

## Research Paper

# Tyrosine phosphorylation regulates ER $\beta$ ubiquitination, protein turnover, and inhibition of breast cancer

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## ABSTRACT

Unlike estrogen receptor  $\alpha$  (ER $\alpha$ ) that predominantly promotes hormone-dependent breast tumor growth, ER $\beta$  exhibits antitumor effects in a variety of cancer types. We recently identified a phosphotyrosine residue in ER $\beta$ , but not ER $\alpha$ , that dictates ER $\beta$  transcriptional activity and antitumor function. We show here that this ER isotype-specific phosphotyrosine switch is important for regulating ER $\beta$  activity in cell proliferation, migration, and invasion. At the mechanistic level, phosphorylated ER $\beta$ , which recruits transcriptional coactivator p300, is in turn targeted by p300 for ubiquitination and proteasome-dependent protein turnover. Furthermore, ER $\beta$ -specific agonists such as S-equol enhance ER $\beta$  phosphorylation, suggesting a crosstalk between ligand- and posttranslational modification-dependent ER $\beta$  activation. Inhibition of xenograft tumor growth by S-equol is associated with reduced tumor Ki-67 expression and elevated ER $\beta$  tyrosine phosphorylation. Taken together, our data support the notion that phosphotyrosine-dependent ER $\beta$  signaling is an attractive target for anticancer treatment.

## INTRODUCTION

The diverse physiological and pathological effects of estrogens are mediated by two estrogen receptors, ER $\alpha$  and ER $\beta$ , which are encoded by different genes (*ESR1* and *ESR2*) [1]. Although these two ER isotypes share homologous protein sequence and similar transcriptional activity, they exhibit quite distinct biological functions in cancer development and progression. ER $\alpha$  is well

documented for its role in promoting estrogen-dependent breast tumorigenesis, whereas ER $\beta$  has been reported to inhibit tumor growth in multiple cancer types including breast and ovarian cancers, melanoma, and glioma [1–7]. In addition to common target genes shared by these two ER isotypes, ER $\beta$  binds to its own transcriptional target genes through either estrogen response elements (ERE) or by tethering to other DNA-binding transcription factors [8–19]. The ER $\alpha$ -independent activity of ER $\beta$  represents

a prevailing mode of ER $\beta$  action in ER $\alpha$ -negative cancers. In ER $\alpha$ -positive cancer cells, ER $\beta$  is also capable of interfering with ER $\alpha$  activity through hetero-dimerization and/or competition for common binding sites [6, 14, 15, 20–30], thus making ER $\beta$  a partial dominant negative receptor for ER $\alpha$  [23, 24, 31]. Clearly, ER $\beta$  functions in transcription and cancer are different from those of ER $\alpha$ .

The disparate functional outcomes of the ER $\alpha$  and ER $\beta$  actions are at least partly due to differences in protein structure between these two ER subtypes. Despite a highly homologous central DNA binding domain (DBD, 96% identity) and carboxyl (C)-terminal ligand-binding domain (AF2 + LBD, 53% identity), the amino (N)-terminal sequence (AF1, 18% identity) is quite divergent between these two ER subtypes. This AF1 domain has been linked to subtype-specific transcription activity of ER $\beta$  [32, 33]. Furthermore, we recently discovered that an ER $\beta$ -specific phosphotyrosine residue in the AF1 domain of ER $\beta$  (pY36) dictates the antitumor activity of ER $\beta$  [34]. This tyrosine residue is highly conserved in all mammalian ER $\beta$  orthologs, but not in ER $\alpha$  (alanine in ER $\alpha$ ). Our published work shows that mutation of Y36 to phenylalanine (Y-F) obliterates ligand-dependent transcription and ER $\beta$  antitumor activity in ER $\alpha$ -negative breast cancer [34]. Of note, ER $\beta$  phosphorylation status strongly correlates with longer survival in breast cancer patients [34]. Thus, this newly identified phosphotyrosine switch offers a potential opportunity to fine-tune ER $\beta$  biological activity in cancer with precision and potency.

In the current work, we investigated the influence of the phosphotyrosine switch on tumor cell proliferation, migration, and invasion. In addition, we determined how abundance of phosphorylated ER $\beta$  protein was regulated in cancer cells. Finally, we found that the ER $\beta$ -specific agonist S-equol induced ER $\beta$  phosphorylation and inhibited tumor growth *in vitro* and *in vivo*. Our study lends further support to the notion that turning on the phosphotyrosine switch in ER $\beta$  is important for mobilizing its antitumor activity.

## RESULTS

### The phosphotyrosine switch is important for antiproliferative activity of ER $\beta$

Our published work demonstrates that the ER $\beta$  phosphotyrosine switch regulates antitumor activity of ER $\beta$  in triple negative breast cancer (ER $\alpha$ /PR-/HER2-) [34]. To determine the generalizability of our previous conclusion, we introduced both wild-type (WT) ER $\beta$  and the functionally inactive tyrosine-to-phenylalanine (Y36F) mutant into ER $\alpha$ -positive breast cancer cells (MCF7), ovarian cancer cells (SKOV3), and glioblastoma cells (U87), which represent three cancer types where ER $\beta$  antitumor activity had been previously demonstrated [1, 4, 35]. Consistent with the

previous reports, WT ER $\beta$  significantly reduced tumor cell viability in all three tumor cell lines (Figure 1A–1B, Supplementary Figure 1). In contrast, cells expressing the Y36F mutant exhibited little suppression of cell viability, suggesting that the phosphorylation switch is required for ER $\beta$  antiproliferative activity in multiple tumor cell types. In addition to cell proliferation, ER $\beta$  can inhibit cell migration and invasion [36]. In this regard, we found that ER $\beta$ -mediated inhibition of both tumor cell migration and invasion were significantly compromised by the Y36F mutation in MCF7 cells (Figure 1C–1D), thus implicating a role of the phosphotyrosine switch in multiple aspects of ER $\beta$ -mediated antitumor function.

