**Title: *ESR1* F404 mutations and acquired resistance to fulvestrant in *ESR1* mutant breast cancer.**

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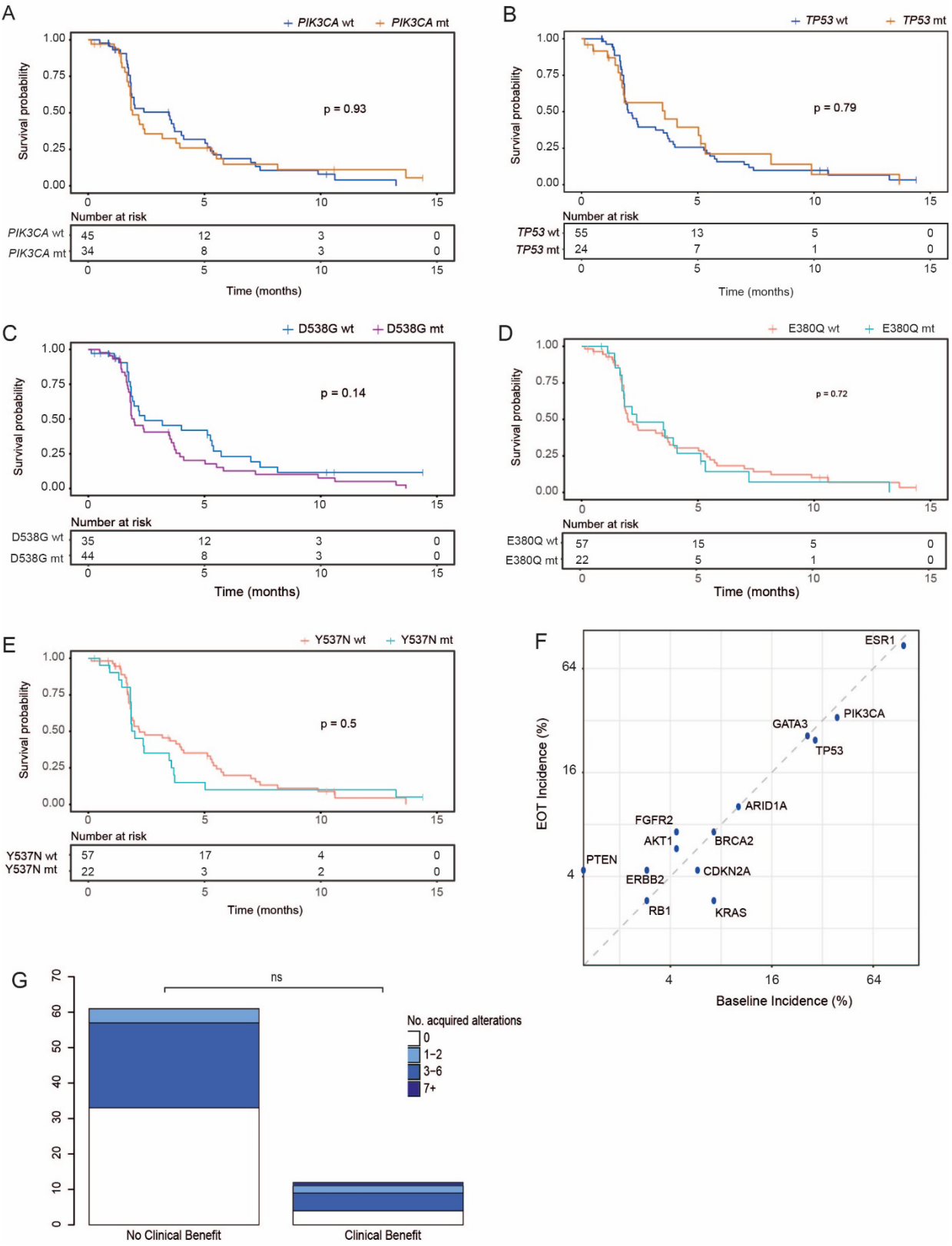
Running title:

Mutations of *ESR1* at F404 confer fulvestrant resistance.

Keywords:

Fulvestrant, acquired resistance, breast cancer.

Supp Figure 1



**Supplementary Figure 1. Progression free survival of patients with selected mutations.**

A. Progression-free survival of patients in Cohort A with baseline *PIK3CA* mutations. p-value from log rank test. HR >1 denotes worse PFS for that group. HR 0.55, 95% CI 0.34 to 0.89. wt, wild type; mt, mutant.

B. Progression-free survival of patients in Cohort A with baseline *TP53* mutations. p-value from log rank test. HR >1 denotes worse PFS for that group. HR 1.79, 95% CI 1.04 to 3.07. wt, wild type; mt, mutant.

C. Progression-free survival of patients in Cohort A with baseline *ESR1* D538G mutations. p-value from log rank test. HR >1 denotes worse PFS for that group. HR 0.81, 95% CI 0.49 to 1.33. wt, wild type; mt, mutant.

