Supplementary data for

RON signaling promotes therapeutic resistance in *ESR1* mutant breast cancer

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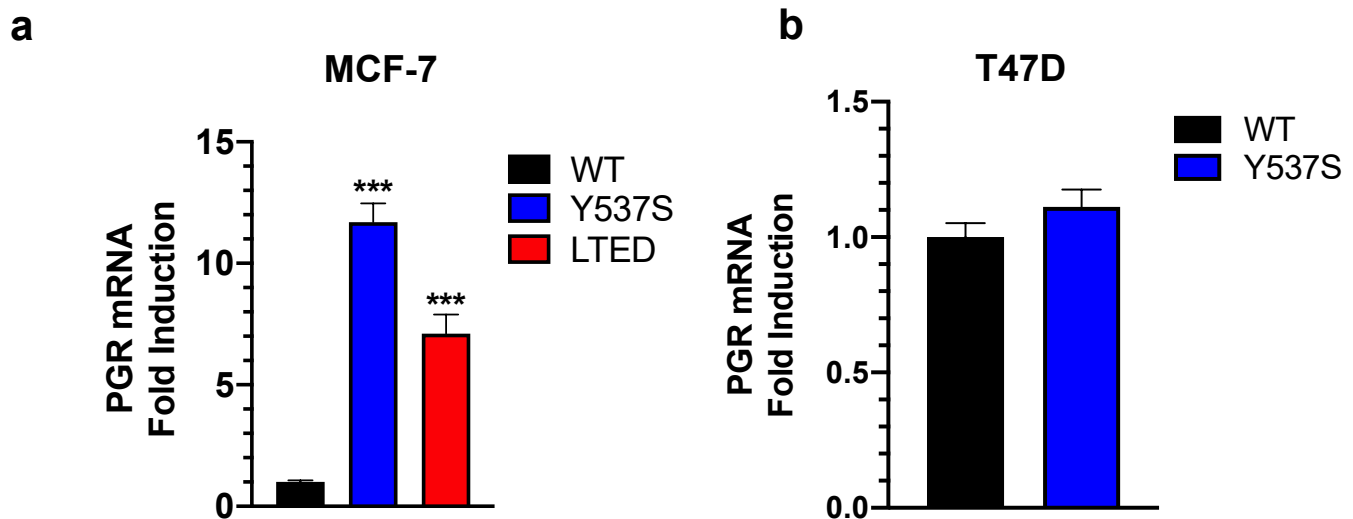
Figure S1. Progesterone Receptor (PGR) mRNA expression in *ESR1* mutant cells

a) MCF-7 WT, *ESR1* Y537S, and LTED cells were cultured in MEM media supplemented with 10% FBS and analyzed by qRT-PCR. PGR was upregulated in *ESR1* Y537S and LTED cells compared to WT. Error bars represent standard error of the mean (N=3 replicates). One-way ANOVA was performed for statistical analysis.

b) T47D WT and *ESR1* Y537S, were cultured in MEM media supplemented with 10% FBS and analyzed by qRT-PCR. Error bars represent standard error of the mean (N=3 replicates). Student's t-test was performed for statistical analysis. P<0.05 was considered statistically significant in all tests. (* p<0.05, ** p<0.01, *** p<0.001)
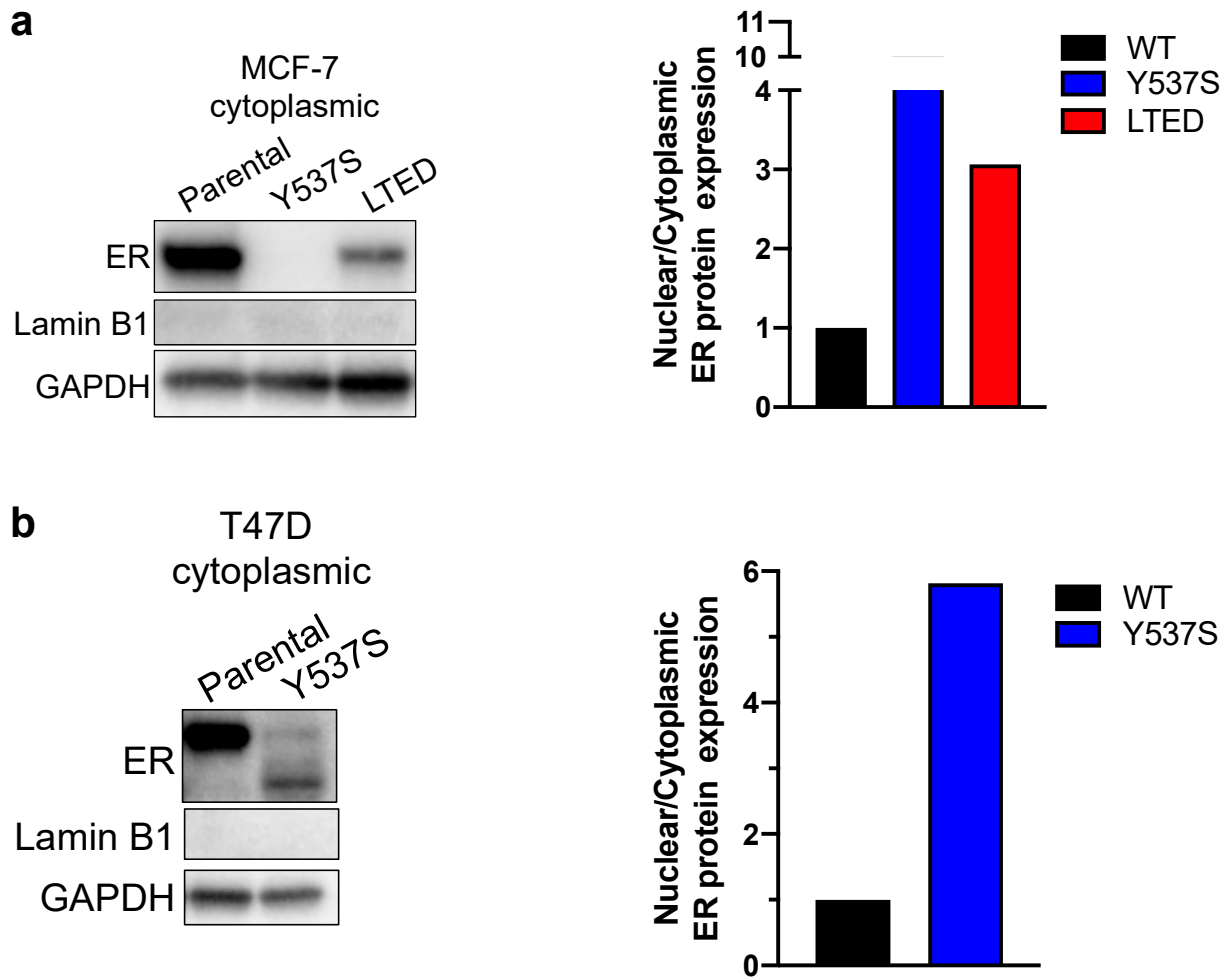
Figure S2. The ER mutant protein is nuclear localized.

