Acquired FGFR and FGF alterations confer resistance to estrogen receptor (ER) targeted therapy in ER+ metastatic breast cancer

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Abstract

Beyond acquired mutations in the estrogen receptor (ER), mechanisms of resistance to ER-directed therapies in ER+ breast cancer have not been clearly defined. We conducted a genome-scale functional screen spanning 10,135 genes to investigate genes whose overexpression confer resistance to selective estrogen receptor degraders. Pathway analysis of candidate resistance genes demonstrated that the FGFR, ERBB, insulin receptor, and MAPK pathways represented key modalities of resistance. In parallel, we performed whole exome sequencing in paired pre-treatment and post-resistance biopsies from 60 patients with ER+ metastatic breast cancer who had developed resistance to ER-targeted therapy. The FGFR pathway was altered via *FGFR1*, *FGFR2,* or *FGF3/FGF4* amplifications or *FGFR2* mutations in 24 (40%) of the post-resistance biopsies. In 12 of the 24 post-resistance tumors exhibiting FGFR/FGF alterations, these alterations were not detected in the corresponding pre-treatment tumors, suggesting that they were acquired or enriched under the selective pressure of ER-directed therapy. *In vitro* experiments in ER+ breast cancer cells confirmed that FGFR/FGF alterations led to fulvestrant resistance as well as cross-resistance to the CDK4/6 inhibitor palbociclib, through activation of the MAPK pathway. The resistance phenotypes were reversed by FGFR inhibitors and, to a lesser extent, MEK inhibitors, suggesting potential treatment strategies.

Significance

A genome-scale overexpression screen revealed a broad spectrum of resistance mechanisms against SERDs, which can provide a resource for researchers studying resistance to ER-directed therapies as well as the biology of estrogen receptor

dependencies in ER+ breast cancer. We demonstrate that activating FGFR/FGF alterations are a mechanism of acquired resistance to ER-directed therapies and CDK4/6 inhibitors in ER+ metastatic breast cancer and can be overcome by combination therapy targeting both the ER and the FGFR pathway. The detection of targetable, clonally acquired genetic alterations in metastatic tumor biopsies highlights the value of serial tumor testing to dissect mechanisms of resistance in human breast cancer and its potential application in directing clinical management.

Introduction

Approximately 70% of breast cancers express the estrogen receptor (ER), and estrogen signaling drives breast cancer cell growth and progression [1]. Endocrine therapies are commonly used to treat ER+ breast cancer and work by reducing estrogen levels or targeting the estrogen receptor through functional inhibition or degradation. Although these endocrine therapies, including tamoxifen, aromatase inhibitors (AI), and the selective estrogen receptor degrader (SERD) fulvestrant have improved survival for ER+ breast cancer patients, within the metastatic setting resistance to endocrine therapies is nearly universal and remains a key challenge in reducing breast cancer morbidity and mortality [2].

Although various resistance mechanisms have been proposed for tamoxifen and aromatase inhibitor resistance, including loss or modification in ER expression (*ESR1* activating mutations and *ESR1* fusions) [3-7], and regulation of alternative signal transduction pathways (PI3K/AKT/mTOR and EGFR/ERBB2/MAPK) [8-10], mechanisms of resistance to SERDs remain understudied. Mechanisms of endocrine resistance identified in patients include acquired mutations in the estrogen receptor itself [4-7], acquired activating mutations in *ERBB2* (HER2) [11, 12], loss of function of *NF1* [13], and other alterations in MAPK pathway genes [14]. Additional mechanisms remain to be identified.

Gain-of-function screens have played a pivotal role in identification of resistance mechanisms to targeted therapies in various cancer types [15-17]. In breast cancer,