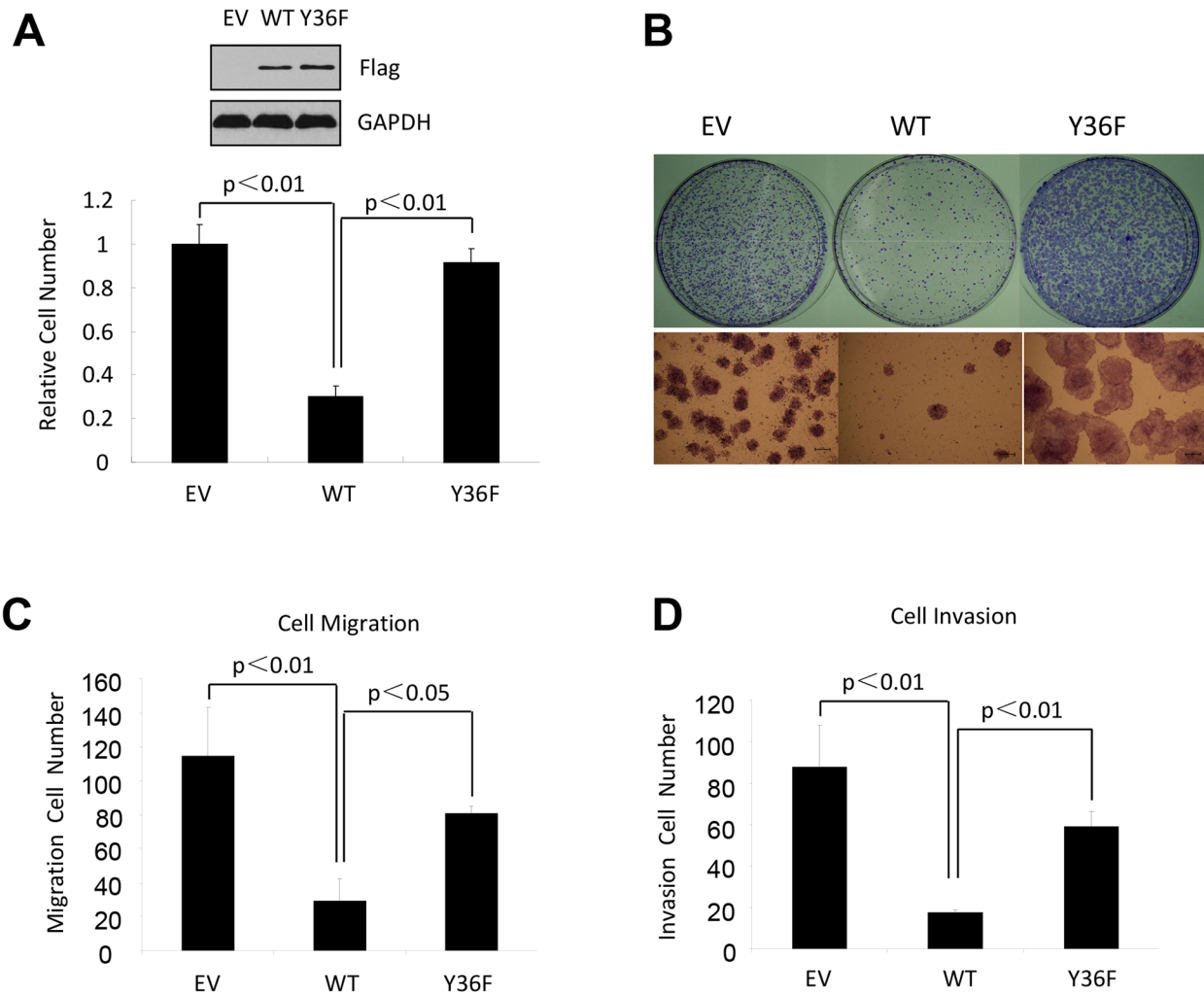
### pY36 facilitates coactivator-dependent ubiquitination and turnover of ER $\beta$

Emerging evidence from studies of eukaryotic transcription suggests a mechanistic coupling between transcriptional activation and ubiquitin-dependent degradation of transcription activators by proteasomes [37, 38]. Consistent with such a notion, AF1 of ER $\beta$  has been implicated in ER $\beta$  ubiquitination and proteasome-mediated degradation [39, 40]. We therefore asked whether pY36 played a role in transcription-coupled ER $\beta$  ubiquitination and turnover. Our previous work indicated that the transcription coactivator p300 is recruited to ER $\beta$  target promoters, including that of MDA7, by ER $\beta$  in a pY36-dependent manner [34]. Here we found that ectopic expression of p300 promoted ligand-dependent transcriptional activation of ER $\beta$ -specific target gene MDA7 by ectopic ER $\beta$  in HEK293T cells (Figure 2A), whereas siRNA-mediated p300 knockdown blunted ER $\beta$  activity (Figure 2B). These data further confirm functional importance of p300 in potentiating ER $\beta$  transcriptional activity. Given the previously reported E4 ubiquitin ligase activity of p300 [41], we used an *in vitro* ubiquitination assay to assess the ability of p300 to ubiquitinate ER $\beta$ . In the presence of ubiquitin and E1/E2 ubiquitin ligases, ER $\beta$  was ubiquitinated by p300 expressed in mammalian cells, but not bacteria (lanes 4 and 5, Figure 2C). This could be due to posttranslational modification of p300 in mammalian cells required for ER $\beta$  ubiquitination. Alternatively, the observed ER $\beta$  ubiquitination could result from the combined action of p300 E4 and a coimmunoprecipitated E3 ubiquitin ligase. We next examined the effect of p300 on ER $\beta$  ubiquitination *in vivo*. We found that p300 knockdown substantially reduced the extent of Flag-tagged ER $\beta$  ubiquitination (compare lanes 2 and 4 in Figure 2D). Furthermore, WT ER $\beta$  was ubiquitinated more extensively than the Y36F mutant (compare lanes 2 and 6 in Figure 2D). This result strongly suggests that the phosphotyrosine switch promotes p300-mediated ER $\beta$  ubiquitination.