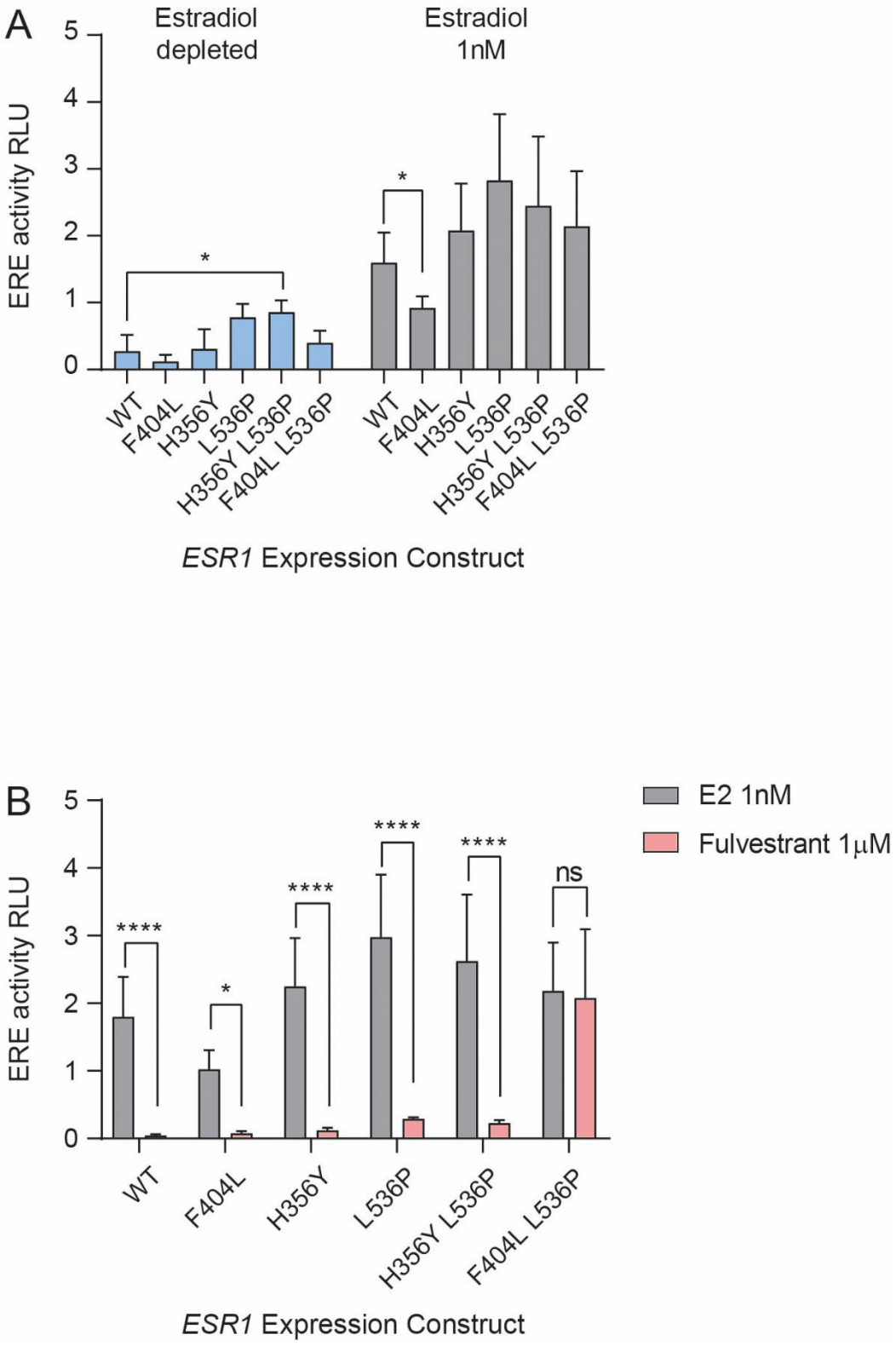
D. Progression-free survival of patients in Cohort A with baseline *ESR1* E380Q mutations. p-value from log rank test. HR >1 denotes worse PFS for that group. HR 1.18, 95% CI 0.69 to 2.03. wt, wild type; mt, mutant.

E. Progression-free survival of patients in Cohort A with baseline *ESR1* Y537N mutations. p-value from log rank test. HR >1 denotes worse PFS for that group. HR 0.91, 95% CI 0.53 to 1.55. wt, wild type; mt, mutant.

F. Incidence of mutations in indicated genes at baseline vs end of treatment.

G. Number of acquired mutations in patients by clinical benefit (CR/PR/SD >= 24 weeks). Comparison by Chi-squared test.

Supp Figure 2



**Supplementary Figure 2. H356Y does not activate estrogen signalling or alter fulvestrant sensitivity in combination with L536P.**

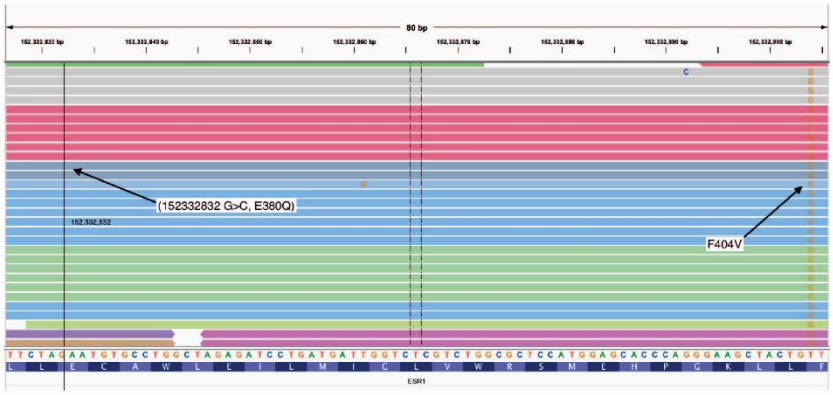
A. MCF7 cells were co-transfected with the indicated ESR1 expression constructs ERE-luciferase reporter and control construct. Cells were treated in either the absence or presence of estradiol (1nM) for 24 hours and ERE-luciferase activity assessed. 2-way repeated measures ANOVA with Dunnett's multiple comparisons test, n=3 mean with SD, *P<0.05, **P<0.01, ****P<0.0001.

B. MCF7 cells were co-transfected with the indicated ESR1 expression constructs ERE-luciferase reporter and control construct. Cells were treated in either the absence or presence of fulvestrant (1μM) for 24 hours and ERE-luciferase activity assessed. 2-way repeated measures ANOVA with Sidak's multiple comparisons test, n=3 mean with SD, *P<0.05, ***P<0.001, ****P<0.0001.