101 several functional screen studies identified *IGF1R*, *KRAS* and *ESR1* as mechanisms of
102 resistance to tamoxifen and/or estrogen deprivation [18-20]. However, genome-scale
103 functional screens for SERD resistance have not been reported.
104
105 We conducted a genome-scale gain-of-function screen in ER+ breast cancer cells
106 spanning 17,255 overexpressed lentiviral open reading frames (ORFs) to investigate
107 genes whose overexpression was sufficient to confer resistance to the SERDs fulvestrant
108 and GDC-0810 [21]. In parallel, we sought to identify endocrine resistance mechanisms
109 of clinical significance through genomic profiling of paired pre-treatment and post-
110 treatment tumor samples from 60 patients with ER+ metastatic breast cancer who
111 developed resistance to endocrine therapy.
112
113 The intersection of top candidate resistance mechanisms from both approaches converged
114 on the fibroblast growth factor receptor (FGFR) pathway. Here, we demonstrate that
115 acquired FGFR/FGF alterations identified in patients with resistant metastatic breast
116 cancer cause resistance to a variety of ER-directed therapies as well as to CDK4/6
117 inhibitors, and that this can be overcome by combination therapy targeting both the ER
118 and the FGFR pathway.
119

Results

A Genome-Scale Gain-of-Function Screen for Resistance to Selective Estrogen Receptor Degraders

To identify the spectrum of genes whose overexpression confers resistance to SERDs *in vitro*, we expressed 17,255 human open reading frames (ORFs), corresponding to 10,135 distinct human genes, in ER+ T47D breast cancer cells in the presence of fulvestrant or GDC-0810. T47D cells were infected with the pooled lentiviral ORF library hORFeome [22]. Fulvestrant, GDC-0810, or vehicle control (DMSO) was added following infection and selection. ORF representation was assessed by sequencing after 21 days of drug exposure. Genes that confer drug resistance will be enriched under drug selection, indicated by a positive log fold change (LFC) for ORF representation before and after DMSO/drug selection.

Using a Z score >3 as a criterion to identify resistance candidates, we identified 64 genes (93 ORFs) that conferred resistance to fulvestrant and 57 genes (83 ORFs) that conferred resistance to GDC-0810 (Fig.1A and Supplemental Table.1). 37 genes (55 ORFs) conferred resistance to both drugs, a degree of overlap which was anticipated given the mechanistic similarities between fulvestrant and GDC-0810. The LFC and corresponding Z score for each ORF in fulvestrant and GDC-0810 treatment arms were highly correlated, with a correlation coefficient of 0.77 (Fig.1A).

To confirm these results, we conducted a secondary screen using a smaller pooled library consisting of 570 ORFs to validate candidates nominated by the primary screen. The

143 secondary screen was performed in both T47D and MCF7 cell lines with a similar screen
144 process as the primary screen. Top resistance genes found in the primary screen were
145 again enriched in the secondary screen, including *FGF* genes, *FOXR1*, *AKT* genes, *PIM*
146 genes and several *GPCR* genes (Supplemental Data Fig.S1). Many top ranked resistance
147 genes (*CSF1R, FGF3, FGF6, FOXR1* and *PIM2*) were shared between T47D and MCF7
148 cells (Supplemental Data Fig.S2). However, distinct resistance genes were also observed
149 in each cell line, suggesting some resistance mechanisms may be cell context-dependent.
150
151 Functional categories of candidate resistance genes include serine/threonine kinases
152 (*PIK3CA*, *AKT1/2/3*, *PIM1/2/3*), receptor tyrosine kinases (*EGFR*, *ERBB2, PDGFRB*),
153 growth factors (*FGF3/6/10/22*), cell cycle regulatory proteins (*CCND1*, *CCND2,*
154 *CCND3*, *CDK6*) and G-protein coupled receptors (*GPCR*) (Fig.1B). As further
155 validation, we overexpressed 13 ORFs belonging to these categories individually in
156 T47D cells and they all conferred resistance to fulvestrant (Fig.1C and Supplemental
157 Data Fig.S3A). Most of the 13 ORFs also conferred resistance to GDC-0810
158 (Supplemental Data Fig.S3B).
159
160 Gene Set Enrichment Analysis (GSEA) of the candidate resistance genes demonstrated
161 enrichment in 4 functional pathways: FGFR signaling, ERBB signaling, insulin receptor
162 signaling, and the MAPK pathway (Fig.1D, Supplemental Data Fig.S4 and Supplemental
163 Table.2). Consistent with this, we recently demonstrated that acquired *ERBB2* activating
164 mutations activate the MAPK pathway and cause endocrine resistance in patients with
165 ER+ metastatic breast cancer [11]. Several recent studies have also shown that alterations

in MAPK pathway genes are enriched in endocrine-resistant tumors [13, 14]. We sought to further examine the role of FGFR and FGF genes in resistance to SERDs in MBC.

