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Supplementary Information for
FKBP52 and FKBP51 Differentially Regulate the Stability of Estrogen Receptor in Breast Cancer

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This PDF file includes:

Figure S1.

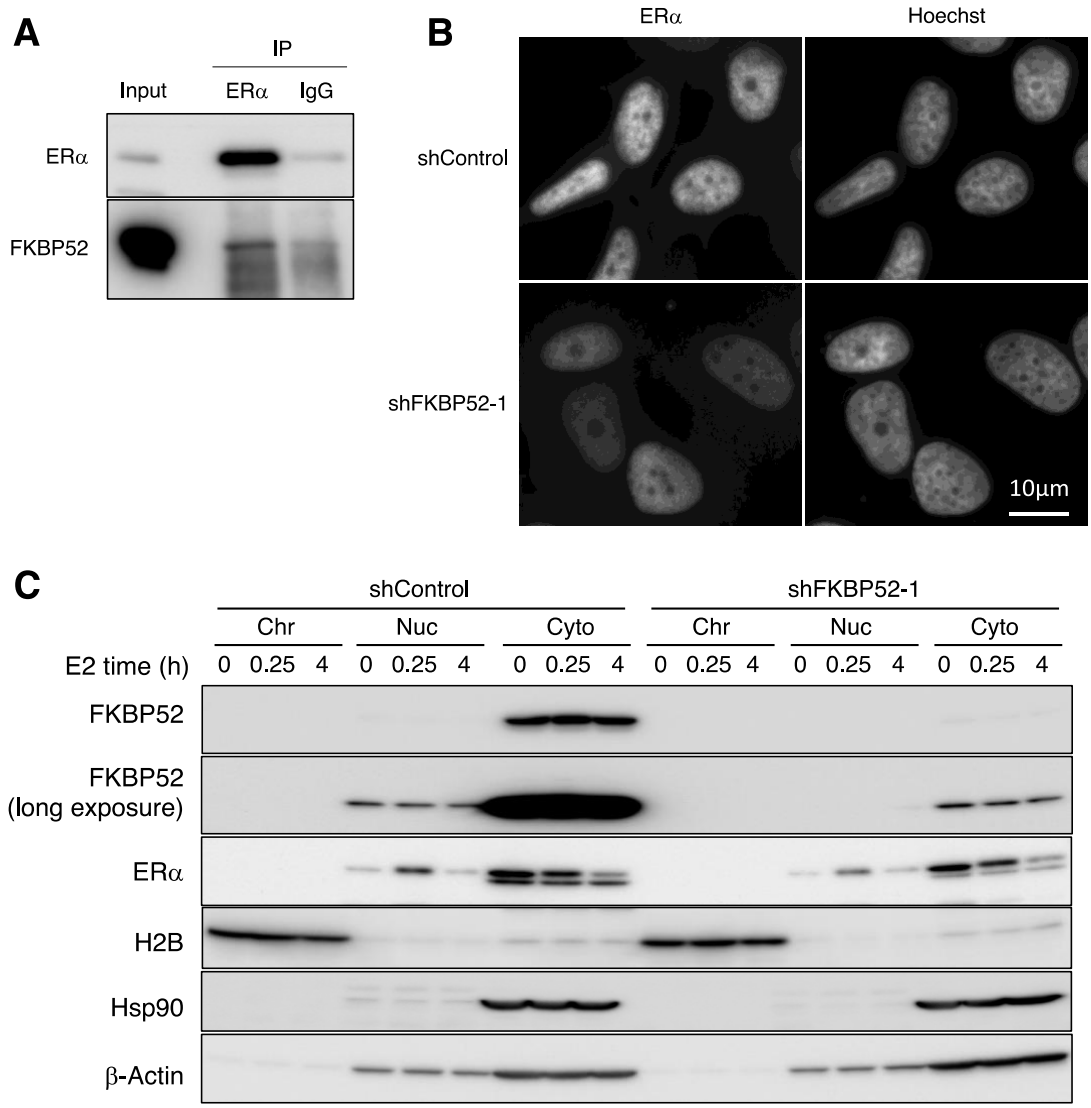

Figure S1. FKBP52 depletion does not affect ERα localization (Related to Fig. 3).