(a) MCF-7 and **(b)** T47D cells were cultured in 5% charcoal-stripped serum supplemented media for 72 hours before harvesting. Subcellular fractionation was performed, and the cytoplasmic and nuclear fractions were analyzed by immunoblot. In the immunoblot densitometry analysis, cytoplasmic fraction was normalized to GAPDH and the nuclear fraction was normalized to Lamin B1.

MCF-7 LTED

a

MCF-7 WT

b

4
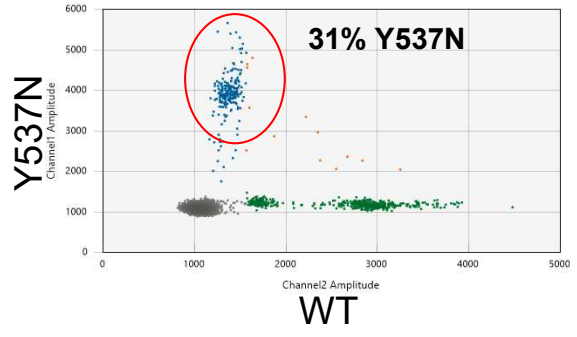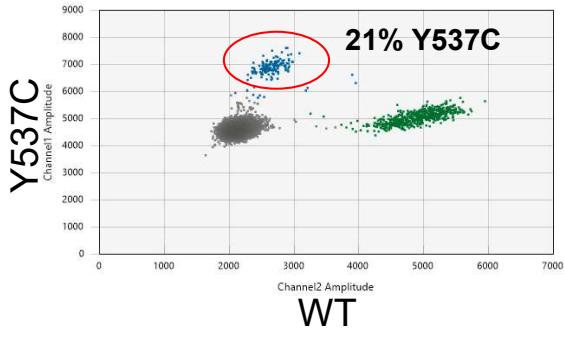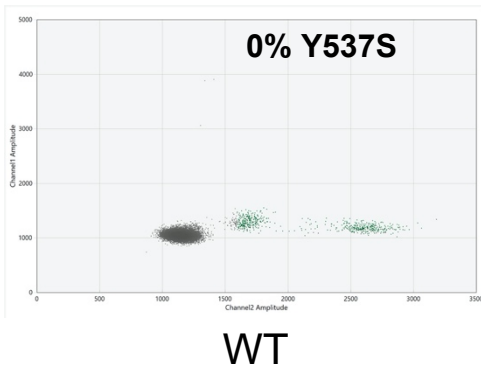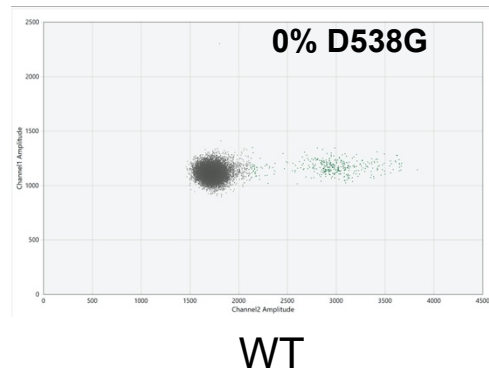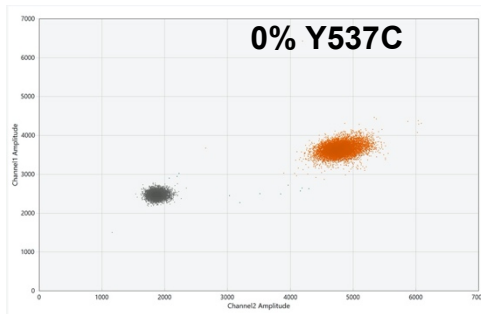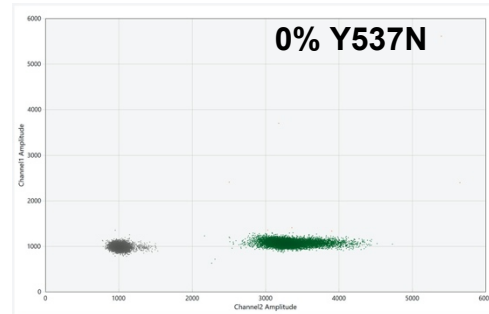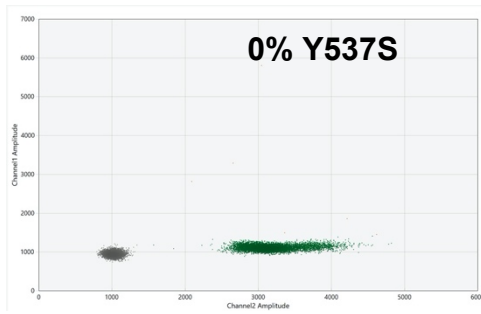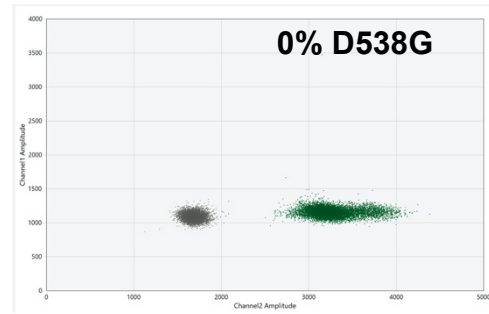
Figure S3. *ESR1* mutations in MCF-7 cells.

