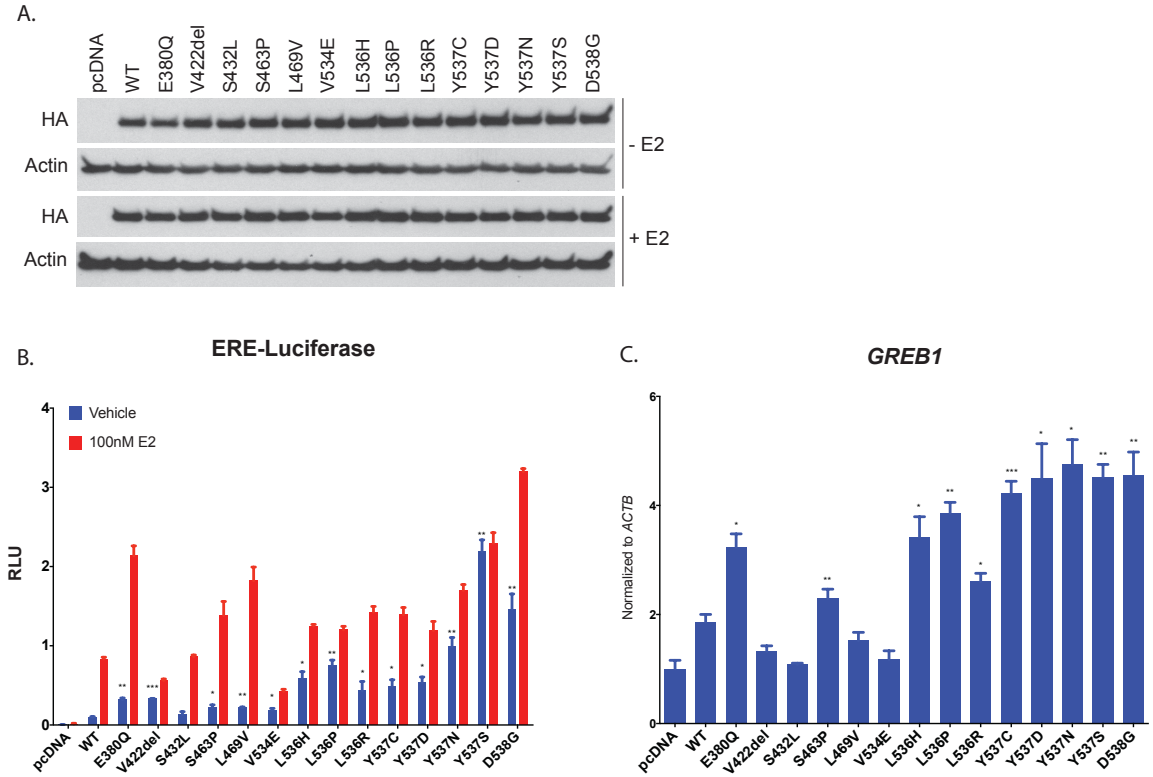
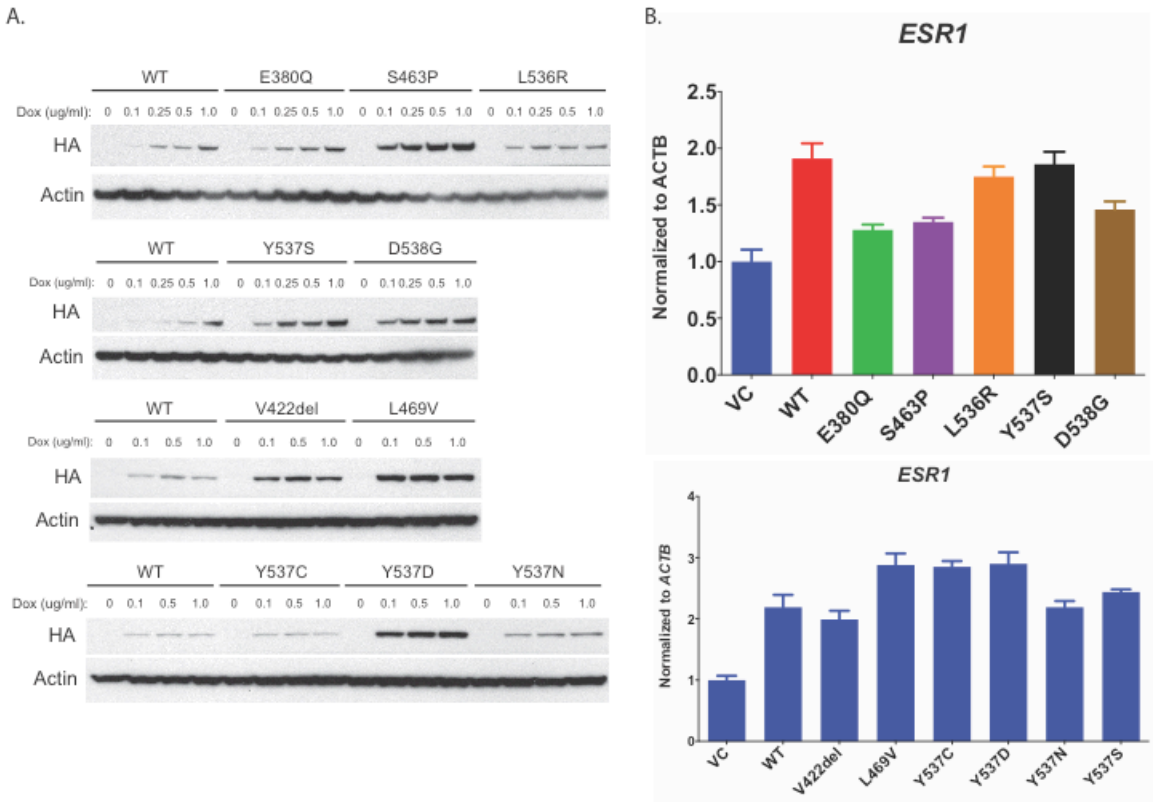
Supplementary Results