Identification of acquired FGFR and FGF alterations in metastatic biopsies from patients with resistant ER+ MBC

To examine the potential role of the FGFR and FGF alterations in the development of endocrine resistance clinically, we analyzed whole exome sequencing (WES) data from paired pre-treatment and post-treatment metastatic tumor biopsies or cell free DNA from 60 patients with ER+ metastatic breast cancer who had received at least one endocrine therapy (tamoxifen, AI, SERDs) for more than 120 days between the two biopsies [23].

Amongst the 60 post-treatment samples, we found *FGFR1* amplifications in 15% (9/60), *FGFR2* amplifications in 5% (3/60), *FGFR2* activating mutations in 3.3% (2/60), and *FGF3/FGF4* amplifications (which are adjacent genes and therefore co-amplified) in 28.3% (17/60) – for a total of 40% (24/60) of the cohort with at least one alteration in one of these four genes (Fig.2A). Overall, the prevalence of *FGFR1*, *FGFR2*, and *FGF3/FGF4* alterations in the resistant metastatic setting seen here is increased compared to what was observed in previously published cohorts of primary ER+ breast cancer, such as The Cancer Genome Atlas (TCGA) [24] (Supplemental Table.3). The incidence of *FGFR2* alterations (6.7%), in particular, is markedly increased compared to primary treatment-naive breast cancer, in which the incidence is less than 2% in TCGA (Supplemental Table.3).

To determine if this enrichment of FGFR/FGF alterations in the metastatic setting was due to acquisition/selection under the selective pressure of endocrine therapy, we compared the WES from the paired pre-treatment and post-treamtent samples for the 24 patients that exhibited FGFR/FGF alterations in their post-treatment samples. These 24 pairs of samples included 23 tumor biopsies and one cell-free DNA sample at the pre-treatment timepoint, and 22 tumor biopsies, and two cell-free DNA samples at the post-treatment timepoint. We performed an evolutionary analysis to evaluate clonal structure and dynamics, including changes in mutations and copy number. The evolutionary inference and clonal dynamics of mutations was based on changes in the estimated fraction of tumor cells harboring each genomic alteration (the cancer cell fraction, CCF) as previously shown for acquired HER2 mutations [11]. The evolutionary inference of copy number changes was based on measuring differences in copy number amplitudes between pre-treatment and post-treatment samples, while accounting for differences in cancer cell fraction ("purity") in the sample and correcting for differences in ploidy. The resultant purity-corrected values provide an estimate of "copy number above ploidy" (CNAP) (see Methods).

For this analysis, we define "acquired" alterations as alterations with higher representation in the post-treatment sample as compared to the pre-treatment sample. For single nucleotide variants (SNVs), this means that the mutation had a substantially higher CCF in the post-treatment sample compared to the pre-treatment sample (including lack of detection in the pre-treatment sample despite having sufficient power to detect the mutation). For copy number changes, this means that there was a substantial increase in

212 the overall copy-number in the post-treatment sample, suggesting a clonal expansion of
213 the amplification. Although we use the term "acquired", we recognize that when the
214 mutation is not detected in the pre-treatment sample, we cannot distinguish between pre-
215 existing alteration that was selected for and clonally enriched versus *de novo* alterations
216 that developed during the treatment.
217
218 In 12 of the 24 patients with FGFR or FGF alterations (50%), the alterations were
219 acquired in the post-treatment sample as compared to the pre-treatment sample (Fig. 2A,
220 marked in red). Five out of nine *FGFR1* amplifications were acquired (55.6%), while all
221 four *FGFR2* alterations were acquired (100%), including one patient (Pt 0300350) with
222 acquisition of both an *FGFR2* mutation and amplification. *FGF3/FGF4* amplifications
223 were acquired in 4 of 17 tumors (23.5%), including one case in which an *FGFR1*
224 amplification was co-acquired. The concurrent acquisition may suggest that the
225 evolutionary selection of both the ligand and receptor provided additional fitness in this
226 tumor. Among the other 12 patients, the alterations in eight patients were shared in both
227 pre-treatment and post-treatment samples (Fig 2A, marked in black), and evolutionary
228 status of alterations in the remaining four patients was inconclusive (Fig 2A, marked in
229 grey). The increase in copy number (corrected for tumor purity and ploidy, Supplemental
230 Table.4) from pre-treatment to post-treatment for *FGFR1*, *FGFR2*, and the *FGF3/FGF4*
231 amplicon in all 12 patients is depicted in Fig.2B.
232
233 Two of the acquired alterations found in these 12 patients were SNVs in the *FGFR2*
234 gene: M538I (chr10:123258070C>T, GRCh37, also denoted as M537I, depending on the