Consistent with a role of proteasome-dependent regulation in ER $\beta$  protein turnover, we reproducibly observed stabilization of Flag-tagged WT ER $\beta$  by the treatment of proteasome inhibitor MG132 (compare lanes 1 and 2 in Figure 2D). To directly compare the half-life of WT and mutant ER $\beta$  protein, we assessed their abundance in the presence of the protein synthesis inhibitor cycloheximide. The Y36F mutant was substantially more stable (half-life > 8 hours, Figure 3A-3B) than its WT counterpart (approximately 1 hour). In a parallel experiment, p300 knockdown also significantly extended WT ER $\beta$  half-life (Figure 3C-3D). Together with our previous findings of pY36-dependent promoter recruitment of p300 by ER $\beta$ , our current data strongly indicate that the phosphotyrosine switch promotes two reciprocal events in ER $\beta$ -mediated transcriptional activation: p300 recruitment by ER $\beta$  and subsequent p300-dependent destruction of ER $\beta$ .

## Endogenous ER $\beta$ exhibits antitumor activity

ER $\beta$  is expressed in a significant percentage of breast tumors across all subtypes [42, 43], making it a potential target in anticancer therapies. In addition, several natural and synthetic ER $\beta$ -selective agonists were well tolerated in clinical trials [44] (ClinicalTrials.gov), further elevating therapeutic feasibility of rallying ER $\beta$  antitumor activity. However, before any ER $\beta$ -targeting therapeutic agents are to be further explored for their clinical utility, it is important to verify their dependence on the presumed therapeutic target. To this end, we used CRISPR-Cas9 to knock out ER $\beta$  in triple-negative breast cancer cells MDA-MB-231. Several out-of-frame mutant clones were identified, two of which were selected for functional studies. The two clones contain an insertion of nucleotide "A" (Mut1) and deletion of "ACAA" (Mut2), respectively, at the

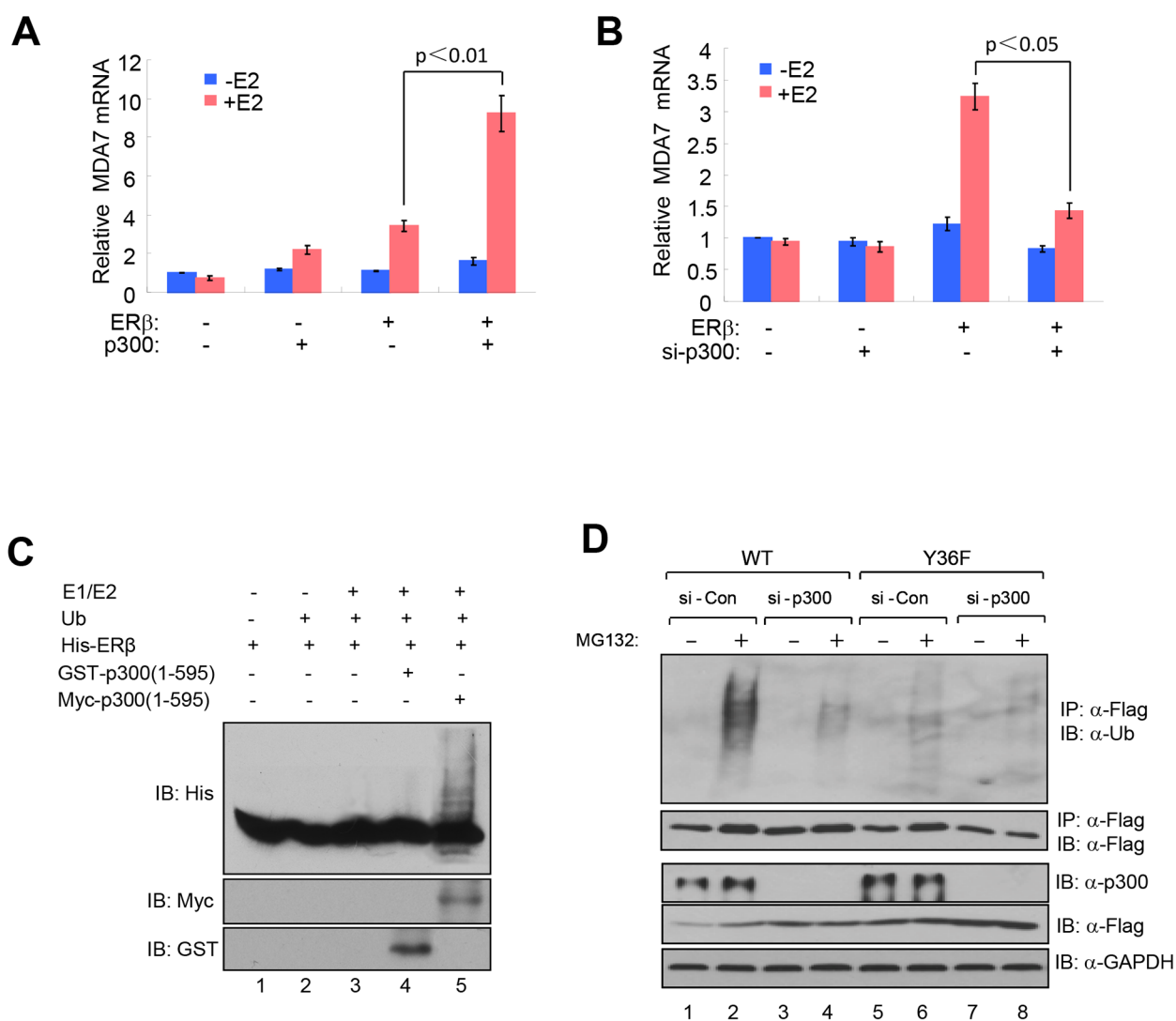


**Figure 1: The phosphotyrosine switch is important for ER $\beta$  activity in multiple cancer types.** *A.* *In vitro* MTT assay using MCF7-derived breast cancer cells that contained empty vector, WT, or Y36F mutant ER $\beta$ . Western blot of Flag-ER $\beta$  proteins in MCF7 stable cell lines. *B.* WT but not Y36F mutant ER $\beta$  overexpression in MCF7 cells reduced colony formation. *C.* and *D.* WT but not Y36F mutant ER $\beta$  overexpression reduced cell migration (*C*) and invasion (*D*). Data here and elsewhere represent average of at least three biological duplicates. Error bars indicate s.e.m.

engineered double strand break in the first protein-coding exon of the *ESR2* locus (Supplementary Figure S2A). Depletion of ER $\beta$  protein in both cell clones was confirmed by immunoblotting (Figure 4A and Supplementary Figure S2B). The resulting knockout cells grew significantly faster than the isogenic ER $\beta$ -expressing cells (Figure 4B–4C, Supplementary Figure S2C). In both Boyden chamber and wound-healing assays, the knockout cells also exhibited enhanced migratory ability versus the control cells (Figure 4D–4E). Furthermore, compared to the parental cells, ER $\beta$  knockout cells were more refractory to a previously characterized ER $\beta$ -selective agonist S-equal [18] (Figure 4F), thus supporting the selectivity of the ER $\beta$ -targeting compound.