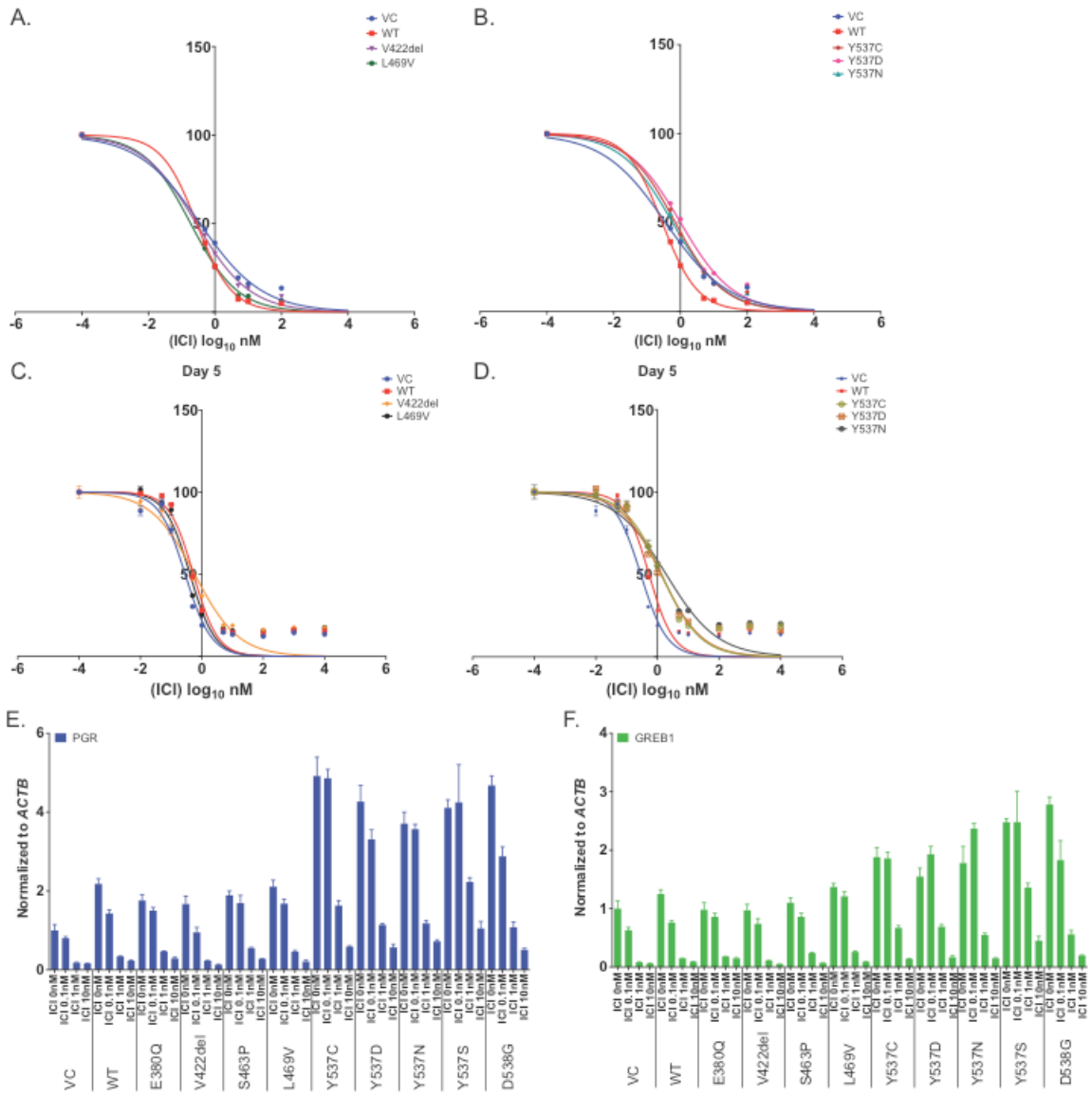
Supplementary Figure 1.