235 isoform) and N550K (chr10:123258034A>T, GRCh37, also denoted as N549K,
236 depending on the isoform). N550K is the most common FGFR2 mutation in breast cancer
237 while M538I was previously identified in lung cancer but has not yet been characterized
238 in breast cancer [25]. Figure 2C illustrates the change in the estimated fraction of tumor
239 cells harboring each genomic alteration (CCF) from the pre-treatment biopsy to the
240 resistant biopsy. In both patients, the *FGFR2* mutations were either not detected in the
241 primary tumor, despite sufficient power to detect mutations at this locus (N550K in Pt
242 0300350) or detected by a single read, inferred in a small fraction (CCF of 2%) of the
243 pre-treatment tumor (M538I in Pt 0300348). In both patients the activating *FGFR2*
244 mutations in the post-treatment biopsies were clonally acquired (CCF of 100%). Pt
245 0300350 was also found to have an acquired *FGFR2* amplification while Pt 0300348 was
246 found to have gained low-level amplification in *FGFR2* post treatment (Fig.2B).
247
248 Notably, the acquired alterations in *FGFR1*, *FGFR2*, and *FGF3/FGF4* were largely
249 mutually exclusive with acquired *ESR1* mutations. *ESR1* mutations are the most common
250 mechanism described for acquired endocrine resistance [26]. Although the overall rate of
251 acquired *ESR1* mutation in this cohort is 22% (13/60), among the 12 cases of acquired
252 FGFR and FGF alterations, only one patient also has an acquired ESR1 mutation (Figure
253 2A).
254
255 *FGF3* and *FGF4* reside in genomic proximity to *FGF19* and *CCND1* and these four
256 genes are often co-amplified. However, here, in 3 out of the 4 cases with acquired
257 *FGF3/FGF4* amplification, *FGF3/FGF4* copies were gained without co-acquisition of

CCND1 amplification, suggesting that this acquisition can occur as an independent genomic event. Similarly, 2 out of the 4 cases with acquired *FGF3/FGF4* did not have co-acquisition of *FGF19* amplification. The relationship of acquisitions of *FGF3*, *FGF4*, *FGF19*, and *CCND1* are depicted in Supplemental Data Fig.S5 and further details about these amplicons are described in Supplemental Methods. Further exploration of the full genomic contexts and other concurrent genetic alterations for the 12 patients with acquired FGFR/FGF alterations are shown in Supplemental Data Fig.S6 and Supplemental Table.5-6.

Figure 3 depicts clinical vignettes for six of the patients with acquired *FGFR1*, *FGFR2*, and/or *FGF3/FGF4* alterations in their post-treatment biopsies. All patients were treated with ER-directed therapy before acquiring FGF or FGFR alterations, including tamoxifen (3 patients), AIs (6 patients), and fulvestrant (3 patients). Vignettes for the other six patients with acquired *FGFR1*, *FGFR2*, and/or *FGF3/FGF4* alterations in their post-treatment biopsies are shown in Supplemental Data Fig.S7. Detailed clinicopathological features and therapy details for all 12 patients are found in Supplemental Table.7.

In addition to these 12 patients in our cohort, we identified several additional patients with acquired *FGFR1* and *FGFR2* activating mutations following the development of resistance to endocrine therapy (Supplemental Data Fig.S8 and Supplemental Table.8). FM patient 1 acquired a clonal *FGFR1* N546K mutation (a known activating mutation paralogous to *FGFR2* N550K) following treatment with an AI. FM patient 2 acquired a subclonal *FGFR2* N550K mutation after treatment with tamoxifen, AI and fulvestrant.