(a) ddPCR 2D droplet plots of defined *ESR1* mutation vs WT in MCF-7 LTED cells. *ESR1* Y537S and D538G were not detected in LTED cells. Detection of 3 out of 1000 positive *ESR1* mutant droplets was defined as positive detection of *ESR1* mutation.

(b) ddPCR 2D droplet plots of defined *ESR1* mutation vs WT in MCF-7 WT cells. *ESR1* Y537C, Y537N, Y537S and D538G mutations were not detected in WT cells. Detection of 3 out of 1000 positive *ESR1* mutant droplets was defined as positive detection of *ESR1* mutation.
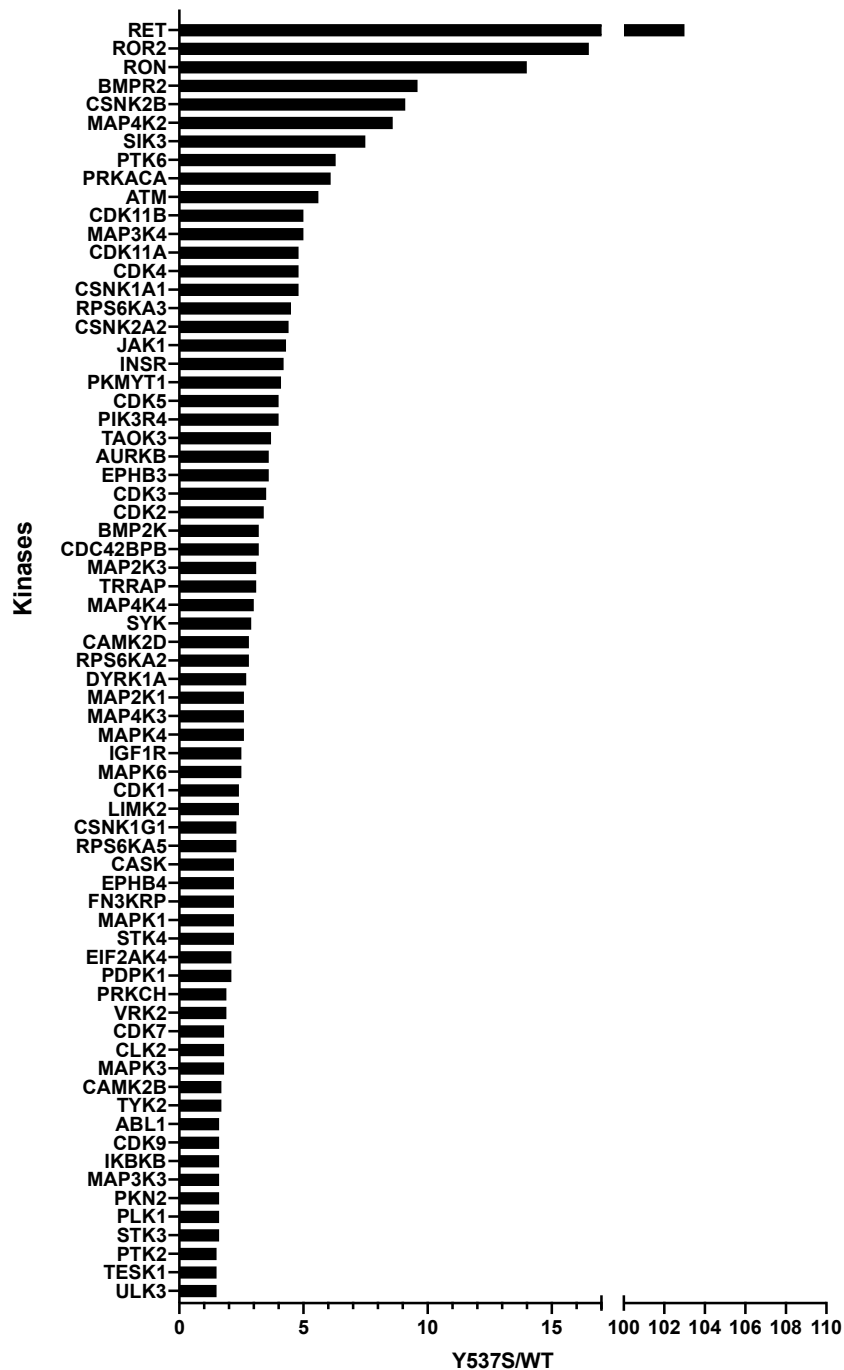
Figure S4. The MCF-7 *ESR1* Y537S cell line had global kinase activation.