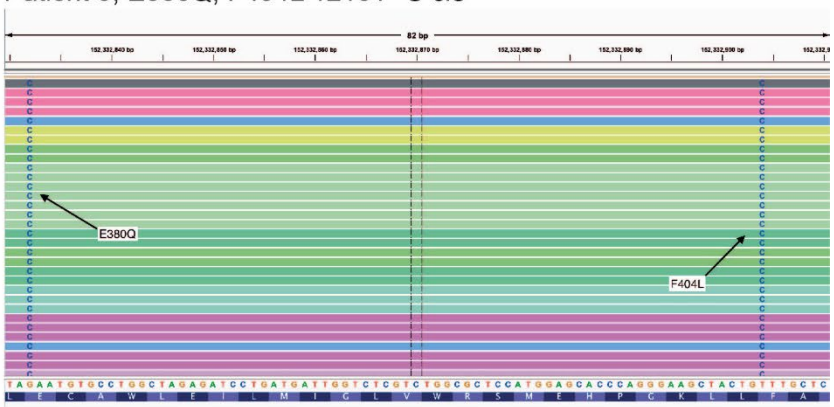
Supp Figure 3

Patient 1, E380Q, F404I 1210T>A *cis*

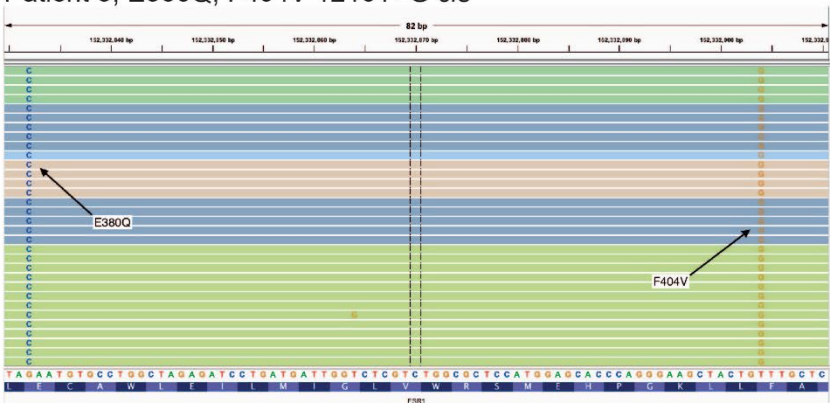

Patient 2, E380Q, F404V *trans*




Patient 3, E380Q, F404L 1210T>C *cis*



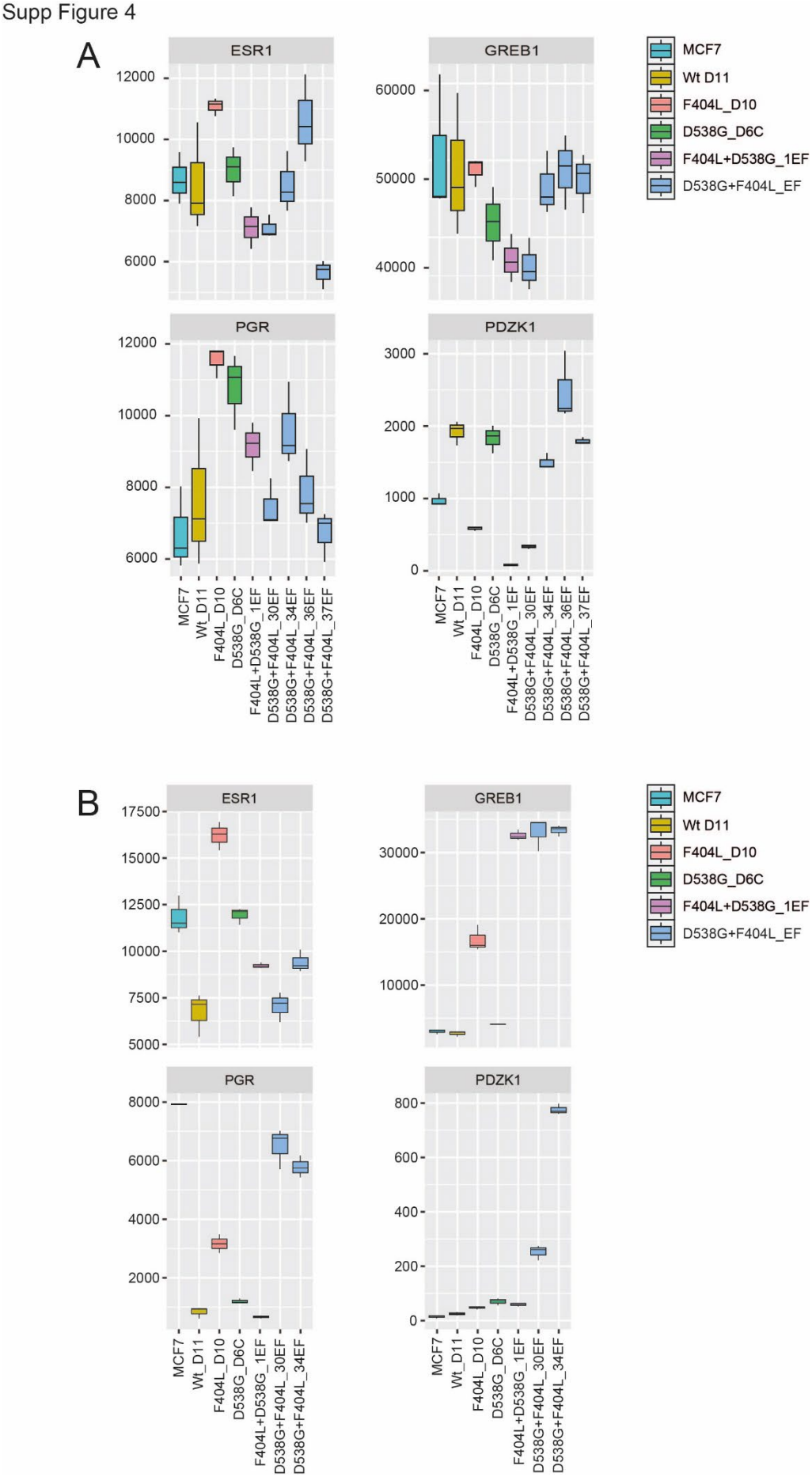

Patient 3, E380Q, F404V 1210T>G *cis*



**Supplementary Figure 3. Acquired *ESR1* F404 mutations in patients treated with Fulvestrant.**

Individual reads visualised in Integrated Genome Viewer (IGV) (1-3)