(A) T47D cell lysates were prepared**, and** ERα was immunoprecipitated. The association between FKBP52 and ERα was analyzed by immunoblotting.

(B) Representative immunofluorescence image of MCF7 cells expressing shControl or shFKBP52 grown on coverslips with Dox for 2 days, stained with the ERα antibody and Hoechst. Scale bar, 10 μm.

(C) MCF7 cells expressing shControl or shFKBP52 were cultured with Dox for 2 days. Cells were collected and subjected to subcellular fractionation. The samples were analyzed by immunoblotting. Histones H2B and Hsp90 were used as markers for chromatin and cytosol, respectively.

Figure S2.

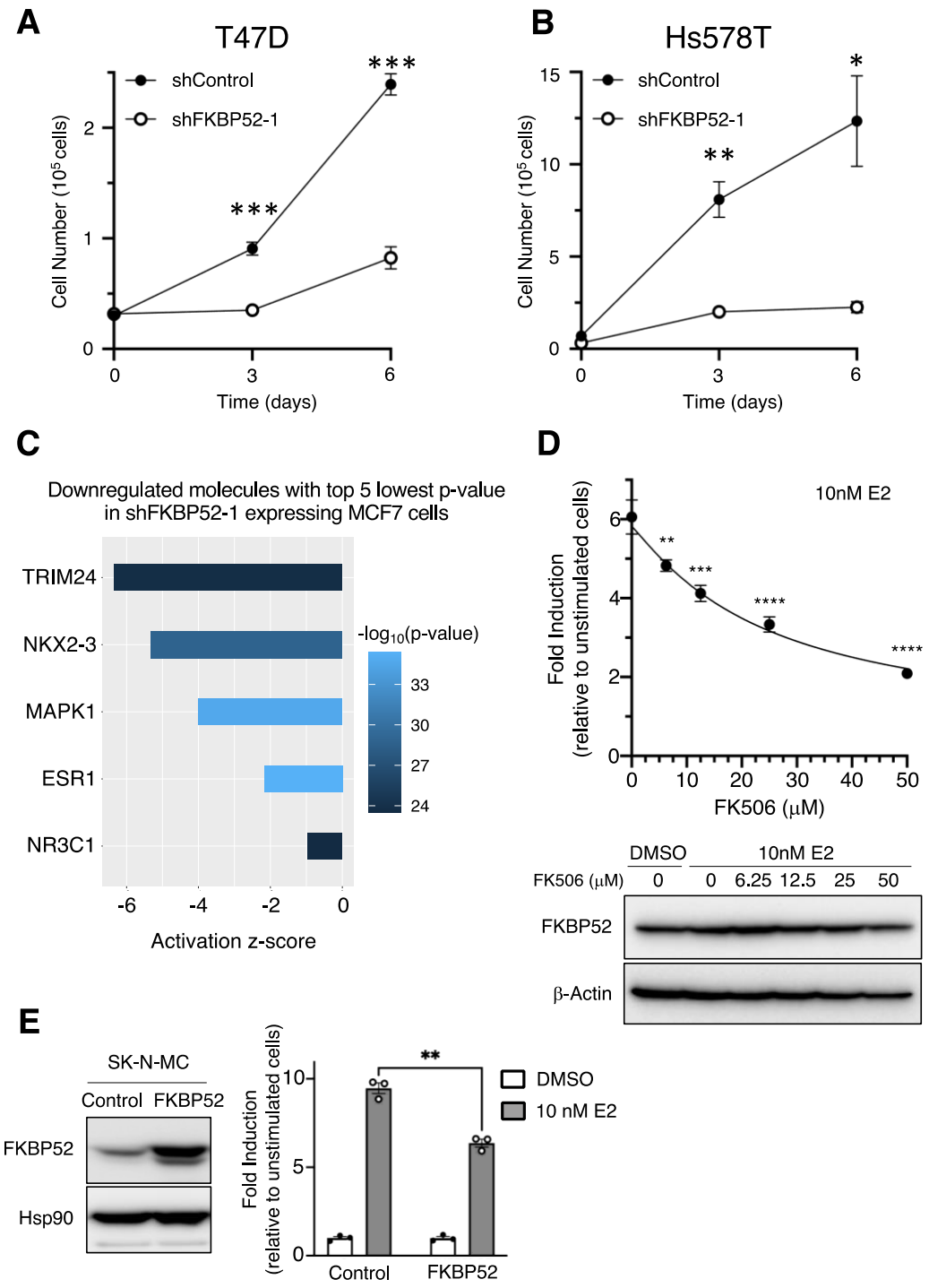

Figure S2. FKBP52 depletion downregulates ERα signaling cascade. (Related to Fig. 4)

(A* and *B) T47D *(A)* and Hs578T *(B)* cells expressing shControl, or shFKBP52 were cultured in the presence of Dox for the indicated number of days. Cells

were collected and counted. Data are expressed as the mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two-tailed Student's t-test.

(C) Downregulated (z-score < 0) upstream molecules with top 5 lowest p-value were identified by IPA upstream regulator analysis in FKBP52-depleted MCF7 cells.

(D) MCF7 cells were cultured in medium containing charcoal-stripped serum for one day. The cells were transiently transfected with ERE-luciferase reporter plasmids. After 24 h of transfection, the cells were treated with the indicated concentration of FK506. After 1 h of FK506 treatment, the cells were treated with 10 nM E2 for a further 24 h and luciferase activity was measured. Data are expressed as the mean ± SEM of three independent experiments. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by Dunnett's test.

(E) SK-N-MC cells stably expressing FKBP52 were cultured for 1 day in medium containing charcoal-stripped serum. The cells were transiently transfected with ERE-luciferase reporter plasmids and FLAG-tagged ERα expression plasmid. Following 24 h of transfection, the cells were treated with E2 for a further 24 h and luciferase activity was measured. The results from three independent experiments are shown. Data are expressed as the mean ± SEM. ** $p < 0.01$ by two-tailed Student's t-test.

Figure S3.