## S-equal stimulates pY36 and inhibits tumor growth *in vivo*

To explore the translational potential of the ER $\beta$  phosphotyrosine switch, we asked whether ER $\beta$  ligand could alter the pY36 status. As shown in Figure 5A, Y36-specific ER $\beta$  phosphorylation was enhanced by the ER $\alpha$ /ER $\beta$  common agonist 17 $\beta$ -estradiol and two ER $\beta$ -specific agonists DPN and S-equal, suggesting a crosstalk between the C-terminal ligand-binding domain and N-terminal phosphorylation site of ER $\beta$ . Furthermore, in a xenograft tumor model, S-equal inhibited MDA-MB-231 cell-derived tumor growth (Figure 5B), accompanied by significant reduction in the number of tumor cells expressing Ki-67, an established marker for cycling cells (Figure 5C), and with concomitant increase in pY36 signal



**Figure 2: pY36 facilitates coactivator-dependent ubiquitylation and turnover of ER $\beta$ .** **A.** Ectopic expression of p300 promoted ligand-dependent transcriptional activation of MDA7 by ectopic ER $\beta$  in HEK293T cells. **B.** siRNA-mediated p300 knockdown blunted ectopic ER $\beta$  activity in HEK293T cells. **C.** *In vitro* ubiquitination assay containing E1/E2 ubiquitin ligases, purified bacterially expressed His-ER $\beta$ , bacterially expressed GST-p300(1-595) or mammalian cell-expressed myc-p300(1-595). **D.** p300 knockdown substantially reduced the extent of ER $\beta$  ubiquitination in HEK293T cells.

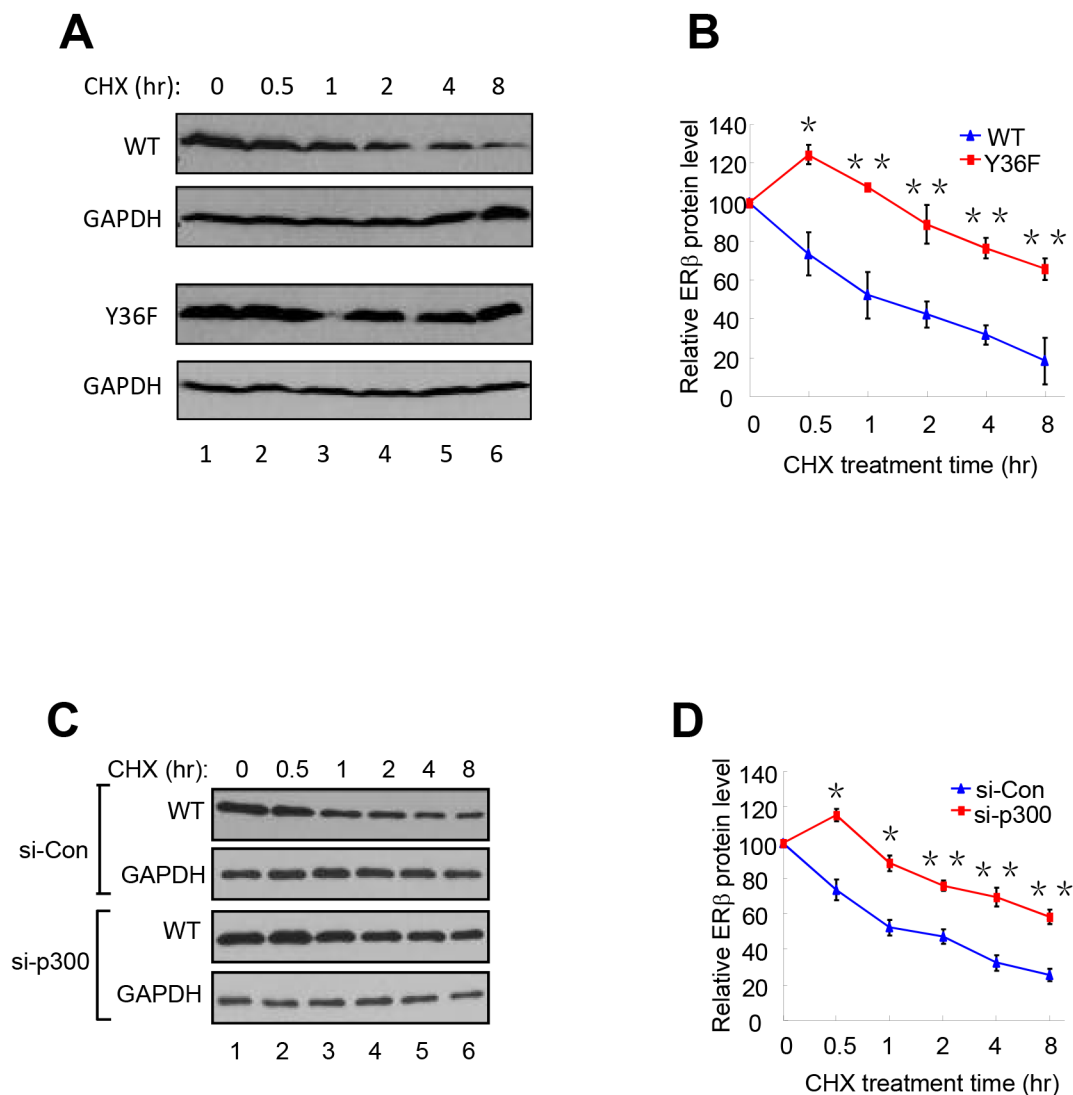
in xenografted tumors (Figure 5D). This *in vivo* finding provides additional supporting evidence for a tumor-intrinsic function of the ER $\beta$  phosphotyrosine switch in therapeutic response to ER $\beta$ -activating compounds.