MCF-7 WT and *ESR1* Y537S cells were cultured in full serum supplemented media and analyzed

by KiP. Kinase hyperactivation was defined as Y537S-unique and ≥1.5 fold in Y537S cells

compared to WT. All kinases ≥1.5-fold hyperactivated are shown.
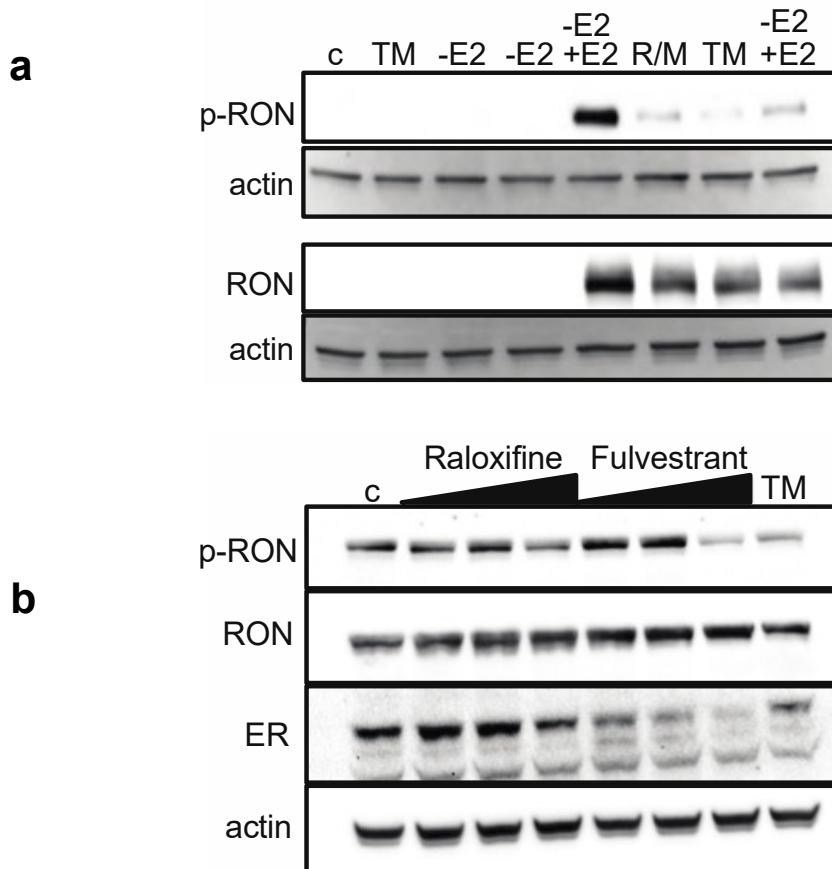
Figure S5. ET reduced p-RON levels in T47D WT cells and in RON/MSP overexpressing T47D cells.

(a) T47D cells with RON/MSP overexpression (R/M) cells were treated with TM or E2 deprivation (with or without E2 add-back) and cell lysates were analyzed by immunoblot. Blocking estrogen signaling reduced p-RON levels in the T47D R/M model. A description of T47D R/M cells is in Cunha et al, Cell Reports 2014.[1]

(b) T47D R/M cells were treated with raloxifene, Ful, and tamoxifen (TM) and cell lysates were analyzed by immunoblot. p-RON levels were reduced with each ET.
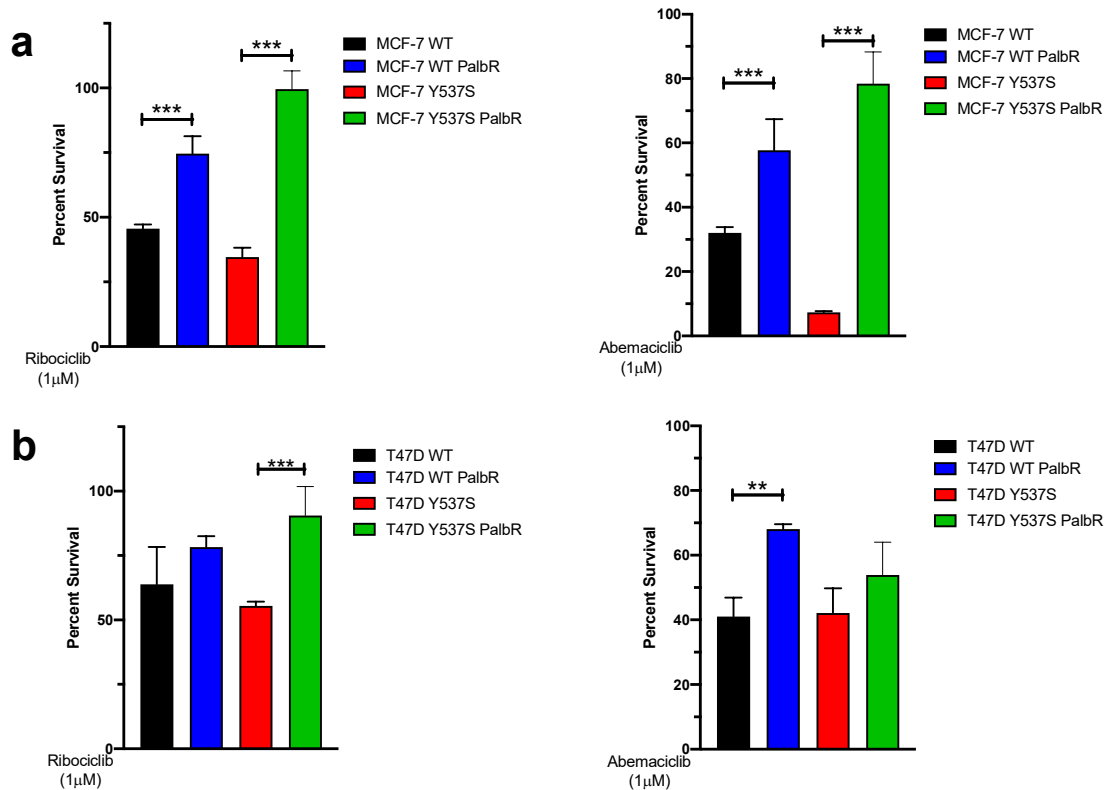
Figure S6. MCF-7 and T47D cell response to CDK4/6 inhibitors ribociclib and abemaciclib.

(a) MCF-7 WT, WT PalbR, *ESR1* Y537S, and *ESR1* Y537S PalbR cells were treated with 1 µM ribociclib or abemaciclib for 7 days and growth was analyzed by MTT. Error bars represent standard deviation (N=3 replicates). Two-way ANOVA was performed for statistical analysis.

(b) T47D WT, WT PalbR, *ESR1* Y537S, and *ESR1* Y537S PalbR cells were treated with 1 µM ribociclib or abemaciclib for 7 days and growth was analyzed by MTT. Error bars represent standard deviation (N=3 replicates). Two-way ANOVA was performed for statistical analysis.