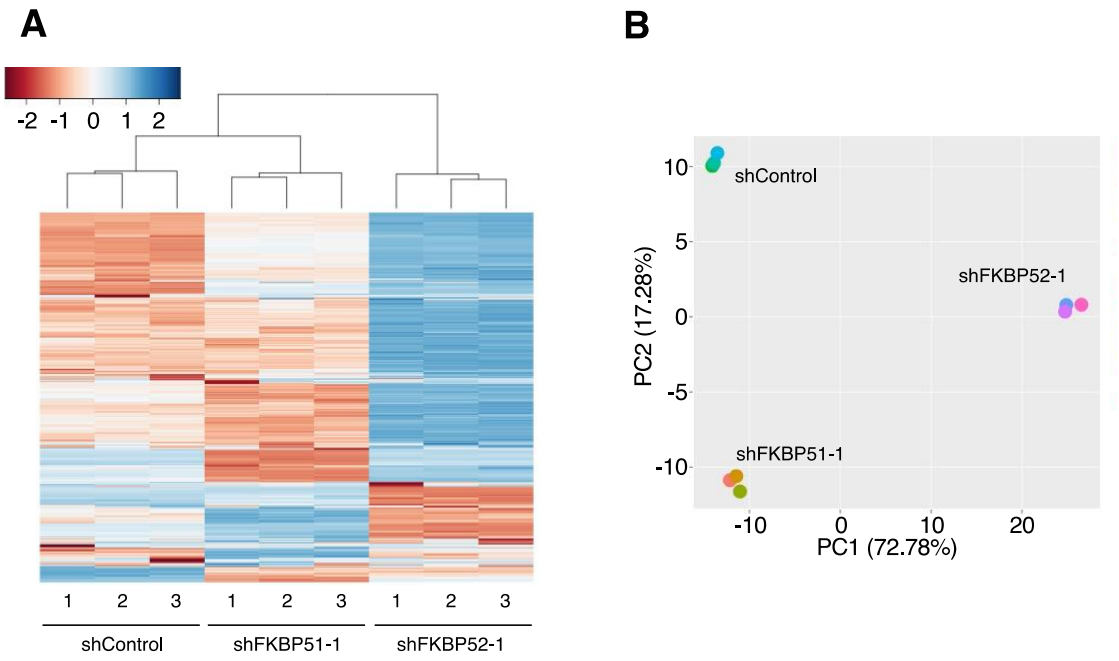

Figure S3. Quality control for RNA-Seq analysis in MCF7 cells expressing shControl, shFKBP52, and shFKBP51.

(A* and *B) Counts of the 500 most-varied genes were analyzed to produce a heatmap *(A)* and principal component analysis (PCA) plot *(B)* using DEBrowser.

Figure S4.

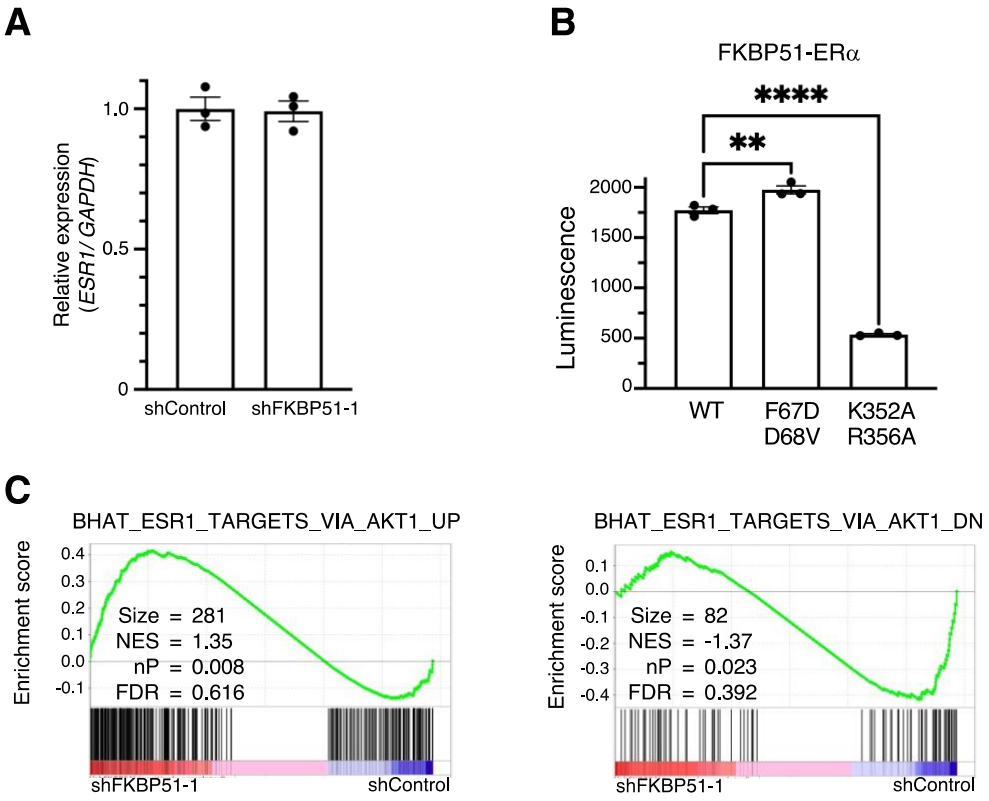

Figure S4. FKBP51 depletion upregulates ERα signaling cascade. (Related to Fig. 5)

(A) RT-qPCR analysis of *ESR1* was performed in MCF10A cells expressing indicated shRNAs. Data are expressed as the mean ± SEM of three independent experiments.