## DISCUSSION

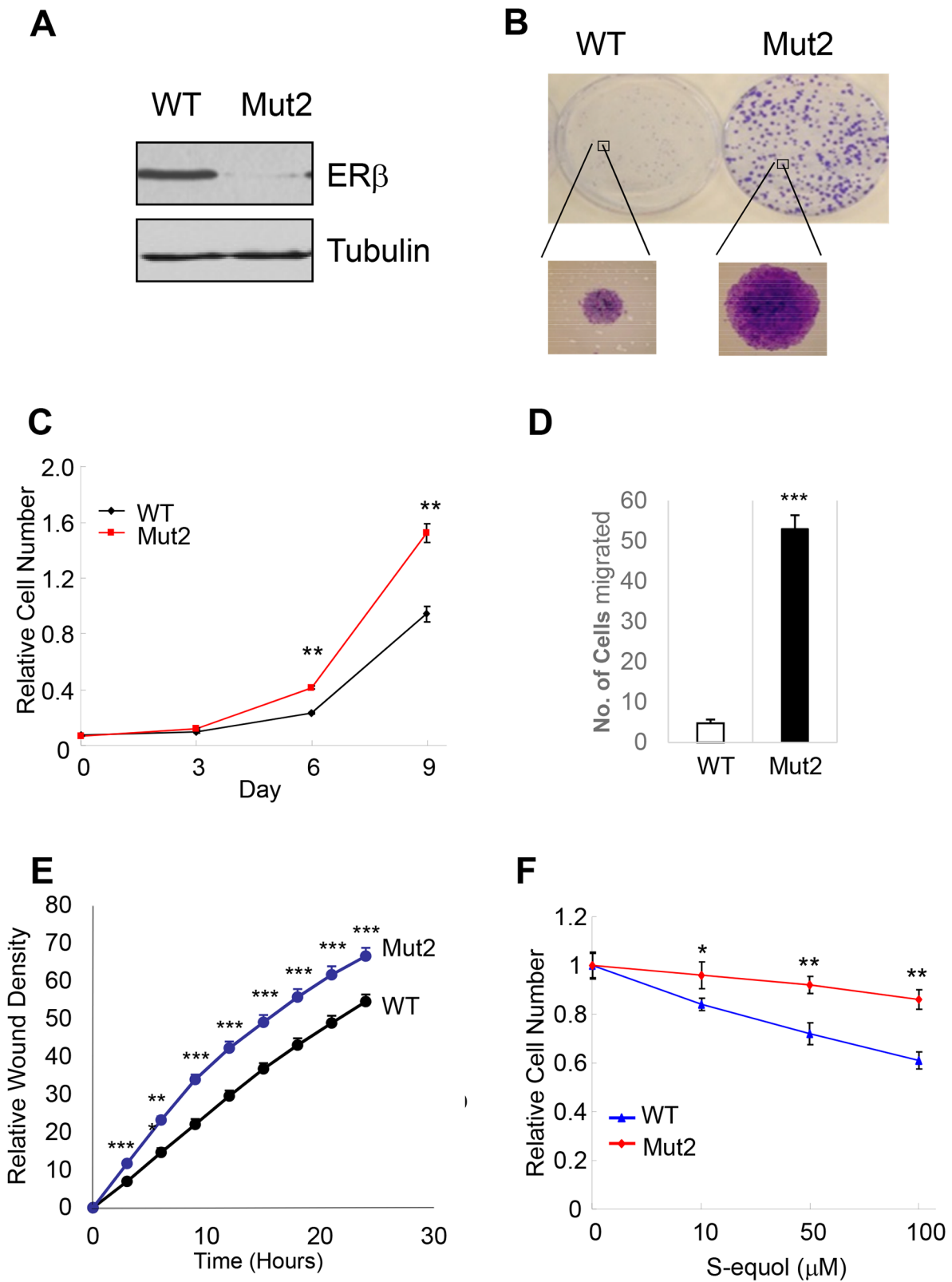
Mobilization of ER $\beta$  antitumor activity is an attractive therapeutic strategy. Our current study bolsters the concept that rallying the antitumor activity of ER $\beta$  through its phosphotyrosine switch is applicable to both ER $\alpha$ -positive and -negative tumor cells of various cancer types. While our work focuses on the phosphotyrosine switch in full-length ER $\beta$  (ER $\beta$ 1), we are aware of ER $\beta$  splicing variants (ER $\beta$ 2-5), which share the same ER $\beta$

AF1 domain containing the phosphotyrosine residue yet have distinct biological activities from ER $\beta$ 1. It will be important to determine in future studies whether the same phosphotyrosine switch regulates the functions of these variants.

Our current work significantly extends mechanistic insights into the pY36-dependent regulation of ER $\beta$  transcriptional activity. In particular, the dual effects of the phosphotyrosine switch on ER $\beta$ -mediated transcriptional activation and ER $\beta$  turnover is reminiscent of accumulating reports of transcription-coupled ubiquitination and proteasome-mediated degradation of transcription factors [37, 38]. For example, the turnover of both ER $\alpha$  and its coactivator SRC3/AIB1 is coupled to their actions in transcriptional activation [45–47]. It remains to be determined whether pY36-