(A) Immunoblot of HA-tagged ER WT and mutants expression from the MCF7 lysates used in the luciferase assay of Figure 2A. (B) SKBr3 cells were transfected with HA-ERα wild type (WT) or mutants, ERE-luciferase and Renilla luciferase reporter constructs in hormone-depleted medium with 100 nM of E2 added for 24 hours where indicated. Relative light units (RLU) were calculated as the ratio of Firefly luciferase over Renilla luciferase activity. Firefly luciferase activity shows increased activity in absence or presence of E2 for certain mutations such as E380Q. Graphs were plotted with the mean ± SD of three biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (C) SKBR3 cells were transfected with empty vector, HA-ERα WT or mutant in hormone-depleted medium and harvested 48 hours post-transfection for mRNA quantification of ER target genes such *GREB1*. Bars represent mean ± SD of triplicate technical replicates normalized to actin (*ACTB*) expression. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.



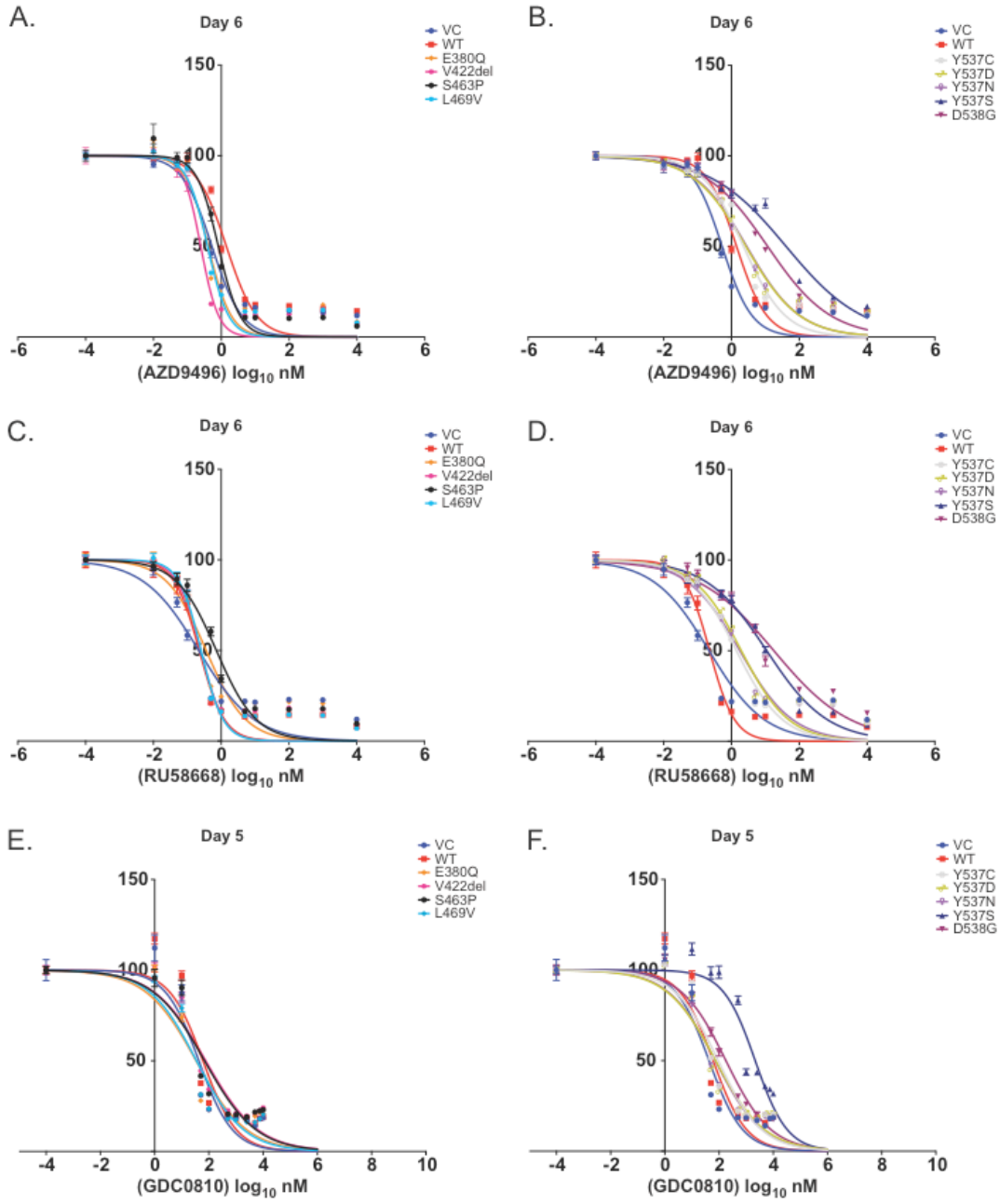
Supplementary Figure 2.