(B) Protein interactions between FKBP51 and ERα were detected using NanoBiT analysis in HEK293T cells. Sm-FKBP51 (WT, F67D/D68V, K352A/R356A) and ERα-Lg expression vectors were co-transfected into HEK293T cells as shown in Fig. 3d. The bar graphs show the level of luminescence 25 min after addition of the Nano-Glo® Live Cell Reagent. The luminescence was monitored with an ARVO X4 (PerkinElmer). Data are expressed as mean ± SEM of three independent experiments. ** p <0.01, **** p < 0.0001 by Dunnett's test.

(C) GSEA profiles of "ESR1 targets via AKT1" comparing MCF7 cells expressing shControl and shFKBP51. Sizes, NES (normalized ES), nP (nominal p-value), and FDR (false discovery rate) are shown. Three biological replicates were analyzed.

Figure S5.

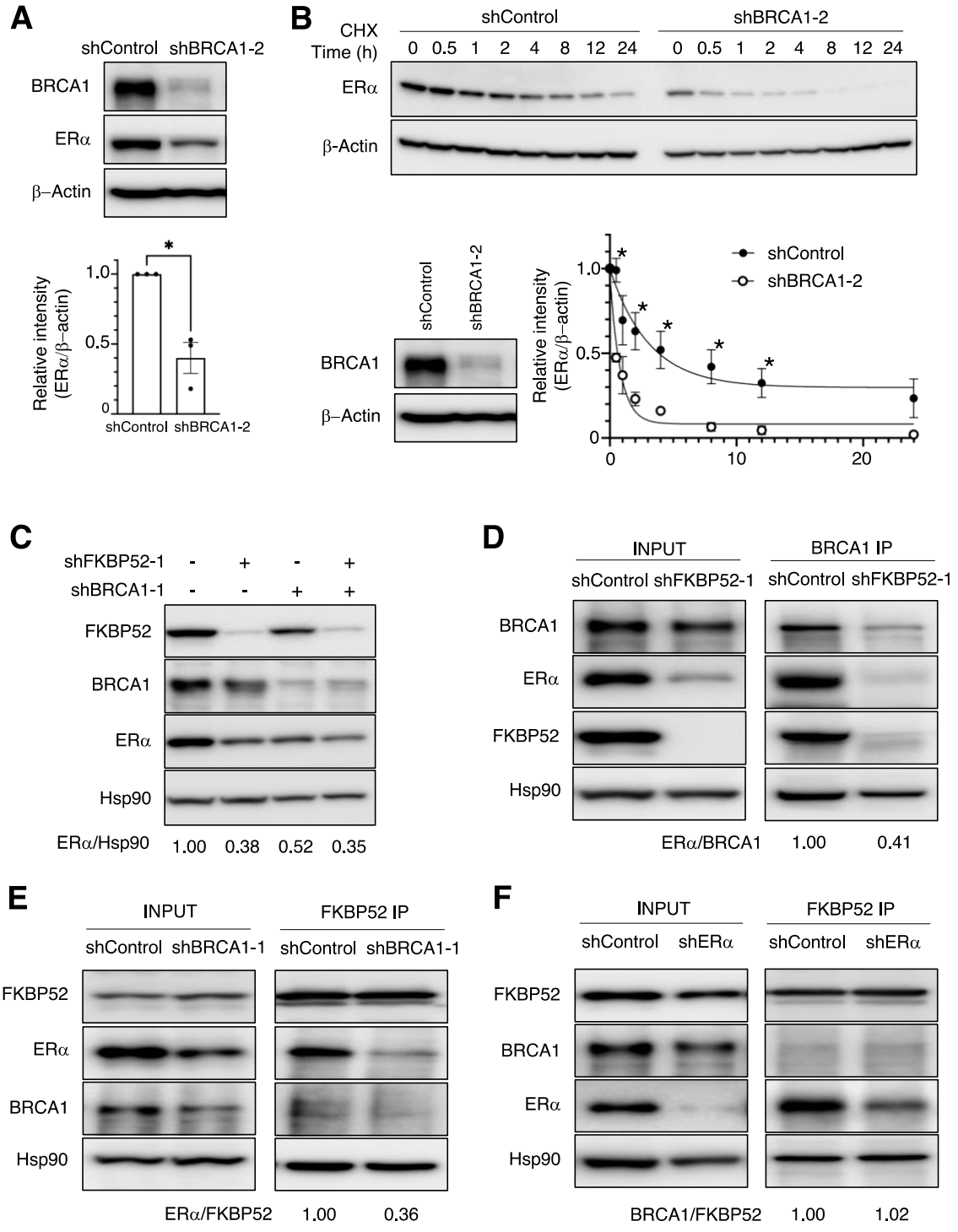

Figure S5. BRCA1 depletion decrease ERα stability. (Related to Fig. 8)
(A) MCF7 cells expressing shControl or shBRCA1-2 were cultured in the presence of Dox for two days. Cells were collected and immunoblotting was

performed. The bar plot shows relative band intensities (mean ± SEM) of three independent experiments. * $p < 0.05$ by two-tailed paired t-test.
(B) MCF7 cells expressing indicated shRNAs were cultured and analyzed as shown in Fig. 2f.
(C) MCF7 cells expressing indicated shRNAs were cultured in the presence of Dox for two days. Cells were collected and immunoblotting was performed. The relative ratios of ERα/β-actin are mentioned at the bottom.
(D-F) Lysates of MCF7 cells expressing shFKBP52 *(D)*, shBRCA1 *(E)*, and shERα *(F)* were prepared, and the indicated proteins were immunoprecipitated. Immunoprecipitants were analyzed by immunoblotting. The relative band intensity is shown at the bottom of the figure.