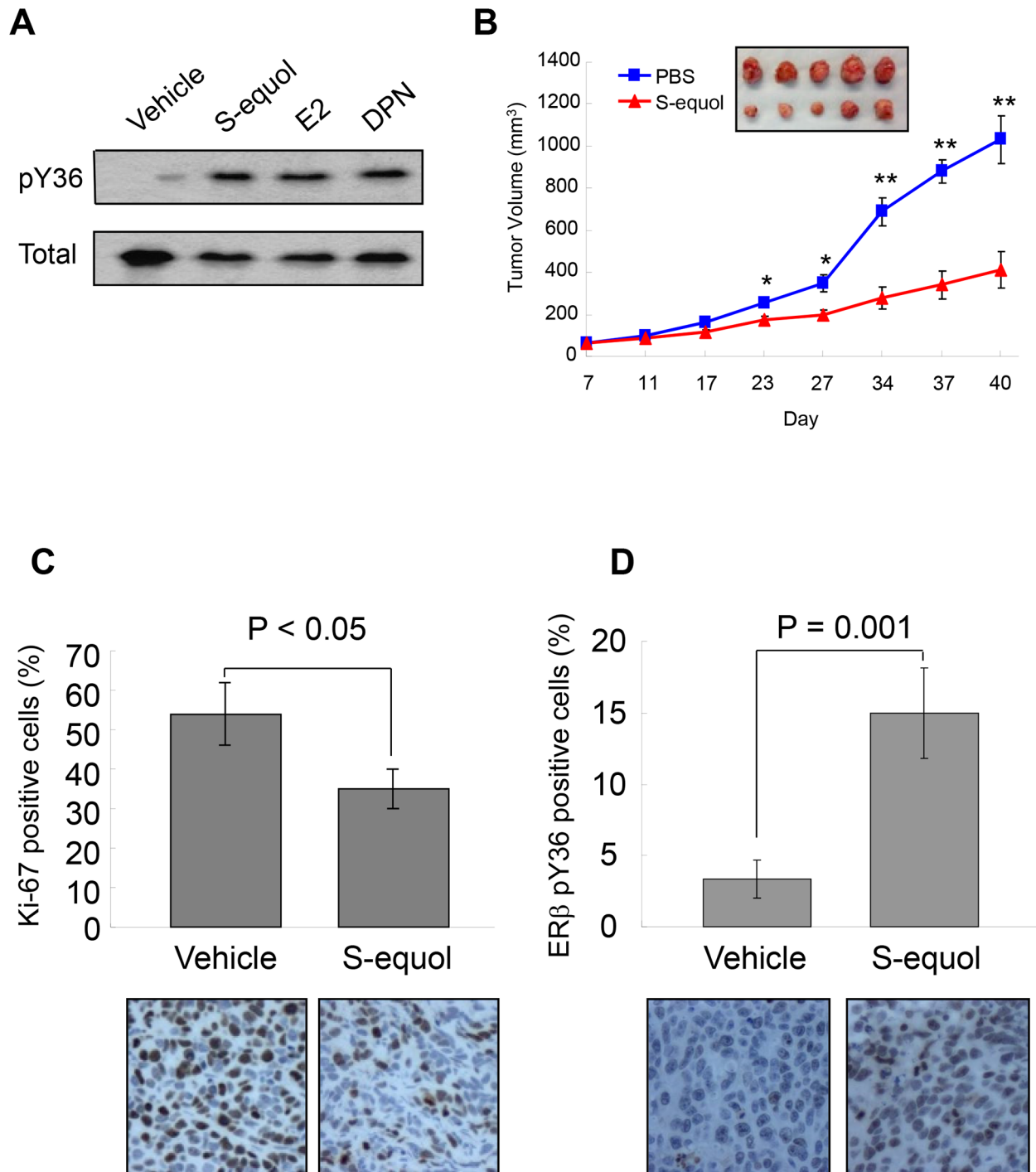
**Figure 3: pY36 facilitates ER $\beta$  protein turnover.** A. and B. The half-lives of WT-ER $\beta$  and Y36F-mutant proteins were analyzed. HEK293T cells were transfected with plasmids encoding WT-ER $\beta$  or Y36F mutant. Cells were treated with cycloheximide (CHX) 24 h after transfection and collected at the indicated time points. The results were quantitated using Image J software. C. and D. WT-ER $\beta$  plasmid was co-transfected with si-Con or si-p300 oligos into HEK293T cells for 48 h. Transfected cells were subsequently treated with CHX for the indicated time.



**Figure 4: Endogenous ERβ exhibits antitumor activity.** **A.** CRISPR/Cas9 genome editing of ERβ-encoding ESR2 gene in MDA-MB-231 breast cancer cells. Western blot of ERβ in MDA-MB-231 cells and one ERβ-edited clone (Mut2). **B.** ERβ KO cells have enhanced colony-forming ability versus parental cells. **C.** ERβ KO cells exhibit accelerated cell growth. **D.** KO cells displays increased migratory ability in a Boyden chamber assay. **E.** Increased cell migration as assessed in a wound-healing assay. **F.** ERβ KO cells are more refractory to ERβ-selective agonist S-equol than parental cells. \* p<0.05, \*\*p<0.01,\*\*\*p<0.001.

dependent ubiquitination of ER $\beta$  occurs on chromatin and subsequent to coactivator recruitment. However, we favor the promoter-specific model, as Y36F mutation or overexpression of its corresponding phosphatase EYA2 increases chromatin occupancy of ER $\beta$  at ER $\beta$ -

specific target promoters [34]. Such an activation-suicide process could ensure a rapid response of the transcription apparatus to fluctuating levels of estrogens and ER $\beta$ -specific stimuli [37, 38]. Alternatively, destruction of ER $\beta$  may be an obligatory part of transcriptional activation,



**Figure 5: S-equol stimulates pY36 and inhibits tumor growth *in vivo*.** **A.** pY36-specific phosphorylation signal was enhanced by the ER $\alpha$ /ER $\beta$  common agonist 17- $\beta$ -estradiol and two ER $\beta$ -specific agonists DPN and S-equol in MDA-MB-231 cells. **B.** S-equol treatment inhibited MDA-MB-231 cell-derived xenograft tumor growth (n = 5). **C.** Expression of Ki-67 in xenograft tumors. **D.** ER $\beta$ -pY36 signal in vehicle- and S-equol-treated xenograft tumor samples. \* p<0.05, \*\*p<0.01.

without which the apparatus would be prevented from proceeding to the subsequent steps of transcription [38]. Regardless of the extent of transcription-ubiquitination coupling, the pY36-dependent functional reciprocity between ER $\beta$  and its coactivators likely results in active

transcription of ER $\beta$  target genes and ultimately inhibition of tumor cell growth (Figure 6).