(A) Immunoblot of HA-tagged ER WT and mutants expression in the doxycycline-inducible stable MCF7 cell lines with various doses of doxycycline. (B) Quantitative PCR detection of *ESR1* transcript levels in the stable MCF7 cells treated with the appropriate doxycycline concentration for the induction of similar expression level as that of the WT. (VC= vector control)



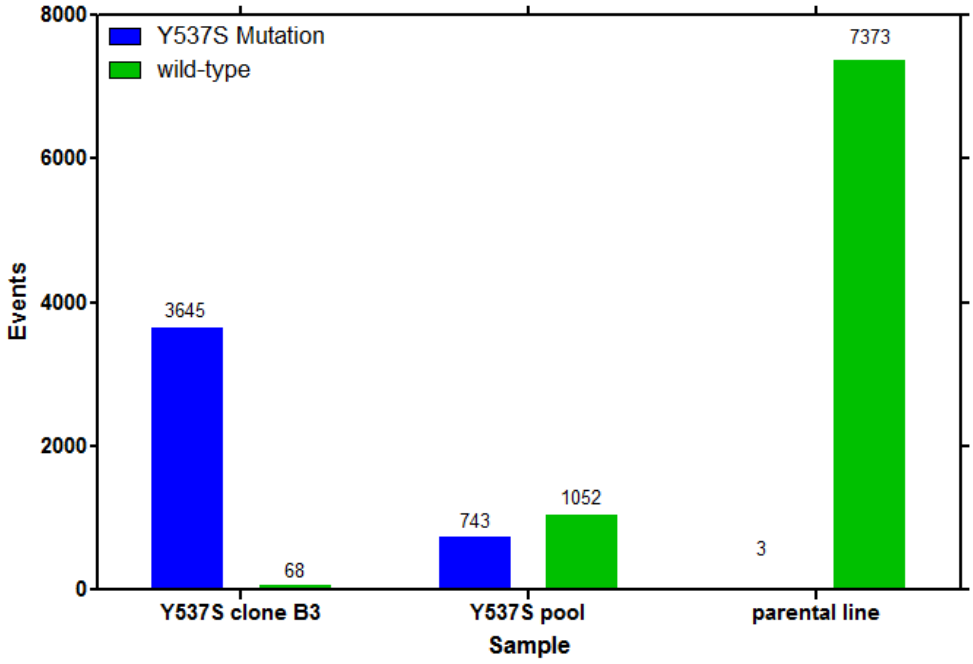
Supplementary Figure 3.

Doxycycline inducible HA-ER WT and mutants expressing MCF7 cells, such as V422del, L469V and Y537C/D/N/S that were treated with various doses of fulvestrant in regular medium demonstrated that certain mutants required higher level of antagonists for complete ERE-luciferase activity (A-B) and growth inhibition (C-D). (E-F) Quantitative PCR detection of ER target genes transcript levels in doxycycline induced HA-ER WT and all mutants expressing cells showed significant reduction in *PGR* and *GREB1* transcript levels when treated with various doses of fulvestrant for 24h in regular media. Graphs were plotted with the mean ± SD of three technical replicates.



Supplementary Figure 4.