281 FM patient 3 acquired a subclonal *FGFR2* K660N mutation, another activating mutation
282 in the kinase domain [27], after treatment with tamoxifen.
283
284 In summary, we observed acquired alterations in FGFR or FGF in 20% (12/60) of
285 patients with endocrine resistant ER+ MBC – comparable to the known frequency of
286 acquired mutations in *ESR1* – highlighting the important role of the FGFR pathway in
287 acquired resistance to ER-directed therapies.
288
289 ***Active FGFR signaling leads to resistance to SERDs through activation of the MAP***
290 ***kinase pathway***
291 To further investigate how FGFR/FGF genes may confer resistance to ER-directed
292 therapy, we treated T47D cells with FGF3, FGF6, FGF10 or FGF22 ligand. Each of these
293 ligands resulted in resistance to fulvestrant (Fig.4A). This effect was reversed by
294 PD173074, a pan-FGFR inhibitor (Fig.4A). The addition of FGF ligand enhanced
295 phosphorylation of ERK and AKT, which was reversed by PD173074 (Fig.4B). The
296 effect of FGF ligands on downstream effectors was enhanced when FGFR1 was
297 simultaneously overexpressed in T47D, which has relatively low expression of FGFR1
298 [28] (Supplemental Data Fig.S9). FGF3, FGF6, FGF10 and FGF22 also reduced
299 fulvestrant sensitivity in MCF7 cells (Supplemental Data Fig. S10 A-B). Similar results
300 have been shown previously for FGF2, which was reported to activate MEK-ERK to
301 drive fulvestrant resistance in ER+ breast cancer cells [29].
302

We next overexpressed FGFR1, FGFR2, or GFP in T47D cells through lentiviral transduction and examined the impact on susceptibility to SERDs. Overexpression of FGFR1 or FGFR2 alone did not affect sensitivity to fulvestrant or GDC-0810. However, with the addition of FGF2 ligand, both FGFR1 and FGFR2 rendered cells highly resistant to the two SERDs (Fig. 4C and Supplemental Data Fig. S11). In comparison, FGF2 ligand alone reduced sensitivity to SERDs in control cells expressing GFP to a much lesser extent than in the FGFR1 or FGFR2 expressing cells, suggesting the potent resistance phenotype requires both FGF ligand and receptor. This requirement for the presence of both FGF ligand and receptor for maximal resistance phenotype may also explain why only FGFs but not FGFR1 or FGFR2 scored in the resistance screen (Fig.1 A-B). The resistance phenotype resulting from FGFR1 and FGFR2 overexpression was completely reversed by the addition of PD173074 (Fig. 4C). Similar results were obtained in MCF7 cells (Supplemental Data Fig.S12 A-B).

FGFR1 and FGFR2 overexpression (in the presence of FGF2 ligand) induced more potent phosphorylation of AKT and ERK than the GFP control (Fig.4D). These results are consistent with previous findings that FGFR1 activation led to MAPK activation and fulvestrant resistance [28]. Activation of downstream effectors p-ERK and p-AKT by FGFR1/2 overexpression was reversed to baseline levels with PD173074 (Fig. 4D and Supplemental Data Fig. S12C). Examination of a larger number of kinases using kinase antibody arrays demonstrated that AKT, ERK and RSK (downstream effector of ERK) were the only kinases of those tested to exhibit increased phosphorylation following FGFR1/2 overexpression and FGF2 stimulation (Supplemental Data Fig.S13).

Collectively, these findings suggest that FGFR1 and FGFR2 cause SERD resistance through the activation of MAPK and/or PI3K/AKT pathways.

We examined the sensitivity of cells overexpressing FGFR1 or FGFR2 to several inhibitors of downstream effectors: the MEK inhibitor trametinib, the AKT inhibitor AZD5363, and the mTOR inhibitor everolimus. FGFR1 or FGFR2 overexpression in the presence of FGF2 led to hypersensitivity to trametinib (Supplemental Data Fig.S14A). In contrast, FGFR1 or FGFR2 overexpression in the presence of FGF2 reduced sensitivity to AKT and mTOR inhibitors (Supplemental Data Fig.S14A).