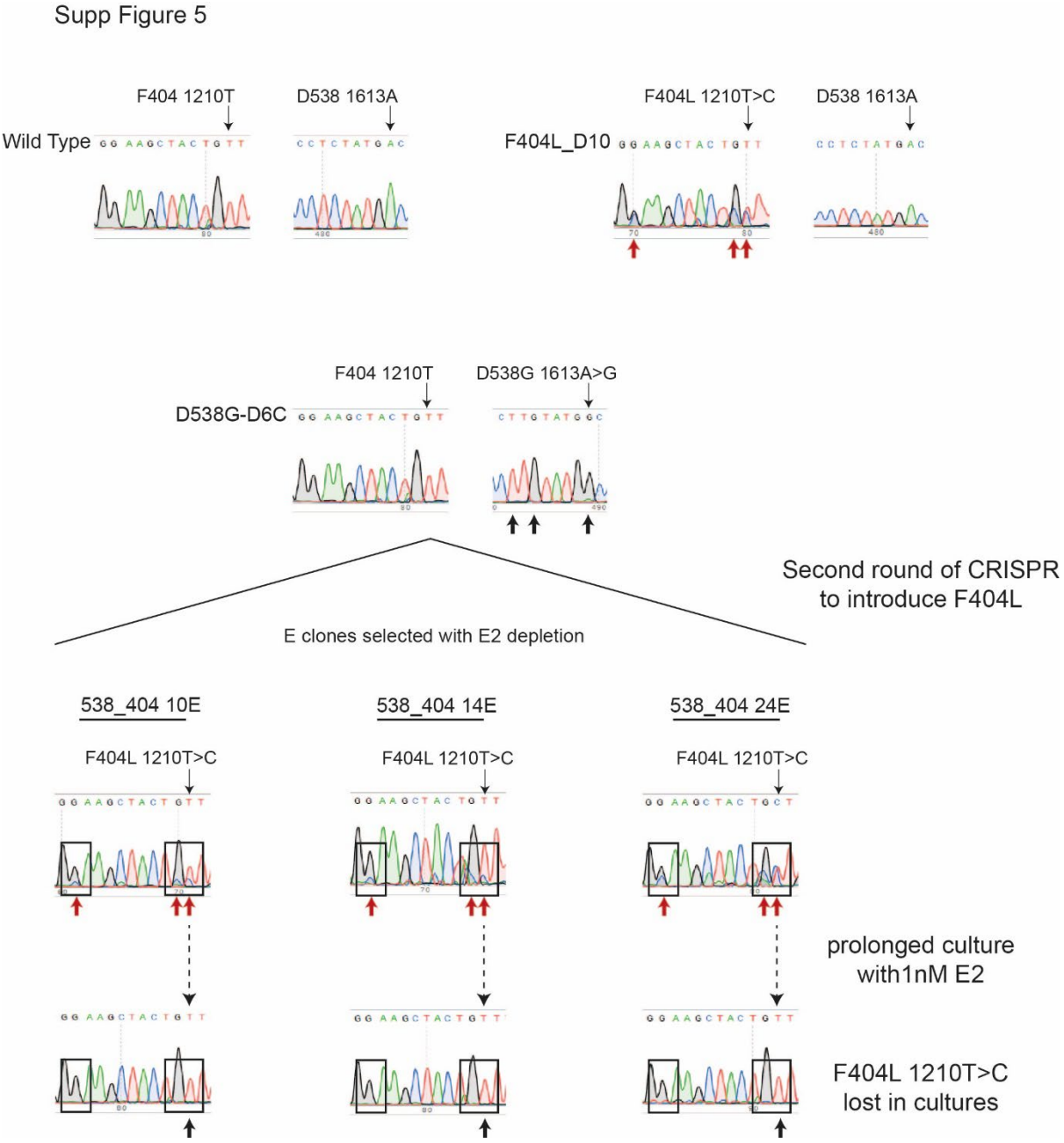
Supp Figure 4



**Supplementary Figure 4. Expression of individual ER target genes in RNAseq experiment**

A. Read counts for selected estrogen response genes ESR1, GREB1, PDZK1 and PGR for the ESR1 mutant cell line models maintained in 1nM estradiol. Box represents the 25% and 75% interquartile range and whiskers represents counts data range.

B. Read counts for selected estrogen response genes ESR1, GREB1, PDZK1 and PGR for the ESR1 mutant cell line models maintained in 1mM Fulvestrant for 24hr. Box represents the 25% and 75% interquartile range and whiskers represents counts data range.