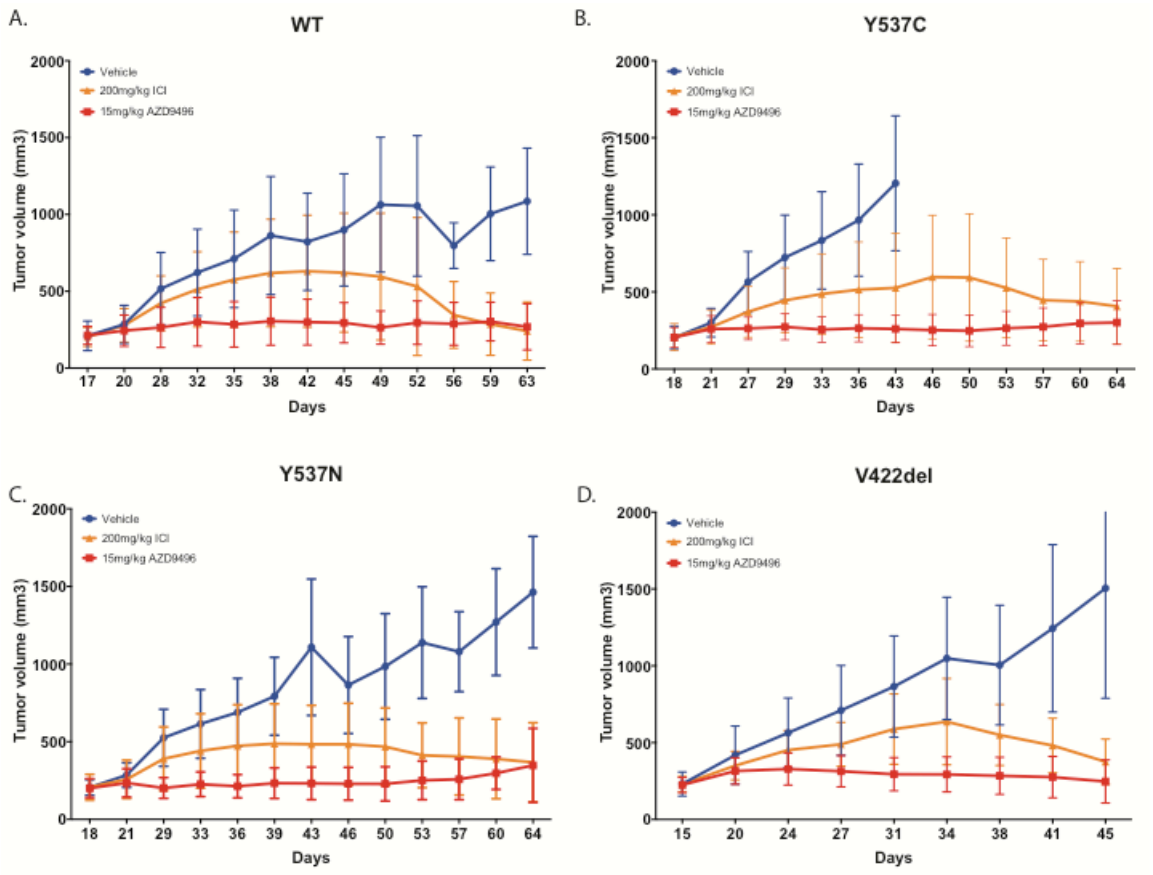
Doxycycline inducible HA-ER WT and mutants expressing MCF7 cells that were treated with various doses of (A-B) AZD9496, (C-D) RU-58668, (E-F) GDC-0810 in regular medium showed that certain mutants required higher level of antagonists for complete growth inhibition.



Supplementary Figure 5.

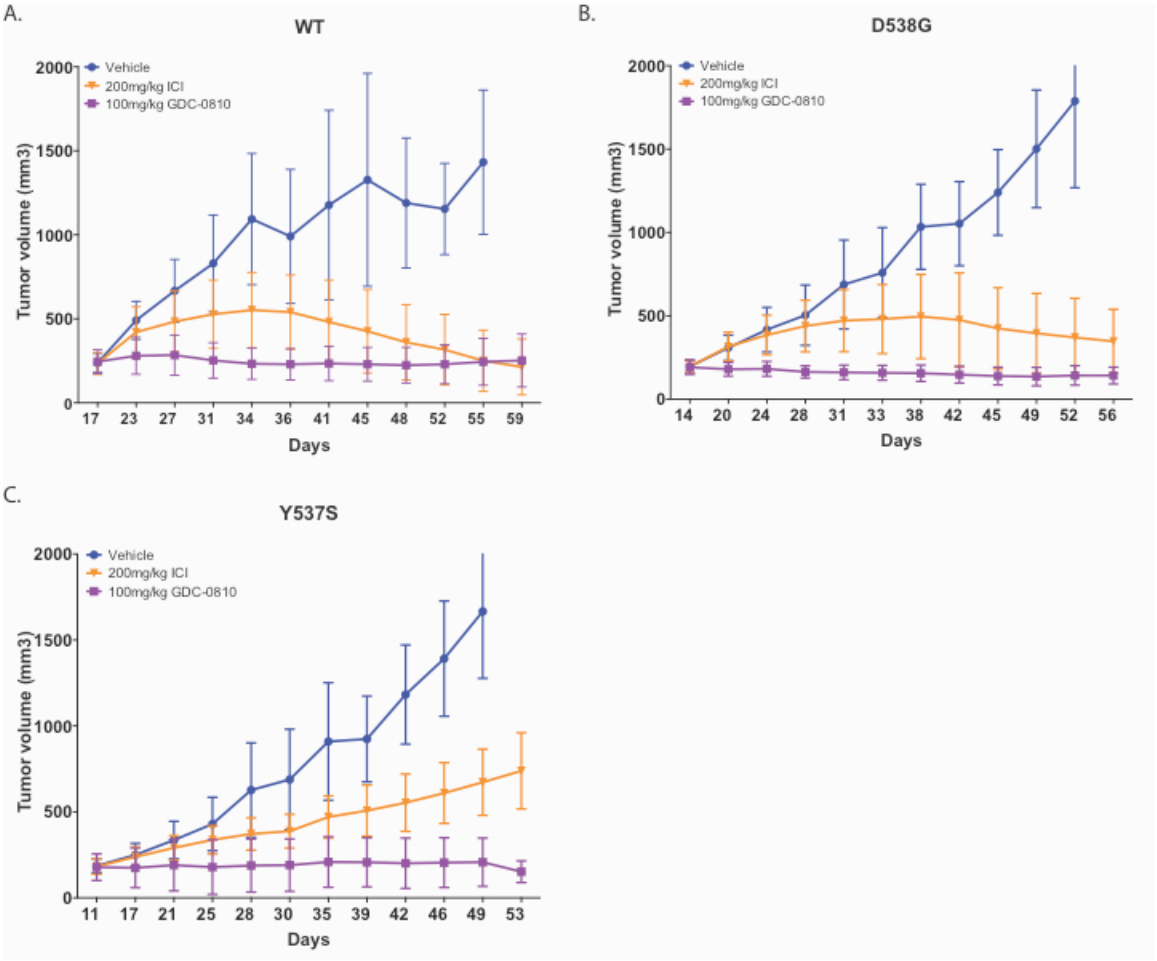
Digital droplet PCR (ddPCR) analysis of the parental, pooled (before single cell sorting) and clone B3 cells indicated that the clone B3 is homozygous for Y537S mutation. (Blue: Y537S probe, Green: wild-type probe)[1]