MCF-7 WT PalbR
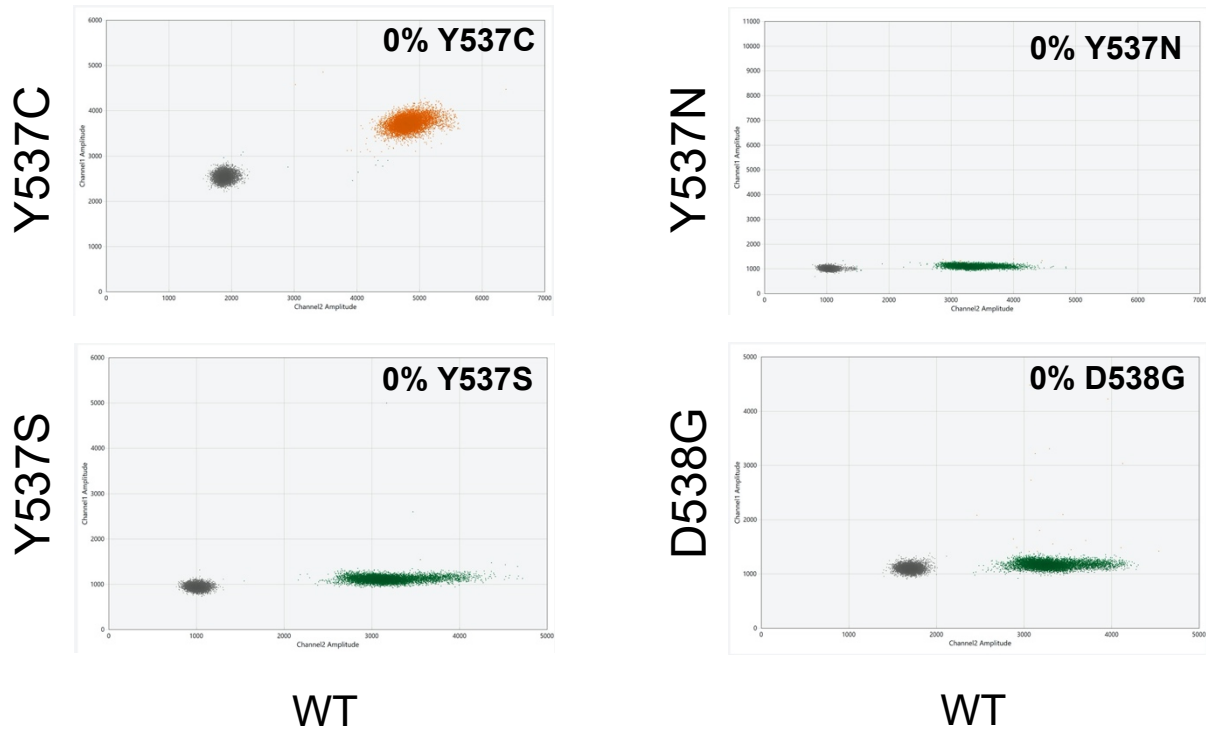
Figure S7. *ESR1* **mutations are not acquired in MCF-7 WT PalbR cells.**

ddPCR 2D droplet plots of defined *ESR1* mutation vs WT in MCF-7 WT PalbR. *ESR1* Y537C, Y537C, Y537N, and D538G were not detected in MCF-7 WT PalbR cells. Detection of 3 out of 1000 positive *ESR1* mutant droplets was defined as positive detection of *ESR1* mutation.
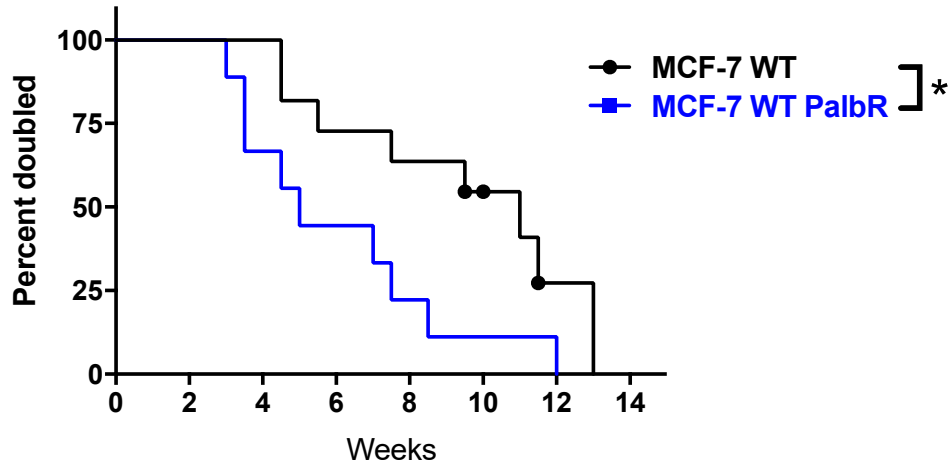
Figure S8. MCF-7 WT PalbR and *ESR1* Y537S PalbR tumor growth, *in vivo*.

MCF-7 WT or WT PalbR xenografts were grown in athymic nude mice that were supplemented with E2 drinking water. When primary tumors reached 350 mm^3, mice were treated with 125 mg/kg palbociclib p.o. 5 days on, 2 days off. The drug effect on primary tumor growth was determined by time to tumor doubling from the start of palbociclib treatment. The Mantel-Cox test was used for statistical analysis. Shaded circles represent censored events representing primary tumors that had not doubled in volume. Median survival is 11.0 weeks for MCF-7 WT (N=11) and 5.0 weeks for MCF-7 WT PalbR (N=9), p-value = 0.0335.