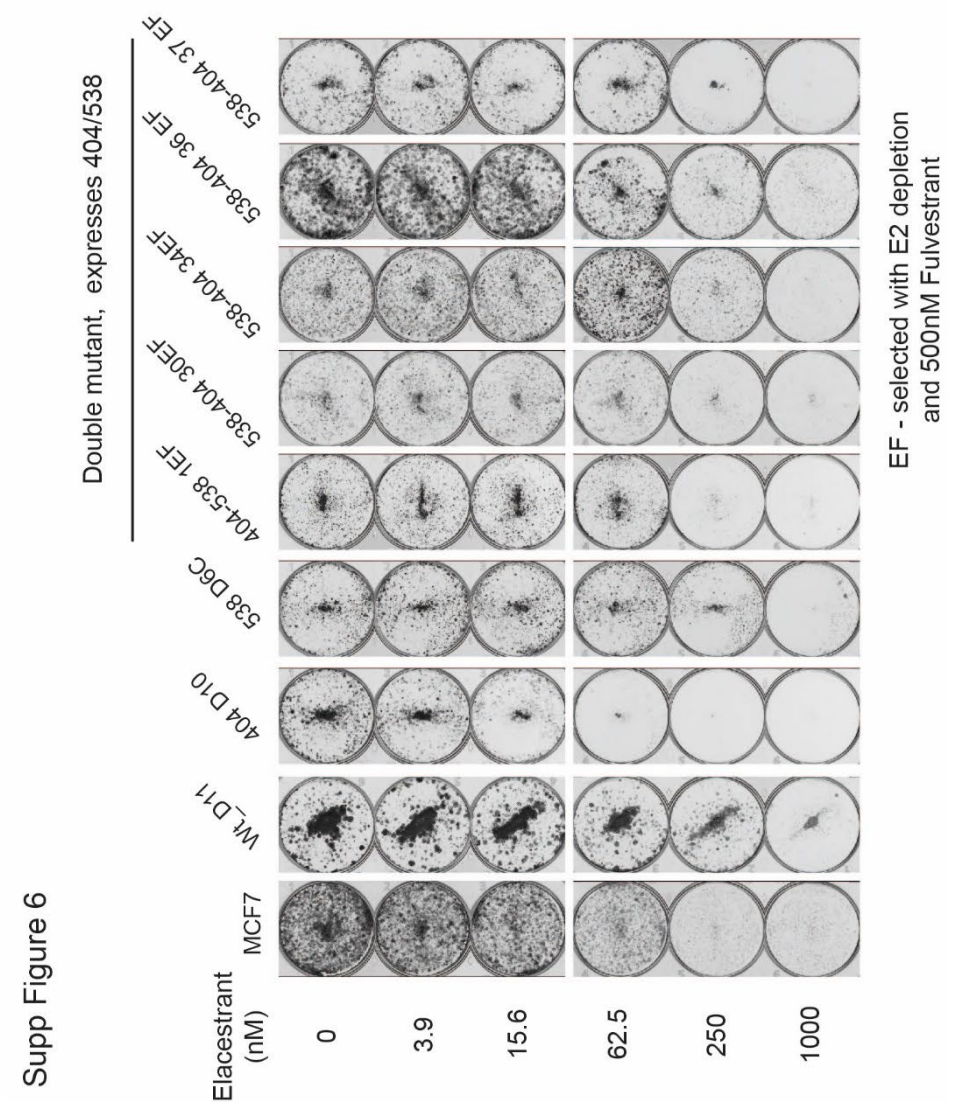


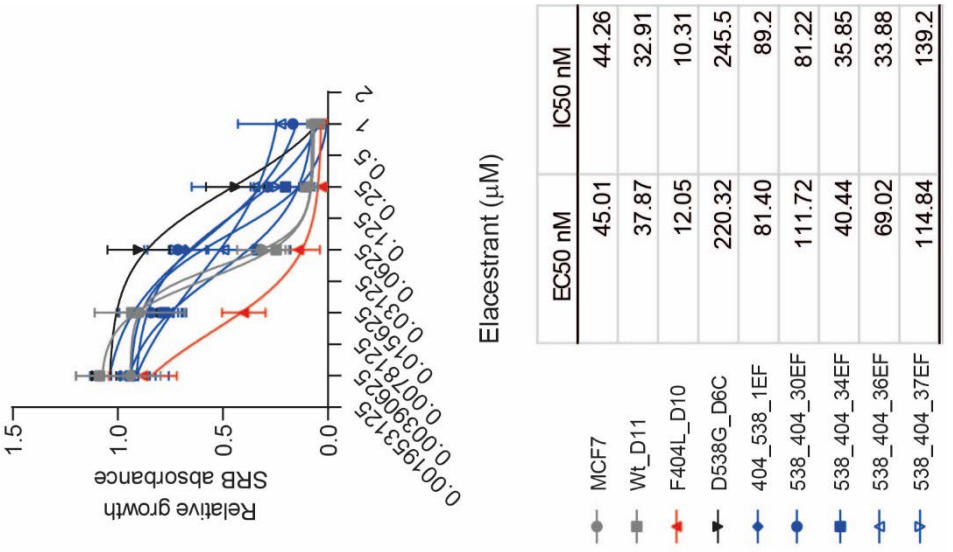

**Supplementary Figure 5. Loss of F404L mutation in long-term culture of "E" mutant clones.**

CRISPR clones of MCF7 cells expressing *ESR1* F404L (1210T>C, CRISPR edit indicated by red arrows) or D538G (1613A>G; CRISPR edit indicated by black arrows) were identified by RT-PCR followed by Sanger sequencing (left hand panels). Each cell line has three mutations, the mutation introduced, and two additional silent mutations that destroy the CRISPR PAM sequence to prevent re-editing. A second

round of CRISPR was used to introduce *ESR1* F404L (1210T>C) into a clone (D6C) that expressed D538G (1613A>G). Clones were selected in the absence of estradiol and expression of F404L confirmed by RT-PCR and Sanger sequencing. With prolonged culture F404L, and the PAM sequence changes, were observed to be lost from all three models.

Supp Figure 6

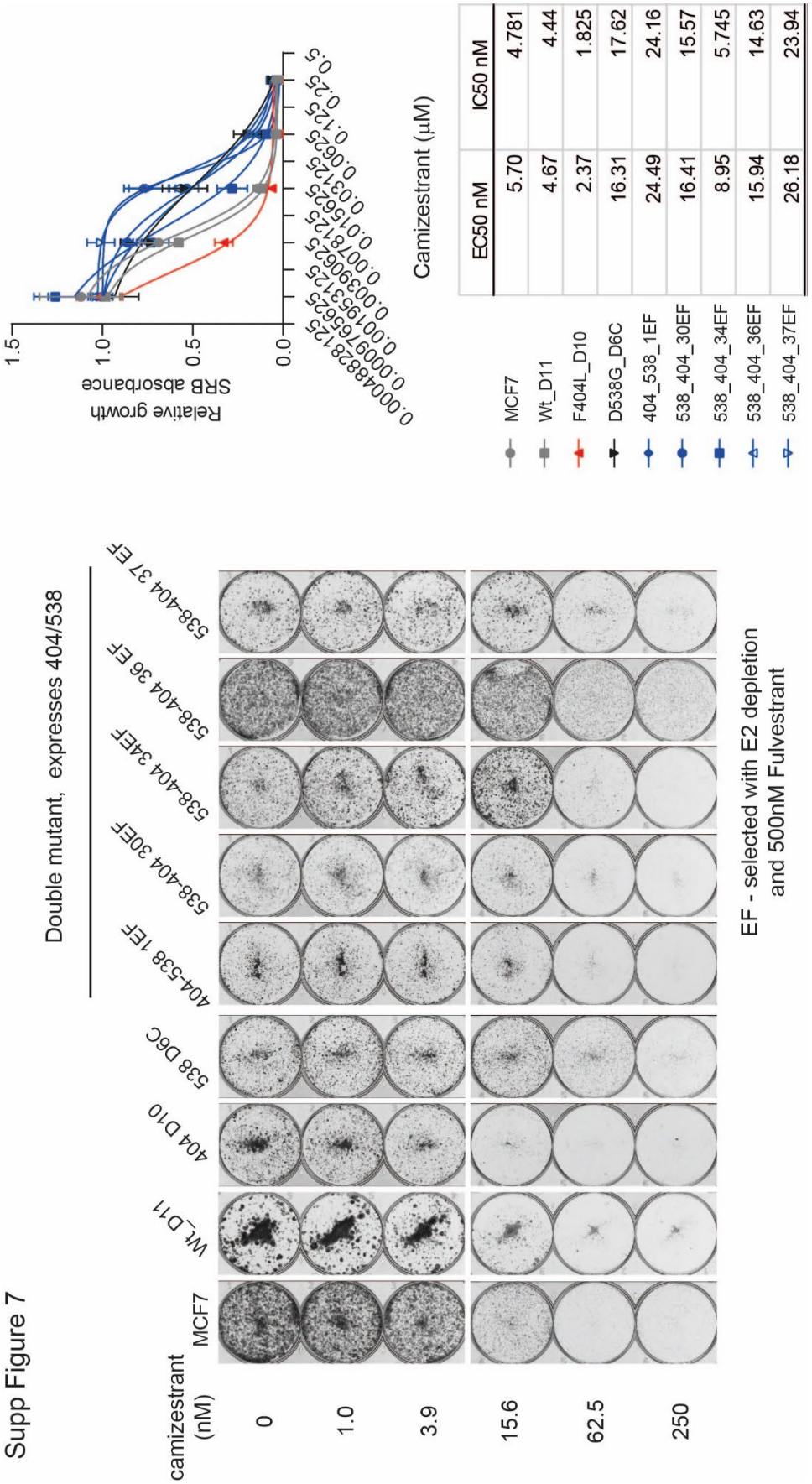



| | EC50 nM | IC50 nM |
|---|---|---|
| MCF7 | 45.01 | 44.26 |
| Wt_D11 | 37.87 | 32.91 |
| F404L_D10 | 12.05 | 10.31 |
| D538G_D6C | 220.32 | 245.5 |
| 404_538_1EF | 81.40 | 89.2 |
| 538_404_30EF | 111.72 | 81.22 |
| 538_404_34EF | 40.44 | 35.85 |
| 538_404_36EF | 69.02 | 33.88 |
| 538_404_37EF | 114.84 | 139.2 |

**Supplementary Figure 6. Response of D538G+F404L mutant models to elacestrant.**

*Lefthand* panel, clonongenic assays grown in indicated concentrations of elacestrant for 14 days. *Righthand* panel, quantification of colony formation assays for *ESR1* mutant models. SRB stained colonies were dissolved and absorbance at 565nm measured. EC50 and IC50 values were calculated from the response curves. Mean with sem, n=3 independent experiments.