[1] Analysis by TLA sequencing indicated that the ESR1 target (TG) sequence has been inserted at the intended location on Chromosome 6. No evidence was found for a multicopy integration. However the SNV and deletion data present in the homology arm of the TG suggest three ESR1 alleles are present, which is further supported by the deviating TG/genome coverage data.
The three alleles are:
- allele 1: contains the mutation of the PAM site and the T537S A>C mutation, which is indicative of the insertion of the donor cassette
- allele 2 and 3: the wildtype sequences have been disrupted by an InDels.



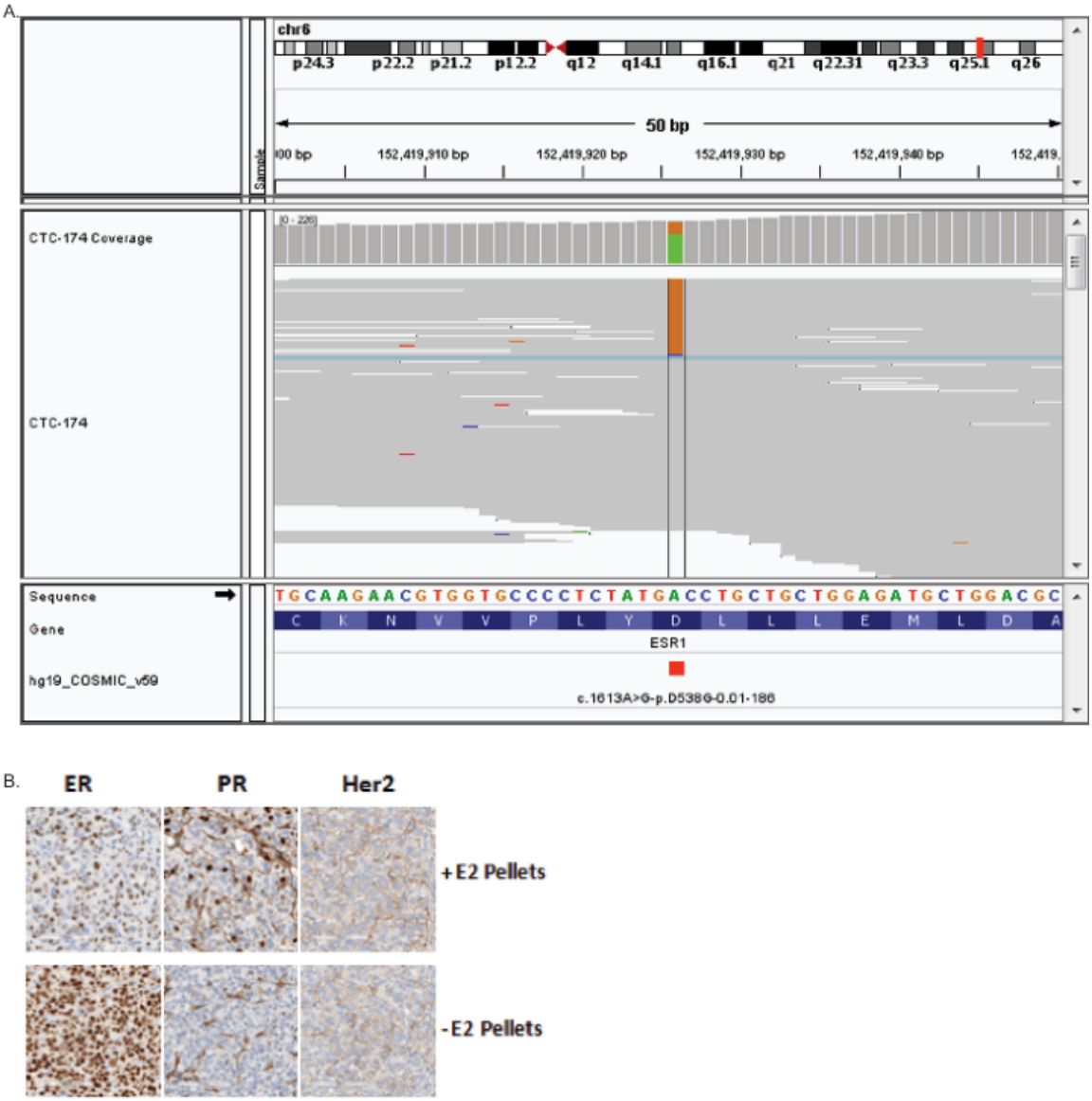
Supplementary Figure 6.