Table S1. MSigDB hallmark pathways upregulated in MCF-7 *ESR1* Y537S vs MCF-7 *ESR1* WT

Gene Set Name	No. Genes	P-value	FDR q-val
HALLMARK_INTERFERON_ALPHA_RESPONSE	92	0.0000	0.0000
HALLMARK_ESTROGEN_RESPONSE_LATE	196	0.0000	0.0000
HALLMARK_INTERFERON_GAMMA_RESPONSE	196	0.0000	0.0000
HALLMARK_ESTROGEN_RESPONSE_EARLY	193	0.0000	0.0000
HALLMARK_E2F_TARGETS	190	0.0000	0.0000
HALLMARK_MTORC1_SIGNALING	193	0.0000	0.0003
HALLMARK_CHOLESTEROL_HOMEOSTASIS	71	0.0000	0.0020
HALLMARK_KRAS_SIGNALING_DN	187	0.0000	0.0024
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	106	0.0020	0.0069
HALLMARK_COAGULATION	136	0.0060	0.0071
HALLMARK_INFLAMMATORY_RESPONSE	197	0.0019	0.0104
HALLMARK_UV_RESPONSE_UP	152	0.0000	0.0180
HALLMARK_ALLOGRAFT_REJECTION	195	0.0571	0.1274
HALLMARK_COMPLEMENT	195	0.0527	0.1302
HALLMARK_MYC_TARGETS_V2	58	0.0610	0.1224
HALLMARK_FATTY_ACID_METABOLISM	152	0.0732	0.1409
HALLMARK_G2M_CHECKPOINT	189	0.0959	0.2002
HALLMARK_IL6_JAK_STAT3_SIGNALING	87	0.0938	0.1896
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	195	0.0886	0.1829
HALLMARK_DNA_REPAIR	141	0.1206	0.2160
HALLMARK_XENOBIOTIC_METABOLISM	197	0.1260	0.2095
HALLMARK_P53_PATHWAY	190	0.1376	0.2016
HALLMARK_KRAS_SIGNALING_UP	192	0.1772	0.2642
HALLMARK_APICAL_JUNCTION	194	0.2073	0.2557
HALLMARK_APOPTOSIS	158	0.2231	0.3029
HALLMARK_BILE_ACID_METABOLISM	112	0.2654	0.3583
HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY	45	0.3430	0.4143
HALLMARK_PI3K_AKT_MTOR_SIGNALING	104	0.3507	0.4249
HALLMARK_PANCREAS_BETA_CELLS	40	0.3758	0.4393
HALLMARK_MYOGENESIS	197	0.4585	0.5205
HALLMARK_APICAL_SURFACE	43	0.4852	0.5556
HALLMARK_ANGIOGENESIS	36	0.6819	0.7387
HALLMARK_SPERMATOGENESIS	132	0.7000	0.7701
HALLMARK_WNT_BETA_CATENIN_SIGNALING	42	0.8485	0.8812
HALLMARK_IL2_STAT5_SIGNALING	194	0.9526	0.9403

Table S2. MSigDB hallmark pathways upregulated in MCF-7 LTED vs MCF-7 *ESR1* WT

Gene Set Name	No. Genes	P-value	FDR q-val
HALLMARK_E2F_TARGETS	190	0.0000	0.0000
HALLMARK_G2M_CHECKPOINT	189	0.0000	0.0000
HALLMARK_MTORC1_SIGNALING	193	0.0000	0.0000
HALLMARK_MYC_TARGETS_V1	193	0.0000	0.0000
HALLMARK_ESTROGEN_RESPONSE_LATE	196	0.0000	0.0027
HALLMARK_CHOLESTEROL_HOMEOSTASIS	71	0.0065	0.0191
HALLMARK_INTERFERON_ALPHA_RESPONSE	92	0.0042	0.0187
HALLMARK_UV_RESPONSE_UP	152	0.0139	0.0344
HALLMARK_DNA_REPAIR	141	0.0350	0.1019
HALLMARK_OXIDATIVE_PHOSPHORYLATION	182	0.0458	0.1136
HALLMARK_MYC_TARGETS_V2	58	0.0509	0.1119
HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY	45	0.0848	0.1597
HALLMARK_KRAS_SIGNALING_DN	187	0.0833	0.1537
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	195	0.1188	0.2170
HALLMARK_ADIPOGENESIS	189	0.1282	0.2220
HALLMARK_FATTY_ACID_METABOLISM	152	0.1385	0.2146
HALLMARK_INTERFERON_GAMMA_RESPONSE	196	0.1619	0.2206
HALLMARK_BILE_ACID_METABOLISM	112	0.3016	0.3754
HALLMARK_PI3K_AKT_MTOR_SIGNALING	104	0.5456	0.6224
HALLMARK_SPERMATOGENESIS	132	0.6577	0.7771
HALLMARK_APICAL_SURFACE	43	0.7228	0.7860
HALLMARK_XENOBIOTIC_METABOLISM	197	0.7908	0.8275
HALLMARK_IL6_JAK_STAT3_SIGNALING	87	0.9864	0.9743

Table S3. MSigDB hallmark pathways upregulated in T47D *ESR1* Y537S vs T47D *ESR1* WT

Gene Set Name	No. Genes	P-value	FDR q-val
HALLMARK_ESTROGEN_RESPONSE_EARLY	193	0.0000	0.0099
HALLMARK_ESTROGEN_RESPONSE_LATE	196	0.0201	0.0909
HALLMARK_COAGULATION	136	0.0368	0.1312
HALLMARK_INTERFERON_ALPHA_RESPONSE	92	0.0326	0.1322
HALLMARK_KRAS_SIGNALING_DN	187	0.0895	0.2218
HALLMARK_IL2_STAT5_SIGNALING	194	0.3191	0.6273
HALLMARK_INTERFERON_GAMMA_RESPONSE	196	0.3452	0.5765
HALLMARK_WNT_BETA_CATENIN_SIGNALING	42	0.3594	0.5226
HALLMARK_NOTCH_SIGNALING	32	0.4412	0.5624
HALLMARK_HYPOXIA	191	0.5193	0.6263
HALLMARK_COMPLEMENT	195	0.6204	0.6997
HALLMARK_HEDGEHOG_SIGNALING	35	0.6719	0.6758