A number of clinically safe natural and synthetic compounds that function as ER $\beta$ -selective agonists have been identified [48]. For example, a synthetic ER $\beta$ -selective agonist LY500307 is being clinically tested for

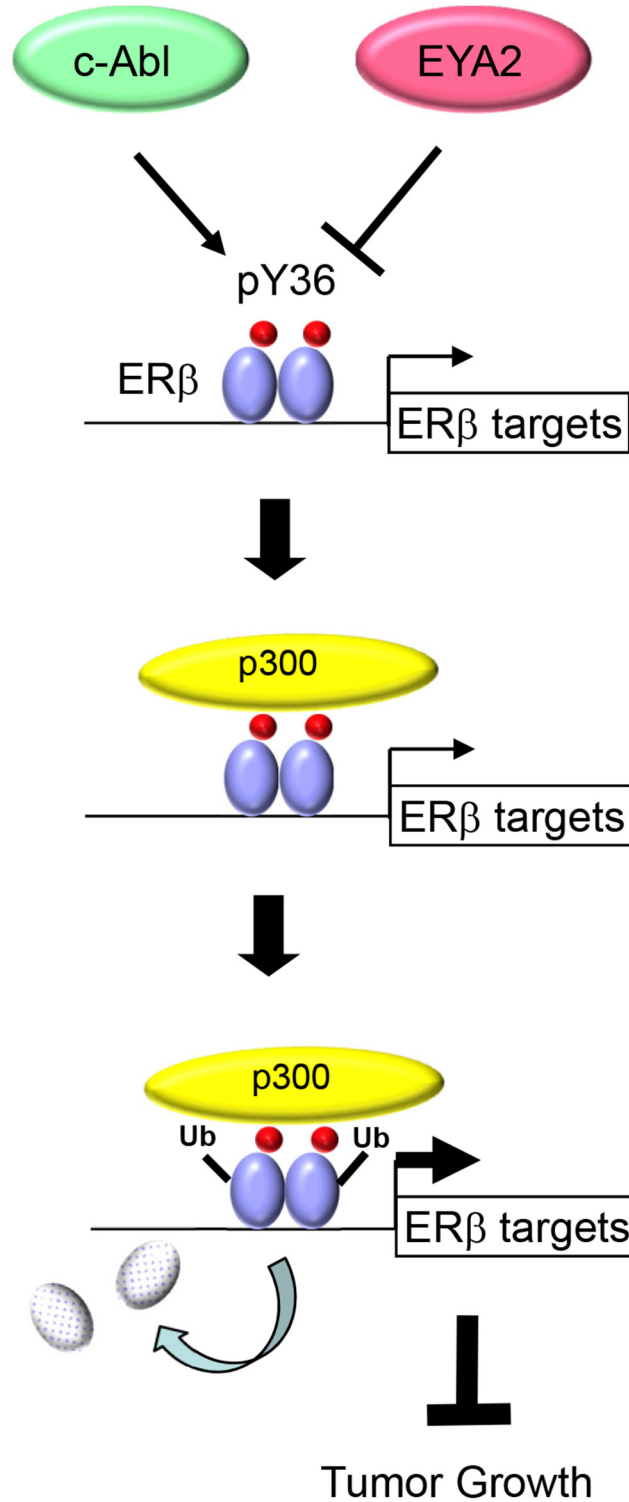


Figure 6: Model for transcription-coupled ER $\beta$  degradation.



benign prostatic hyperplasia (BPH) and schizophrenia [49]. In addition, S-equol is clinically safe and well tolerated in humans, based on multiple phase I and II clinical trials [50–53]. As these compounds likely have additional targets *in vivo* besides ER $\beta$ , it is critical to distinguish their ER $\beta$ -dependent and -independent effects. The differential effects of S-equol on the proliferation of parental and ER $\beta$  knockout tumor cells observed in the current work strongly suggest that the antiproliferative action of S-equol is at least partly mediated by ER $\beta$ . Our findings are also consistent with previously reported antitumor effect of S-equol on MCF7-derived xenografted tumors [54]. Of note, stimulation of ER $\beta$  phosphorylation by S-equol observed both *in vitro* and *in vivo* raises the distinct possibility of crosstalk between ligand- and posttranslational modification-dependent activation of ER $\beta$ . While the exact mechanism underlying the N- (AF1) and C-terminal (AF2) crosstalk awaits future investigation, we speculate that this could be mediated by transcriptional coactivators that interact with both ER $\beta$  activation domains. Of note, previous studies of ER $\alpha$  suggest functional communications between AF1 and AF2 activation domains in ER $\alpha$ -mediated transcriptional activation and biological functions *in vivo* [55–60]. We therefore envision that simultaneous targeting of ER $\beta$  tyrosine phosphorylation and ligand binding could achieve maximal activity of ER $\beta$  for treating those ER $\beta$ -expressing cancers with an intact pY36 signaling circuitry. In summary, ER $\beta$ -selective agonists, together with the known kinase and phosphatase targeting this switch, provide multiple novel and druggable targets for activating the subtype-specific function of ER $\beta$ .

## MATERIALS AND METHODS

### Cell lines and reagents

The expression vectors for WT and mutant ER $\beta$  were described previously [34]. The plasmid for p300 expression was kindly provided by Dr. Zhi-Min Yuan (Harvard T.H. Chan School of Public Health). Cell lines were originally purchased from the American Type Culture Collection and cultured per their instructions. 17 $\beta$ -estradiol (E2), DPN, and MG132 were obtained from Tocris, Inc. S-equol was generously provided by Ausio Pharmaceuticals. The following antibodies were purchased commercially: anti-Flag M2 (A8592 and F3165, Sigma-Aldrich), anti-ER $\beta$  for immunoblotting (14C8, GeneTex; 9.88, Abcam), anti-ER $\beta$  for immunoprecipitation (IP) (EPR3777, Novus), anti-p300 (sc-584, Santa Cruz Biotechnology Inc.), anti-GAPDH (G9295, Sigma-Aldrich), anti-FLAG-HRP (A8592, Sigma-Aldrich), anti-Flag M2 agarose (A2220, Sigma-Aldrich), and anti-Ki67 (GTX16667, GeneTex). The rabbit polyclonal anti-pY36 antibody was raised as previously described [34]. Oligonucleotides si-Con (non-targeting) (L-001810-10),

si-p300 (L-003486-00), ON-TARGETplus smart pool siRNA duplexes were purchased from Dharmacon.

### MTT assay

Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) using the manufacturer's protocol (ATCC). Briefly, cells were plated in 96-well microtiter plates, allowed to attach overnight, and treated with concentrations of S-equol as indicated for 72 h at 37°C. MTT was added to the culture medium to yield a final concentration of 0.5 mg/ml following cell treatment, and the incubation was continued for 1 h at 37 °C. The pellets were dissolved with dimethyl sulfoxide at room temperature for 10 min. Cell viability was determined by measuring the absorbance of the converted dye at a wave length of 570 nm.