Mice bearing MCF7 inducible HA-ER WT (A), Y537C (B), Y537N (C) or V422del (D) tumors were randomly assigned to treatment groups of either 15 mg/kg of AZD9496, daily orally or 200 mg/kg of Fulvestrant twice weekly, s.c. Tumors treated with AZD9496 showed greater growth inhibition as compared to those treated with Fulvestrant. The result was presented as average tumor volume measured for each group ± SEM (n = 10 mice/group).



Supplementary Figure 7.

Mice bearing MCF7 inducible HA-ER WT (A), D538G (B) or Y537S (C) tumors were randomly assigned to treatment groups of either 100 mg/kg of GDC-0810, daily orally or 200 mg/kg of Fulvestrant twice weekly, s.c. Tumors treated with GDC-0810 showed greater growth inhibition as compared to those treated with Fulvestrant. The result was presented as average tumor volume measured for each group ± SEM (n = 10 mice/group).



Supplementary Figure 8.

(A) RNA-seq analysis of the patient-derived xenograft (PDX) model, CTC-714, indicated that the D538G mutant allele frequency is 31% (orange bar) while WT is 69% (green bar). The depth of coverage is 180 reads. (B) Immunohistochemistry staining indicating the expression of ER, PR and HER2 in the PDX model, CTC-714.

	ESR1 Wild Type		ESR1 mutated			
	Therapy		Therapy			
	No	Yes	No	Yes	OR (95% CI)*	P value
Adjuvant or Metastatic						
AI or Tamoxifen	427 (51.2%)	407 (48.8%)	9 (9.5%)	86 (90.5%)	10.0 (4.9-22.9)	2e-16
AI	533 (63.9%)	301 (36.1%)	13 (13.7%)	82 (86.3%)	11.3 (6.0-22.2)	2e-16
Tamoxifen	561 (67.3%)	273 (32.7%)	31 (32.6%)	64 (67.4%)	4.2 (2.6-6.9)	1e-10
Adjuvant						
AI or Tamoxifen	516 (61.9%)	318 (38.1%)	37 (38.9%)	58 (61.1%)	2.5 (1.6-4.0)	2e-05
AI	650 (77.9%)	184 (22.1%)	59 (62.1%)	36 (37.9%)	2.2 (1.3-3.4)	0.0013
Tamoxifen	612 (73.4%)	222 (26.6%)	49 (51.6%)	46 (48.4%)	2.6 (1.6-4.1)	4e-05
Metastatic						
AI or Tamoxifen	634 (76%)	200 (24%)	21 (22.1%)	74 (77.9%)	11.1 (6.6-19.5)	2e-16
AI	677 (81.2%)	157 (18.8%)	31 (32.6%)	64 (67.4%)	8.9 (5.5-14.6)	2e-16
Tamoxifen	773 (92.7%)	61 (7.3%)	68 (71.6%)	27 (28.4%)	5.0 (2.9-8.6)	1e-08

Supplementary Table 1.

Odds ratio and exact 95% confidence intervals for prior exposure to therapy comparing *ESR1* mutant vs wild type. Restricted to patients with HR+/HER2- metastatic breast cancer. AI = aromatase inhibitor