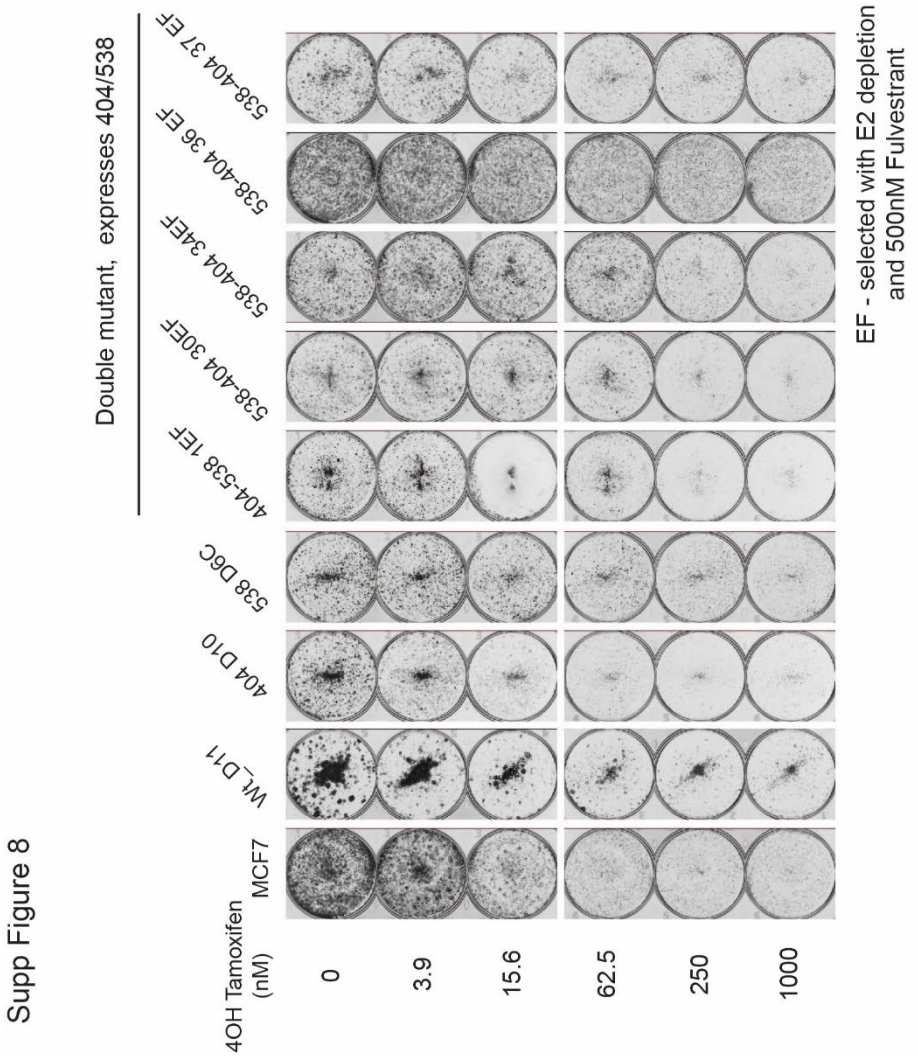


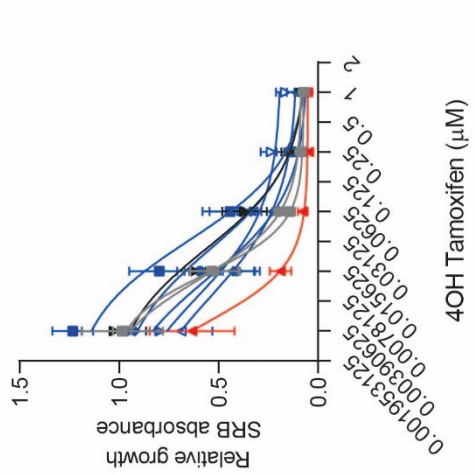

| | EC50 nM | IC50 nM |
|---|---|---|
| MCF7 | 5.70 | 4.781 |
| Wt_D11 | 4.67 | 4.44 |
| F404L_D10 | 2.37 | 1.825 |
| D538G_D6C | 16.31 | 17.62 |
| 404_538_1EF | 24.49 | 24.16 |
| 538_404_30EF | 16.41 | 15.57 |
| 538_404_34EF | 8.95 | 5.745 |
| 538_404_36EF | 15.94 | 14.63 |
| 538_404_37EF | 26.18 | 23.94 |

**Supplementary Figure 7. Response of D538G+F404L mutant models to camizestrant.**

*Lefthand* panel, clonongenic assays grown in indicated concentrations of camizestrant for 14 days. *Righthand* panel, quantification of colony formation assays for *ESR1* mutant models. SRB stained colonies were dissolved and absorbance at 565nm measured. EC50 and IC50 values were calculated from the response curves. Mean with sem, n=3 independent experiments.