### Cell migration/invasion assays

For assessing MCF7 cell migration, cells were grown to 80% confluence and harvested from the plate using 0.05% trypsin-EDTA. Cells were collected, washed with serum-free medium twice, and resuspended in serum-free medium. Twenty-four-well transwell chambers (BD BioCoat Cat.# 354575) with 8.0  $\mu$ m pore size polycarbonate membranes were used. Cells were plated at  $1 \times 10^5$  cells/well in 0.5 mL in the inserts, which were then placed into chambers containing growth medium with 10% FBS. After incubation at 37°C, for 24 h, inserts were removed and cells were fixed in 4% paraformaldehyde and stained with 0.1% crystal-violet. Cells on the upper membrane surface were removed with a cotton swab. Air-dried membranes were viewed under 10 x magnification and migrated cells were counted in five randomly chosen fields/membrane. Each cell line was assayed at least 3 times and assays were performed in duplicate. Error bars show s.e.m. For invasion assays, Matrigel-coated inserts with 8.0  $\mu$ m pore size membranes (BD BioCoat Cat.# 354480) were used.

For assessing MDA-MB-231 cell migration, cells were grown to 80% confluency, serum-starved for 3 h, and then harvested from the plate using 0.05% trypsin-EDTA. Cells were seeded at  $5 \times 10^4$  cells/200  $\mu$ l of serum-free medium per insert, which were then placed into 24-well transwell chambers (BD BioCoat Cat.# 354575, 8.0  $\mu$ m pore size), containing growth medium with 10% FBS. After incubation at 37°C for 4 h, the cells were fixed and processed as mentioned above. For the wound-healing assay, cells were seeded in 96-well image lock plate (Essen BioScience Cat.#4379) in triplicates, at 70,000 cells/well in growth medium with 10% FBS. After incubation at 37°C for 6 h, a scratch was made and wound healing was assessed in time lapse for 24 h using IncuCyte Live-Cell Imaging System (IncuCyte ZOOM®, Essen Bioscience Inc.).

## ***In vitro* ubiquitination assay**

Recombinant His-ER $\beta$  or GST-p300(1-595) was constructed, expressed and purified from *E. coli* BL21 cells expressing PET32a-ER $\beta$  or GST-p300(1-595) according to the manufacturer's instructions (Qiagen and Amersham). Myc-p300(1-595) protein immunoprecipitates were immunoprecipitated with Myc beads from 293T cells transfected with Myc-p300(1-595) and eluted with a Myc peptide. *In vitro* ubiquitination assays were performed in ubiquitination reaction buffer (25 mM Hepes pH 7.4, 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.05% Triton X-100, 0.5 mM DTT, 3 mM Mg-ATP (B-20, Boston Biochem)) with 100 ng E1 (E-305, Boston Biochem), 50 ng E2 (ubch5a, E2-616, Boston Biochem), 5  $\mu$ g ubiquitin (Ub, U-100Pf, Boston Biochem), and were incubated for 60 min at 37°C. The samples were subjected to SDS-PAGE and analyzed with indicated antibodies.

## ***In vivo* ubiquitination assay**

293T cells were transfected with WT or mutant ER $\beta$ . Twenty-four h after transfection, cells were re-seeded and transfected with control siRNA or p300 siRNA with Lipofectamine RNAiMAX transfection reagent (Invitrogen). Two days following transfection, cells were treated with MG132 (5  $\mu$ M) for 6 h. Immunoprecipitation was performed as previously described [61].

## **Protein half-life assay**

HEK293T cells were transfected with WT and mutant ER $\beta$ . 24 h after transfection, fresh medium containing cycloheximide (Sigma) was added to a final concentration of 50  $\mu$ M. Cells were harvested at indicated time points. ER $\beta$  steady-state levels were analyzed by Western blotting. Each result was derived from at least three independent experiments assessing densitometry-based protein ER $\beta$  quantification using GAPDH as the internal control.

## **Ligand treatment**

For ligand stimulation, cells were cultured in phenol red-free medium containing 5% charcoal stripped (CS) FBS for 3 days, re-seeded in Nunclon plates, and transfected with various vectors as indicated with Lipofectamine 2000 (Invitrogen). Six h after transfection, cells were treated with either vehicle or ligand at the indicated final concentration.

## **Generation of ER $\beta$ CRISPR knockout cells**

ER $\beta$  specific sgRNA target sequences were cloned into the CRISPR v2 vector (Addgene plasmid #52961). The 20-bp target sequences of the indicated sgRNAs were as follows: sgER $\beta$ -1, GGATTGACTGCAGTTGTAGG;

sgER $\beta$ -2, GAAGGAGAATTAAGGCTAGA. Three days after transfection, cells were selected with puromycin (Sigma) at 1  $\mu$ g/ml for 3 weeks. Genomic DNA was extracted using the PureLink Genomic DNA Mini Kit (K1820-00, Life Technologies) according to the manufacturer's instructions. Drug-resistant cell clones were propagated and screened for mutations at nuclease target sites by PCR amplification of genomic sequences followed by DNA sequencing.

## **Xenografts**

All animal experiments were performed after obtaining University of Texas Health Science Center at San Antonio (UTHSCSA) IACUC approval, and all methods were carried out in accordance with the IACUC approved guidelines. 5 x 10<sup>6</sup> MDA-MB-231 cells were injected orthotopically into mammary gland fat pads of 6 week-old female athymic nude mice (Harlan). When the tumor masses reached 50 to 80 mm<sup>3</sup> (about one week after the inoculation), the mice were given daily subcutaneous injections of S-equol (20 mg/kg per day) or PBS as a vehicle control. Tumor development was followed by caliper measurements along two orthogonal axes: length (L) and width (W) and volume (V) was estimated by the formula  $V = [L \times (W^2)]/2$ .

## **Immunohistochemistry**

Xenograft tumors harvested from mice were fixed in 10% neutral-buffered formalin, dehydrated, embedded in paraffin, and sectioned at 3  $\mu$ m thickness. Representative tumor sections from vehicle control and S-equol-treated mice were tested for Ki-67 expression to assess cell proliferation, and for ER $\beta$  pY36.

## **Statistics**

Statistical significance in the experiments was assessed by two-tailed Student's *t* test. In all assays,  $p < 0.05$  was considered statistically significant.

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## CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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