Table S4. Uniquely activated kinases identified in KiP assay

WT-unique	Y537S-unique
CDC7	ADCK5
CDK12	BMPR1A
CHKA	CDC42BPG
CLK1	CDK10
CSNK1G2	CHEK1
DYRK2	EIF2AK2
KIAA1804	EPHA4
MAP3K5	EPHB1
MAP3K6	FGFR4
MET	LYN
PRKACB	MAP3K7
SRPK2	NEK7
STRADA	PHKG2
	PRKX
	RPS6KA6
	SIK2
	TP53RK
	WNK1

Table S5. Kinome response to Tam in MCF-7 *ESR1* Y537S cells by KiP analysis

Kinase	Y537S/WT	Y537S Tam/Y537S	Kinase	Y537S/WT	Y537S Tam/Y537S
RET	103.33	0.07	DYRK1A	2.64	0.33
ROR2	16.71	1.28	MAP2K1	2.63	0.64
MST1R	14.00	0.26	MAPK4	2.62	0.55
BMPR2	9.40	0.13	MAP4K3	2.55	0.63
MAP4K2	8.60	0.00	MAPK6	2.50	0.58
SIK3	7.00	0.93	IGF1R	2.47	0.38
PTK6	6.35	0.65	LIMK2	2.44	0.71
PRKACA	6.06	0.53	CDK1	2.41	0.79
HSPB8	5.60	0.30	CSNK1G1	2.33	0.00
ATM	5.56	0.14	RPS6KA5	2.33	0.04
CDK11B	5.00	0.00	MAPK1	2.23	0.91
MAP3K4	5.00	0.00	STK4	2.19	0.64
CDK4	4.78	0.38	PDPK1	2.18	0.63
CSNK1A1	4.75	0.55	CASK	2.18	0.18
RPS6KA3	4.55	0.33	EPHB4	2.17	0.31
CSNK2A2	4.38	0.90	BRD4	2.07	0.20
ATR	4.33	0.00	EIF2AK4	2.05	0.00
JAK1	4.27	0.16	PRKCH	2.00	2.13
INSR	4.18	0.46	EIF2AK3	1.94	0.00
PKMYT1	4.15	0.80	CDK7	1.83	0.62
CDK5	4.00	0.63	VRK2	1.80	0.94
PIK3R4	4.00	0.00	CLK2	1.77	0.72
TAOK3	3.67	0.72	MAPK3	1.76	0.66
EPHB3	3.61	0.37	TYK2	1.74	0.41
AURKB	3.60	1.24	CAMK2B	1.64	0.64
CDK3	3.46	0.00	ABL1	1.64	0.42
CDK2	3.45	0.94	STK3	1.63	0.57
BMP2K	3.33	0.91	MAP3K3	1.61	0.72
CDC42BPB	3.20	0.00	IKBKB	1.60	0.09
TRRAP	3.14	0.27	PLK1	1.59	0.27
MAP2K3	3.10	0.42	CDK9	1.58	0.90
MAP4K4	3.04	0.48	PKN2	1.57	0.00
SYK	2.92	0.50	TESK1	1.55	0.69
RPS6KA2	2.81	0.36	ULK3	1.51	0.74
CAMK2D	2.80	0.82			

Extended Materials and Methods

Cell Culture

MCF-7 cells were cultured in MEM (Corning, Tewksbury, MA) supplemented with 10% FBS (Gemini BioProducts, West Sacramento, CA), 100U/mL penicillin, 100 µg/mL streptomycin, and 1X NEAA. T47D cells were cultured in RPMI (Corning, Tewksbury, MA) with 10% FBS, 100U/mL penicillin, 100 µg/mL streptomycin, and 1X NEAA. For studies using hormone and growth factor-reduced conditions, cells were cultured in phenol red-free media supplemented with 5% charcoal-stripped FBS. Cells were maintained in a 37°C incubator with 5% CO2.

Generation of *ESR1* mutant cell lines

CRISPR/Cas9 knock-in was performed as previously described.[2] To introduce point mutations into the endogenous ER locus in MCF-7 and T47D cell lines, we first constructed sgRNA vectors based on px458.[3] Based on the corresponding sgRNA sequence, we then synthesized anti-sense oligonucleotide(s) (ssODNs) with the intended mutation as well as silent mutations that serve to prevent Cas9 re-cutting and facilitate screening.

For each ER mutation site, the sgRNA vector (7 µg) and corresponding ssODN template (3 µl of 100 µM) were electroporated into cells using the Neon electroporation system from LifeTechnologies. GFP+ cells were subsequently sorted on an Aria III (Cytometry and Cell Sorting Core) and plated into 96-well plates for further culturing and screening analysis.

Individual clones that grew back were expanded for genomic DNA extraction and PCR amplification of the regions that encompass each intended KI site. Successful KI of each mutation was confirmed by Sanger sequencing of the PCR products. sgRNA sequence for Y537S: 5'-TCCAGCAGCAGGTCATAGAG. The sequence for the anti-sense ssODNs for the Y537S mutation is:

5'cctccacggctagtgggcgcatgtaggcggtgggcgtccagcatctccagcagcag*Atca***G**a*C*agggg*T*accacgttcttgcacttcat gctgtacagatgctccatgcctttgttactgt (intended mutation in bold silent mutations in italics)

MCF-7 LTED cells were generated by culturing MCF-7 cells in phenol red-free MEM media supplemented with 5% charcoal-stripped FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1X NEAA for six months.

Subcellular Fractionation

MCF-7 and T47D cells were cultured in 5% charcoal-stripped FBS supplemented media for 72 hours prior to harvesting. Cellular fractionation was performed using the Thermo Scientific Subcellular Protein Fractionation Kit (Thermo Fisher Cat No. PI78840) using the manufacture's protocol. Cytoplasmic and nuclear fractions were analyzed by immunoblot.