Supp Figure 8



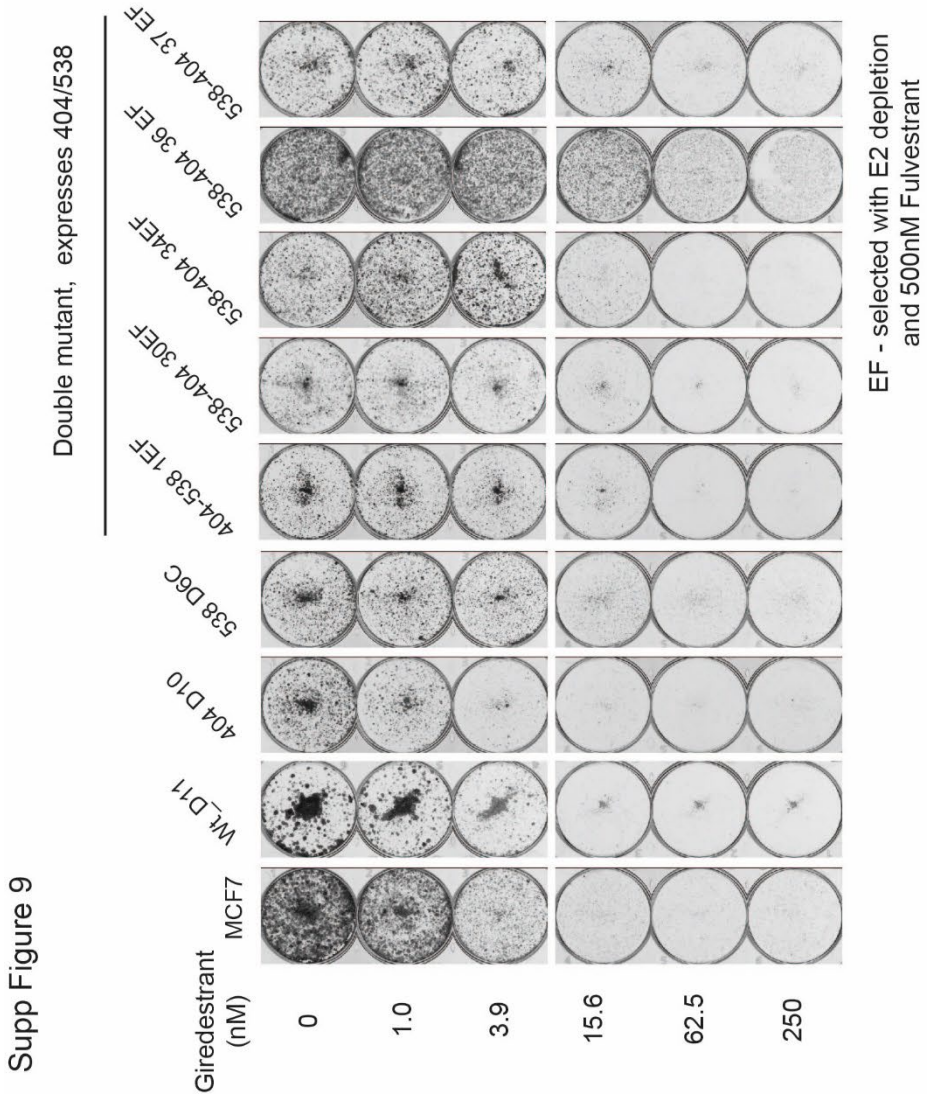



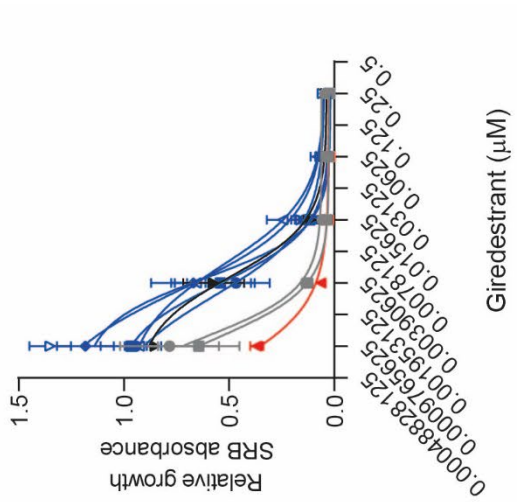

| | EC50 nM | IC50 nM |
|---|---|---|
| MCF7 | 17.24 | 13 |
| Wt_D11 | 17.04 | 14.89 |
| F404L_D10 | 5.56 | 4.925 |
| D538G_D6C | 33.50 | 26.95 |
| 404_538_1EF | 16.46 | 13.49 |
| 538_404_30EF | 31.22 | 26.33 |
| 538_404_34EF | 51.31 | 32.15 |
| 538_404_36EF | 10.75 | 8.579 |
| 538_404_37EF | 14.88 | 8.825 |

**Supplementary Figure 8. Response of D538G+F404L mutant models to 4OH tamoxifen.**

*Lefthand* panel, clonongenic assays grown in indicated concentrations of 4OH tamoxifen for 14 days. *Righthand* panel, quantification of colony formation assays for *ESR1* mutant models. SRB stained colonies were dissolved and absorbance at 565nm measured. EC50 and IC50 values were calculated from the response curves. Mean with sem, n=3 independent experiments.

Supp Figure 9



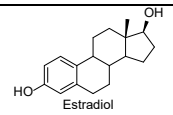
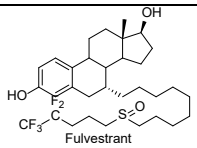
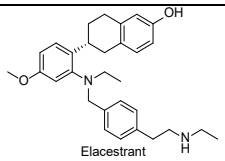
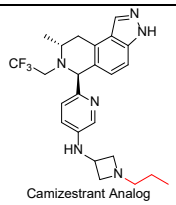
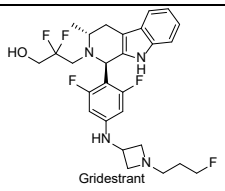




| | EC50 nM | IC50 nM |
|---|---|---|
| MCF7 | 1.49 | 1.186 |
| Wt_D11 | 1.32 | 1.194 |
| F404L_D10 | 0.64 | 0.5992 |
| D538G_D6C | 4.44 | 4.546 |
| 404_538_1EF | 5.33 | 4.388 |
| 538_404_30EF | 3.61 | 2.979 |
| 538_404_34EF | 4.28 | 3.892 |
| 538_404_36EF | 6.37 | 5.93 |
| 538_404_37EF | 5.84 | 3.35 |

**Supplementary Figure 9. Response of D538G+F404L mutant models to giredestrant.**

*Lefthand* panel, clonongenic assays grown in indicated concentrations of giredestrant for 14 days. *Righthand* panel, quantification of colony formation assays for *ESR1* mutant models. SRB stained colonies were dissolved and absorbance at 565nm measured. EC50 and IC50 values were calculated from the response curves. Mean with sem, n=3 independent experiments.