AA change	Type	Ref	Var	Allele Freq (T)	Allele Freq (N)
Y537S	Missense	A	C	0.23	NA
Y537S	Missense	A	C	0.25	NA
Y537S	Missense	A	C	0.62	0
Y537S	Missense	A	C	0.13	NA
Y537S	Missense	A	C	0.19	NA
Y537S	Missense	A	C	0.47	NA
Y537S	Missense	A	C	0.41	NA
Y537S	Missense	A	C	0.23	NA
Y537S	Missense	A	C	0.04	NA
Y537S	Missense	A	C	0.06	NA
Y537S	Missense	A	C	0.31	0
Y537S	Missense	A	C	0.4	NA
Y537S	Missense	TA	AG	0.12	NA
Y537N	Missense	T	A	0.14	NA
Y537N	Missense	T	A	0.25	NA
Y537N	Missense	T	A	0.17	0
Y537N	Missense	T	A	0.06	NA
Y537N	Missense	T	A	0.26	NA
Y537D*	Missense	T	G	0.39	NA
Y537C	Missense	A	G	0.47	NA
Y537C	Missense	A	G	0.27	NA
Y537C	Missense	A	G	0.21	NA
Y537C	Missense	A	G	0.05	NA
Y537C	Missense	A	G	0.39	NA
Y537C	Missense	A	G	0.39	0
V534E	Missense	T	A	0.21	NA
V478L*	Missense	G	C	0.07	NA
V422del	IF del	TGG	-	0.26	NA
V422del	IF del	ATGG	A	0.22	NA
V418E*	Missense	T	A	0.43	NA
S463P	Missense	T	C	0.49	NA
S432L*	Missense	C	T	0.26	NA
S432L*	Missense	C	T	0.22	NA
S329Y*	Missense	C	A	0.33	NA
N532K*	Missense	C	G	0.61	NA
L536R	Missense	T	G	0.6	0
L536P*	Missense	T	C	0.26	NA
L536P*	Missense	T	C	0.12	NA
L536H*	Missense	T	A	0.19	NA
L536H*	Missense	T	A	0.37	NA
L536H*	Missense	T	A	0.33	NA
L536H*	Missense	T	A	0.51	NA
L466Q*	Missense	T	A	0.29	NA
G521Rfs*18*	FS ins	-	A	0.18	NA
G442R*	Missense	G	C	0.33	NA
G344D*	Missense	G	A	0.05	NA
F461V*	Missense	T	G	0.27	NA
E542G*	Missense	A	G	0.05	NA
E380Q	Missense	G	C	0.63	NA
E380Q	Missense	G	C	0.15	NA
E380Q	Missense	G	C	0.34	NA
E380Q	Missense	G	C	0.06	0
E380Q	Missense	G	C	0.68	NA
E380Q	Missense	G	C	0.21	NA
E380Q	Missense	G	C	0.36	NA
E380Q	Missense	G	C	0.65	0
E380Q	Missense	G	C	0.12	NA
E380Q	Missense	G	C	0.42	NA

AA change	Type	Ref	Var	Allele Freq (T)	Allele Freq (N)
E380Q	Missense	G	C	0.63	NA
E380Q	Missense	G	C	0.15	NA
E380Q	Missense	G	C	0.34	NA
E380Q	Missense	G	C	0.06	0
E380Q	Missense	G	C	0.68	NA
E380Q	Missense	G	C	0.21	NA
E380Q	Missense	G	C	0.36	NA
E380Q	Missense	G	C	0.65	0
E380Q	Missense	G	C	0.12	NA
E380Q	Missense	G	C	0.42	NA
D538G	Missense	A	G	0.18	NA
D538G	Missense	A	G	0.23	NA
D538G	Missense	A	G	0.2	NA
D538G	Missense	A	G	0.59	NA
D538G	Missense	A	G	0.47	NA
D538G	Missense	A	G	0.05	NA
D538G	Missense	A	G	0.38	NA
D538G	Missense	A	G	0.08	NA
D538G	Missense	A	G	0.24	NA
D538G	Missense	A	G	0.33	NA
D538G	Missense	A	G	0.38	NA
D538G	Missense	A	G	0.21	NA
D538G	Missense	A	G	0.06	0
D538G	Missense	A	G	0.22	NA
D538G	Missense	A	G	0.35	NA
D538G	Missense	A	G	0.21	NA
D538G	Missense	A	G	0.19	NA
D538G	Missense	A	G	0.58	NA
D538G	Missense	A	G	0.11	NA
D538G	Missense	A	G	0.43	0.01
D538G	Missense	A	G	0.23	NA
D538G	Missense	A	G	0.41	NA
D538G	Missense	A	G	0.21	NA
D538G	Missense	A	G	0.32	NA
D538G	Missense	A	G	0.21	0.01
D538G	Missense	A	G	0.5	NA
D538G	Missense	A	G	0.23	NA
D538G	Missense	A	G	0.36	NA
D538G	Missense	A	G	0.32	NA
D538G	Missense	A	G	0.37	NA
D538G	Missense	A	G	0.26	NA
D538G	Missense	A	G	0.15	NA
D538G	Missense	A	G	0.1	NA
D538G	Missense	A	G	0.64	NA
D538_L539insHD*	IF ins	-	ATGACC	0.12	NA
A546D*	Missense	C	A	0.25	NA
A546D*	Missense	C	A	0.41	NA

Supplementary Table 2.

ESR1 LBD mutations detected in ER+ breast cancer patients and their respective allelic frequencies. (* indicate mutations previously not reported) IF = in frame; FS = frameshift; ins = insertion; del = deletion.

ER mutants	Apo - t½ (min)	E_2 - t½ (min)
Wild type	3	14
E380Q	11	23
S463P	42	very stable
L536R	9	48
Y537S	83	1490
D538G	13	very stable

Supplementary Table 3.

Half-life (t½) values of the trypsin treatment of ER mutants calculated based on the measurements taken from time resolved-FRET assays. The data, representing 2–3 replicate experiments, were analyzed using GraphPad Prism 4.