Digital Droplet PCR (ddPCR)

ddPCR primer/probe sets for *ESR1* were used to detect *ESR1* mutations in MCF-7 LTED cells using the BioRad QX100™ Droplet Digital™ PCR system as previously described.[4] *ESR1* Y537C ID dHsaMDS732897750, Y537N ID dHsaMDS296069817, ESR1 Y537S ID dHsaMDS975379796, D538G ID dHsaMDS460485301. Detection of 3 out of 1000 positive *ESR1* mutant droplets was defined as positive detection of *ESR1* mutation.

Microarray Analysis

To determine the hormone-independent transcriptome of MCF-7 *ESR1* WT, *ESR1* Y537S, and LTED cells, cells were plated in regular serum-containing media for 24 hours and then starved for 48 hours in 5% charcoal-stripped serum. For MCF-7 *ESR1* WT and *ESR1* Y537S PalbR models, cells were plated in regular serum-containing media for 72 hours. RNA was extracted using the RNeasy kit (Qiagen, Germantown, MD). Labeled cRNA was hybridized onto Affymetrix GeneChip Human

Genome U133 Plus 2.0 Arrays (Life Technologies, Carlsbad, CA) in triplicate. Microarray data were processed using two different methods. First, expression values were estimated using RMA (Robust Multi-array Average) method. Group comparison was run using ANOVA analysis performed with Partek Genomics Suite software (www.partek.com/softwareoverview). Differentially expressed genes with fold >1.5 and P value <0.05 were used for GSEA and MSigDB analysis (http://software.broadinstitute.org/gsea/msigdb) (HALLMARK gene sets). Probe intensities were normalized using RMA (Robust Multi-Array Average) method. The mean of individual probes belonging to the same gene were used for GSEA analyses. 'BiomaRt' R package was used to convert the probe ID to HGNC (HUGO Gene Nomenclature Committee) gene symbols. The differential gene expression analysis was performed with 'limma' R package.

ChIP-seq

ChIP-Seq experiments were conducted as described previously, and were done in triplicate.[5] Briefly, chromatin from approximately 1×10^7 fixed cells, cultured in 5% CSS media was sonicated using Bioruptor Standard Sonicator (Diagenode) to a size range of 200-600 bp. Solubilized chromatin was subjected to immunoprecipitation with the ER antibody HC-20 (Santa Cruz Biotechnology, Dallas, TX). A fraction of the sample was not exposed to antibody to be used as control (input). The samples were reverse-crosslinked, treated with proteinase K, and DNA was purified. Subsequent library construction with the TruSeq ChIP Library Preparation Kit (Illumina) and sequencing on the NextSeq 500 (Illumina) of the purified input and immunoprecipitated DNA were conducted by the UT Southwestern McDermott Sequencing Core. ER ChIP-Sequencing data was assessed for quality using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and mapped using Bowtie2[6] to the human genome build UCSC hg38. Enriched regions were determined using MACS2 software [7] with significance achieved at q<0.05. Peak overlaps were determined using BEDTools.[8] Genes associated with peaks were determined using BEDTools and the GENCODE[9] gene model. Signal density plots for ChIP-Seq signals were generated using the HOMER software suite[10] and

visualized using Python scientific libraries. Signal tracks were generated using BEDTools, and visualized using the UCSC genome browser[11] and the Integrated Genome Viewer (IGV).[12]

ChIP-PCR

ChIP assay was modified from a previously described method.[13] Y537S cells were plated at 4 x 10^6 cells per 10 cm plate in duplicate and cultured in MEM supplemented with 10% (v/v) FBS overnight. Cells were washed with PBS and serum starved in 10 mL of phenol red-free MEM supplemented with 5% (v/v) CSS for 48 hrs. Cells were crosslinked with 0.75% (v/v) formaldehyde for 10 min at room temperature and crosslinking was quenched with 0.125 mM glycine for 5 min. Cells were washed with cold PBS twice and collected in 1 mL of PBS containing protease inhibitors (cOmplete™ Mini EDTA-free Protease Inhibitor Cocktail, Sigma). Cells were pelleted and re-suspended in a 1:1 ratio of lysis buffer (1% sodium dodecyl sulfate (SDS), 10 mM EDTA, 50 mM Tris-HCl, pH 8.0.) and PBS supplemented with protease inhibitors. Chromatin was fragmented using a Branson sonicator (Branson Ultrasonics, Danbury, CT, model S450D) to achieve a DNA fragment size in the range of 200-500 bp. Fragmented soluble chromatin was diluted 5-fold with dilution buffer (0.55% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl) and 5 % was reserved as the input fraction. The remaining lysate was precleared with 25 µL of 1:1 mixture of Protein-A and -G Dynabeads (Invitrogen, Life Technologies). Soluble chromatin was immunoprecipitated overnight at 4 ºC with 4 µg of ERα (Millipore, 06-935) or rabbit anti-mouse IgG (Invitrogen, Life Technologies).

Immunoprecipitated complexes were captured by incubating each sample with 50 µL of a 1:1 mixture of Protein-A and -G Dynabeads at 4 ºC with rotation for 2 hrs. Precipitates were each washed sequentially for 10 minutes twice with cold low salt buffer (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl.) and then high salt buffer (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl). Precipitates were then washed three times with cold TE and extracted three times with elution buffer (1% SDS, 0.1 M $NaHCO_3$). Filtered 1 M $NaHCO_3$ was added to the input

samples to a final concentration of 0.1M $NaHCO_3$. Input and eluted protein-DNA complexes were de-crosslinked for 6 hrs at 65 ºC and DNA was purified with QIAquick spin columns (QIAGEN). Enrichment of DNA fragments after ChIP was measured by qPCR in triplicate using the primer sequences in Table 1. GAB2 primers: Forward: 5'-AAGAACACCATCTCCGCTAGG -3', Reverse: 3'-TCGTATGCATCAAGGTCAGGA-5'.

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