**Supplementary Table 1. Comparison of Potential Binding Energy and Distance of Pi-Pi Stacking Interaction with F404 and mutant modes.**

| PDB ID[a] | Ligand Structure | Binding Free Energy with ER F404 | Binding Free Energy with ER F404L | Binding Free Energy with ER F404I | Binding Free Energy with ER F404V |
|---|---|---|---|---|---|
| **3UUD** (Y537S) |  Estradiol | -75.31 kcal/mol | -68.09 kcal/mol | -65.90 kcal/mol | -66.28 kcal/mol |
| **Docking** (Y537S) |  Fulvestrant | -95.58 kcal/mol | -92.49 kcal/mol | -92.23 kcal/mol | -93.18 kcal/mol |
| **7TE7** (L536S) |  Elacestrant | -108.75 kcal/mol | ***-110.01 kcal/mol*** | -105.90 kcal/mol | -98.33 kcal/mol |
| **6ZOR** (L536S) |  Camizestrant Analog | -69.40 kcal/mol | ***-74.31 kcal/mol*** | ***-73.82 kcal/mol*** | ***-72.93 kcal/mol*** |
| **7MSA** (L372S /L536S) |  Gridestrant | -81.36 kcal/mol | ***-82.42 kcal/mol*** | ***-85.77 kcal/mol*** | -80.98 kcal/mol |

[a] Numbers in parentheses are the location of mutations elsewhere in the ER LBD needed to assist in crystallization.

**Supplementary Table 2. Clinicopathological features of PlasmaMATCH Cohort A.**

| | n=84 | |
|---|---|---|
| | n | % |
| **Age group (years) at registration** | | |
| <50 | 18 | 21.4 |
| 50-59 | 36 | 42.9 |
| 60-69 | 20 | 23.8 |
| >=70 | 10 | 11.9 |
| | | |
| **Metastatic disease present at diagnosis** | 18 | 21.4 |
| | | |
| **Time since primary diagnosis (years)** | | |
| <1 year | 2 | 2.4 |
| 1-3 years | 11 | 13.1 |
| 3-5 years | 17 | 20.2 |
| >=5 years | 54 | 64.3 |
| | | |
| **Tumour characteristics at initial diagnosis** | | |
| Pathological invasive tumor size (cm) | | |
| <=2cm | 18 | 21.4 |
| 2-5 cm | 29 | 34.5 |
| >5cm | 11 | 13.1 |
| Not known/Missing | 26 | 31.0 |
| | | |
| **Nodal status** | | |
| N0 | 18 | 21.4 |
| N1-3 | 24 | 28.6 |
| N4+ | 20 | 23.8 |
| Not known/Missing | 22 | 26.2 |
| | | |
| **Histological type** | | |
| Ductal | 63 | 75.0 |
| Lobular | 9 | 10.7 |
| Mixed ductal and lobular | 5 | 6.0 |
| Other invasive | 1 | 1.2 |
| DCIS | 1 | 1.2 |
| Not known/Missing | 5 | 6.0 |
| | | |
| **Tumor grade** | | |
| G1 | 7 | 8.3 |
| G2 | 37 | 44.0 |
| G3 | 28 | 33.3 |

| | | |
|---|---|---|
| Not known/Missing | 12 | 14.3 |
| | | |
| **Molecular subtype** | | |
| HR+, HER2- | 80 | 95.2 |
| HR+, HER2+ | 3 | 3.6 |
| HR+, HER2 Unknown | 1 | 1.2 |
| | | |
| **Disease sites** | | |
| Visceral | 78 | 92.9 |
| Soft tissue/nodal | 6 | 7.1 |
| | | |
| **Treatment received for locally advanced/metastatic disease prior to study registration** | | |
| | | |
| **Chemotherap**y | | |
| 0 lines | 29 | 34.5 |
| 1 line | 26 | 31.0 |
| 2 lines | 13 | 15.5 |
| >2 lines | 16 | 19.0 |
| | | |
| **Endocrine therapy** | | |
| 0 lines | 5 | 6.0 |
| 1 line | 34 | 40.5 |
| 2 lines | 36 | 42.9 |
| 3 lines | 8 | 9.5 |
| 4 lines | 1 | 1.2 |
| | | |
| **Total lines of treatment received (chemotherapy and endocrine therapy combined)** | | |
| 0 | 2 | 2.4 |
| 1 | 15 | 17.9 |
| 2 | 23 | 27.4 |
| 3 | 16 | 19.0 |
| 4 | 12 | 14.3 |
| 5 | 11 | 13.1 |
| >5 | 5 | 6.0 |
| | | |
| **Other systemic therapy** | | |
| Anti-HER2 therapy | 3 | 3.6 |
| mTOR inhibitor (everolimus, vistusertib) | 18 | 21.4 |
| CDK 4/6 inhibitor (palbociclib, ribociclib, abemaciclib) | 8 | 9.5 |
| Immunotherapy (atezolizumab, pembrolizumab) | 0 | 0.0 |
| Denosumab | 11 | 13.1 |
| Bisphosphonate | 5 | 6.0 |

## Supplementary Methods

### Computer modeling of SERDs pi-stacking with ER

Docking of Fulvestrant with Bazedoxifene-ERα complex (Y537S mutant, PDB ID: 6PSJ) in Schrodinger: Download PDB file of Bazedoxifene- ERα complex from Protein Data Bank (https://www.rcsb.org/), The protein was prepared with Schrodinger Protein Preparation Wizard module. Fulvestrant structure was prepared with Ligprep module. Grid of receptor (6PSJ Chain B) was generated with Receptor Grid Generation module with H-bond constraint between E353 and Hydroxyl of A-ring. Docking Fulvestrant in the receptor grid with H-bond constrain and standard precision. Posture of Fulvestrant-ERα (Y537S) complex was obtained.

Preparation of mutant modes: PDB files of Estradiol (PDB ID: 3UUD, Y537S), Elacestrant (PDB ID: 7TE7, L536S), Camizestrant analog (PDB ID: 6ZOR, L536S) and Giredestrant (PDB ID: 7MSA, L372S/L536S) were downloaded from Protein Data Bank, and prepared with Schrodinger Protein Preparation Wizard module. Chain A of 3UUD, Chain B of 7TE7, Chain B of 6ZOR and Chain C of 7MSA were selected based on the integrality of protein and ligands. Using Mutate Residues module in Schrodinger to convert F404 to F404L, F404I and F404V. Refinement of these mutant modes with Refine Protein-Ligand Complex module of Schrodinger within 7.0 Å distance from the ligands.

Calculation of Potential Binding Energy: Split refined complex with ligands and receptors. Calculating potential binding energy with MM-GBSA module of Schrodinger, using take complexes from separated ligand and protein option, and setup flexible residue distance from ligand as 6.0 Å. The potential binding energy (MMGBSA dG Bind) of complexes were obtained.

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