Supplementary Materials and Methods

Cell culture and reagents

MCF7 (HTB-22, ATCC), T47D (HTB-133, ATCC), Hs578T (HTB-126), and HEK293T (632180, Takara) cells were cultured in Dulbecco's modified Eagle's medium (044-29765, Wako) supplemented with 10% fetal bovine serum (FBS) (173012, Sigma) and antibiotic-antimycotic solution (15240062, Thermo Fisher Scientific). SK-N-MC (HTB-10, ATCC) cells were cultured in Eagle's minimal essential medium (055-08975, Wako) supplemented with 10% FBS and antibiotic-antimycotic solution. MFR were provided by Dr. Noriko Saitoh (The Cancer Institute of Japanese Foundation for Cancer Research) and cultured in RPMI-1640 medium (187-02705, Wako) supplemented with 10% FBS, antibiotic-antimycotic solution, and Fulvestrant (1047, Tocris). The cells were treated with FK506 (063-06191, Wako), MG132 (474790, Merck), Cycloheximide (C1988, Sigma), or Fulvestrant (1047, Tocris). All of which were dissolved in dimethyl sulfoxide (DMSO). FK506, MG132, Cycloheximide, and Fulvestrant were used at concentrations of 6.25-50 μM, 10 μM, 50 μg/mL, and 100 nM, respectively.

β-Estradiol treatment

Cells were cultured in minimum essential medium (MEM) α (41061-029, Gibco) containing charcoal-stripped FBS (A3382101, Gibco) for 2 days. β-Estradiol (E8875, Sigma-Aldrich) was added to the cells at a concentration of 10 nM and cells were harvested at the indicated times.

Construction of expression vectors

Stable mammalian expression plasmids for human FKBP52 and FKBP51 were prepared by directional cloning of the full open reading frame (ORF) into CSII-CMV-MCS-IRES2-Bsd.

