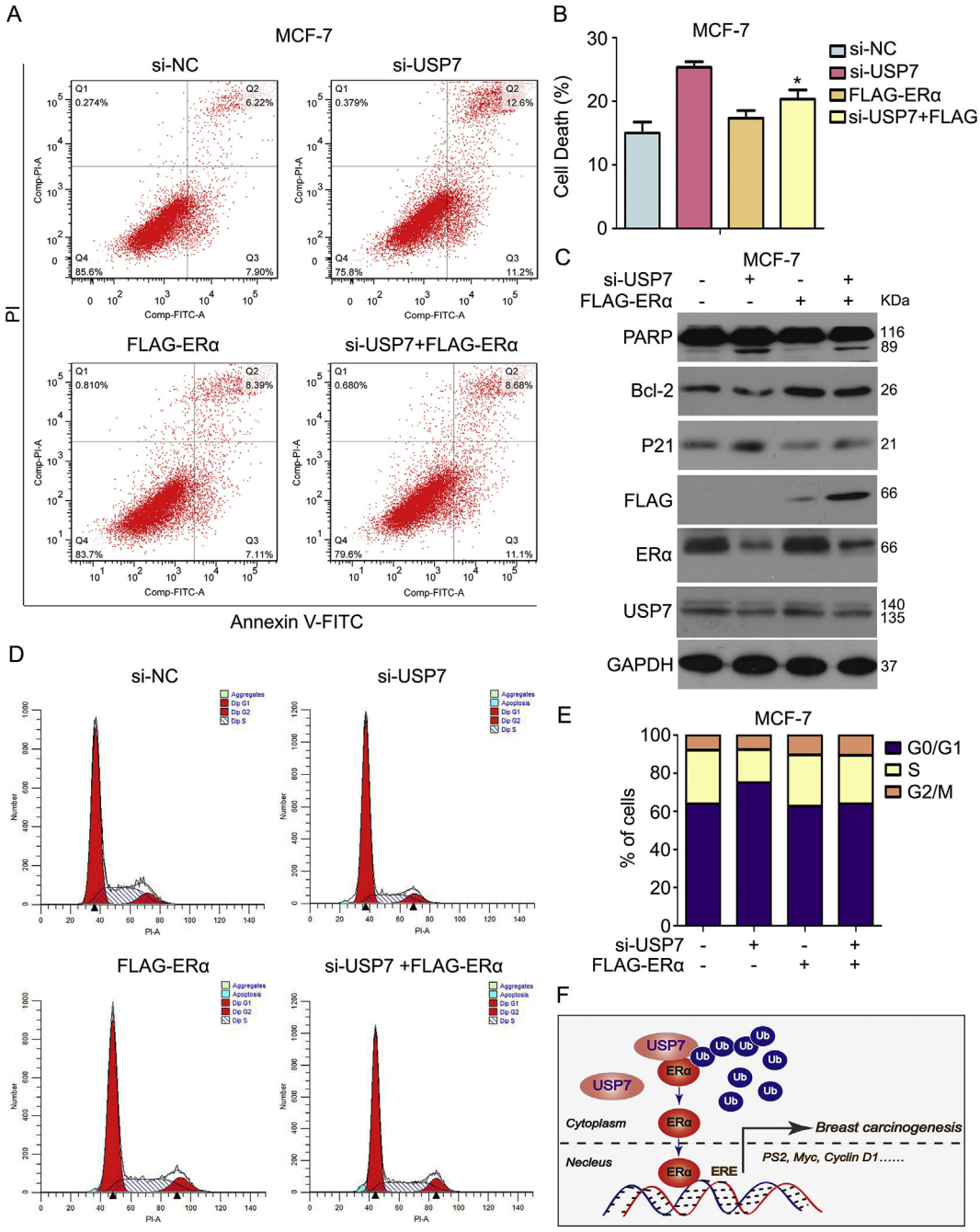

Fig. 7. USP7-promoted breast carcinogenesis depends on ERα status. MCF-7 cells were transfected with FLAG-tagged ERα and USP7 siRNA for 48 h. (A) Flow cytometry analysis was performed by Annexin V-FITC/PI staining to calculate the cell apoptosis. (B) Cell death population was shown from the three independent replicates. *P < 0.05 vs. USP7 siRNA treatment group. (C) The treated cells were prepared for western blotting analysis. PARP, Bcl-2, p21, FLAG, ERα and USP7 expression were detected. (D) Flow cytometry was used to test cell distribution. (E) The ratio of cells was analyzed. (F) A proposed mechanism for USP7 to regulate the ERα level in breast cancer.

expression upon USP7 silence. The current study also demonstrated that USP7 removes the K48-ubiquitin chain but not K63-ubiquitin chain on ERα, resulting in inhibiting proteasome-mediated ERα degradation. We also generated a series of USP7 truncations, and found that the domain of USP7, amino acids 1–560, regulates its interaction with ERα. Additionally, compared with USP7/WT, catalytically inactive mutant of USP7 (USP7/C223S) upregulated the level of ubiquitination on ER, suggesting that USP7 promoted-ERα stability is a consequence of the enzymatic active site of USP7 catalyzed-ERα deubiquitination.

It has been suggested that USP7 plays a functional role in promoting cancer progression [27,29]. In the current study we confirmed the molecular mechanisms by which USP7 contributes to growth and progression of ERα-positive breast cancer. USP7 silencing or inhibition significantly suppresses growth of ERα-positive breast cancer through inducing G_0/G_1 arrest and apoptosis. In particular, cell apoptosis and cell cycle arrest induced by USP7 knockdown can be rescued by ERα overexpression. Overexpressing ERα inhibits PARP cleavage, decreased Bcl-2 and increased p21 expression in MCF-7 cells. These findings indicate that inhibition of ERα deubiquitination is definitely required for USP7 deletion-induced cell death and growth arrest.

In conclusion, this work strongly suggests an interaction between ERα and the DUB USP7, and reports USP7 as the first deubiquitinase

identified to mediate ERα expression. Furthermore, we suggest that USP7 may drive breast tumorigenesis with expression of ERα and that USP7 is a potential target for breast cancer intervention.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.09.003.

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