Transient mammalian expression plasmids for human ERα were prepared by directional cloning of the ORF into pCMV FLAG vector (Agilent Technology). NanoBiT-fused protein expression constructs were prepared by directional

cloning of the full ORF regions of FKBP52, FKBP51, and ERα into NanoBiT® vectors (pFN33K LgBiT TK-neo Flexi Vector, pFC34K LgBiT TK-neo Flexi Vector, pFN35K SmBiT TK-neo Flexi Vector, and pFC36K SmBiT TK-neo Flexi Vector; N2015, Promega). All directional cloning was performed using the In-Fusion® HD Cloning Kit (639648, Clontech).

Construction of shRNA

To generate lentivirus-based shRNA constructs, a 21-base shRNA-coding fragment with an ACGTGTGCTGTCCGT loop was introduced into pENTR4-H1 digested with BglII. The pENTR4-H1-shRNA vectors were incubated with CS-RfA-ETBsd or CS-RfA-ETHyg vectors and Gateway™ LR™ Clonase Enzyme Mix (11791019, Invitrogen) for 2 h at 25°C to produce the CS-RfA-ETBsd-shRNA vector. The target sequences for the lentivirus-based shRNA were FKBP51-1: GGAAGATAGTGTCCTGGTTAG, FKBP51-2: GGAACAGACAGTCAAGCAATG, FKBP52-1: GCGGAATCATTCGCAGAATAC, FKBP52-2: GCAAGGACAAATTCTCCTTTG, BRCA1-1: GAAGGAGCTTTCATCATTC, BRCA1-2: ACTGATACTGCTGGGTATAAT, ERα: CGCTCTAAGAAGAACAGCC; and Luciferase: CGTGCGTGGAATGCTTCGA. Unless otherwise stated, shFKBP51-1 or shFKBP52-1 was used for the experiments. The shBRCA1 construct was kindly provided by Dr. Tomohiko Ohta (1).

Site-directed mutagenesis

FKBP52 mutants (F67D/D68V and K354A) and FKBP51 mutants (F67D/D68V and K352A/R356A) were generated using the KOD-Plus-Mutagenesis Kit (SMK-101, Toyobo). The finished constructs were sequenced to confirm that they were correct. The mutagenesis primer sequences were as follows: FKBP52 F67D/D68V F: GTATCCAGTCTGGATCGCAAGGA, FKBP52 F67D/D68V R: ATCCTTTGTGCCATCTAATAGCCAG, FKBP52 K354A F: CTCGTTGTTGCTGTCCAGTTCTAGG, FKBP52 K354A R: GCGGGCCTCTTCCGCCG, FKBP51 F67D/D68V F: GTTTCCAGTCATGATAGAAATGAACC, FKBP51 F67D/D68V R:

GTCCTTCTTTCCATTTGACAATTTTCC, FKBP51 K352A/R356A F: GCTTGTATGCGAGGGGTGAAGCCCAGCTGC, and FKBP51 K352A/R356A R: CTGCCTCATTGGCACTGTCCAGTCCAAGG.

Cell growth

To determine cell growth, 4×10^4 cells were plated in a 3.5 cm culture dish. The day after seeding was considered day 0, on which the medium was replaced with the medium containing drugs of interest. The cells attached to the dish were trypsinized, then counted.

Immunofluorescence staining

MCF7 cells were fixed in 4% paraformaldehyde for 5 min. Cells were then washed three times with PBS and incubated with 0.5% Triton/PBS for 10 min, followed by washing and blocking in 5% goat serum for 30 min. Cells were then incubated with primary antibodies (1:200) for 1 h to detect ERα and FKBP52. Anti-rabbit or anti-mouse secondary antibodies conjugated to fluorescein isothiocyanate (FITC; 1:200; 1 h) were used to visualize proteins of interest. Photomicrographs were taken at 400× magnification using a DeltaVision Elite (GE Healthcare). We confirmed that ERα and FKBP52 signals were diminished in ERα- and FKBP52-knockdown cells, respectively.

Subcellular fractionation

MCF7 cells were collected and subjected to perform subcellular fractionation using Subcellular Protein Fractionation Kit for Cultured Cells (78840, Thermo Fisher Scientific), according to the manufacturer's protocol.

NanoBiT assay

Large-BiT or small-BiT fusion protein expression constructs were prepared as described above. In this article, we refer to the expression construct containing the c-terminal large-BiT-fused FKBP52 as FKBP52-Lg. HEK293T cells were cultured in MEM α containing 5% charcoal-stripped FBS at a density of 2×10^4

cells/well in 96-well plates for 24 h. Cells were transfected with 50 ng of large-BiT fusion construct and 50 ng of small-BiT fusion construct using 200 ng of PEI MAX. Cells were transfected with either Sm-FKBP52 or Sm-FKBP51, as well as ERα-Lg to measure the interaction between FKBP and ERα. Forty-eight hours after transfection, the luminescence that was caused by the approach of large-BiT and small-BiT was measured using the Nano-Glo® Live Cell Assay System (N2011, Promega) and an ARVO X4 multi-label reader (PerkinElmer).

RNA-seq expression database analysis

Breast cancer patient RNA-seq data from the TCGA BRCA database were obtained using the University of California at Santa Cruz Xena (2). Data were filtered to include only primary tumor data. “illuminaHiSeq” was used as the expression value, “ER_Status_nature2012” was used to divide samples into ERα positive and negative, and “Solid Tissue Normal” was used as a normal tissue control. Violin plots were generated using GraphPad Prism 9, and significance was calculated using the Mann-Whitney U test, with $p < 0.05$ considered significant.

For tumors other than breast cancer, violin plots comparing RNA-seq expression data between normal tissues and tumors were generated using TNMplot (3).

Dual luciferase reporter assay

The 3×ERE-TATA-pGL3 (firefly luciferase) and pCMV Rluc (Renilla luciferase) constructs were kindly provided by Dr. Maiko Okada.

T47D or MCF7 cells were cultured in MEM α containing 5% charcoal-stripped FBS at a density of 1×10^4 cells/well in a 96-well plate for 24 h. Cells were transfected with 74 ng of 3×ERE-TATA-pGL3 and 1 ng of pCMV Rluc using 187.5 ng of PEI MAX. Twenty-four hours after transfection, the cells were treated with 10 nM E2 or DMSO for 24 h. For SK-N-MC cells, the protocol was modified as follows: seeding density, 3×10^4 cells/well; transfection, 50 ng 3×ERE-TATA-pGL3, 1 ng pCMV Rluc, 24 ng pCMV FLAG ERα, and 225 ng PEI MAX. Firefly luciferase and Renilla luciferase luminescence was measured using the Dual-

Luciferase® Reporter Assay System (E1910, Promega) and an ARVO X4 multi-label reader. Fold-induction was calculated by dividing firefly luminescence by Renilla luminescence. Dose-response curves of FK506 were generated using "[Inhibitor] vs. response –Variable slope (four parameters)" on the nonlinear regression of GraphPad Prism 9.

SI References

1. T. Kuroda, M. Okada, K. Tsugawa, T. Ohta, Establishment of A Doxycycline-inducible BRCA1 Deficient and Estrogen Receptor Expressing Human Breast Cell Line. *Marianna Medical Journal* **43**, 151-162 (2015).
2. M. J. Goldman *et al.*, Visualizing and interpreting cancer genomics data via the Xena platform. *Nat Biotechnol* **38**, 675-678 (2020).
3. Á. Bartha, B. Győrffy, TNMplot.com: a web tool for the comparison of gene expression in normal, tumor and metastatic tissues. *bioRxiv* 10.1101/2020.11.10.376228 (2020).