We attempted to reverse FGFR-induced resistance to fulvestrant by inhibiting the MAPK pathway. Treatment of FGFR1 overexpressing cells with trametinib partially resensitized cells to fulvestrant, and treatment of FGFR2 overexpressing cells with trametinib fully resensitized the cells to fulvestrant (Fig.4E-F). Treatment with the mTOR inhibitor everolimus also partially blocked resistance conferred by FGFR1 or FGFR2 overexpression (Supplemental Data Fig.S14B). We also performed long-term colony formation assays and observed similar results (Supplemental Data Fig.S14C).

Together, these results suggest that the MAPK pathway is the primary downstream effector of FGFR activation resulting in endocrine resistance. This is consistent with the pathway analysis of resistance genes in our initial overexpression screen, which demonstrated enrichment in MAPK pathway genes (Figure 1D), as well as our prior findings of MAPK activation through acquired HER2 mutations in endocrine

resistance—adding further support to the idea that the MAPK pathway may be a common node of endocrine resistance [11, 14].

FGFR activation confers cross-resistance to CDK4/6 inhibitors

Since the combination of endocrine therapy and CDK4/6 inhibitors is now a standard of care treatment for patients with ER+ metastatic breast cancer, we also examined the effect of FGFR signaling on sensitivity to the combination of fulvestrant and the CDK4/6 inhibitor palbociclib. In T47D cells, FGFR1 and FGFR2 overexpression in the presence of FGF2 also conferred resistance to combination treatment of fulvestrant and palbociclib (Fig. 5A). The resistance phenotype was again abrogated by PD173074 (Fig. 5A). Resistance to fulvestrant and palbociclib was also partially reversed by trametinib (Fig. 5B and Supplemental Data Fig. S14D), further providing the support for the role of MAPK pathway activation in FGFR-mediated drug resistance. The reversal of resistance by trametinib was accompanied by reduced ERK phosphorylation (Fig. 5C). Similar results were achieved in MCF7 cells, although everolimus was more effective than trametinib in reversing the resistance phenotype by FGFR1 or FGFR2 overexpression (Supplemental Data Fig.S15 and Fig.S16).

In the presence of fulvestrant and palbociclib, FGFR1 or FGFR2 overexpression was accompanied by increased p-Rb and CCND1 levels, both of which were partially reversed by trametinib (Fig.5C). CCND1 knockdown in cells overexpressing GFP, FGFR1 or FGFR2 impaired cell proliferation similarly across all three cell lines without affecting the IC50 of fulvestrant (Supplemental Data Fig.S17). This suggests that the

proliferation advantage provided by active FGFR signaling is partially dependent on CCND1. This is consistent with prior results suggesting that CCND1 was involved in FGF2-mediated drug resistance [29].

Clinical evidence also supports the finding that FGFR alterations can cause resistance to CDK4/6 inhibitors. Following the acquisition of *FGFR2* N550K (along with *FGFR2* amplification), Pt 0300350 did not respond to the combination of letrozole and palbociclib (Fig.3), suggesting that *FGFR2* alterations may lead to intrinsic resistance to the combination of endocrine therapy and CDK4/6 inhibitors. Another patient with an *FGFR2* N550K mutation (FM Patient 2) also did not respond to the combination of fulvestrant and palbociclib (Supplemental Data Fig.S8). Collectively, this suggests targeting the FGFR pathway may also be a viable strategy to overcome FGFR/FGF-mediated resistance to SERDs and CDK4/6 inhibitors.

FGFR2 mutations found in patients are activating and can be targeted with irreversible FGFR inhibitors

We identified 3 acquired mutations in the kinase domain of FGFR2 in patients who developed resistance to endocrine therapy. Two of these, FGFR2 N550K and K660N, are known activating FGFR2 mutations that have been previously identified in breast cancer [25, 27]. FGFR2 N550K is part of the molecular brake at the kinase hinge region, which allows the receptor to adopt an active conformation more easily (Fig.6A). N550K is a recurring hotspot mutation reported to confer resistance to several FGFR inhibitors including PD173074 and dovitinib [30]. FGFR2 K660N is located in a conserved region

in the tyrosine kinase domain and has been confirmed to increase kinase activity [27, 30]. The third mutation, FGFR2 M538I, has not been previously reported in breast cancer. Based on its location, M538I appears to stabilize the active kinase confirmation by strengthening the hydrophobic spine of the FGFR2 kinase [30] (Fig.6A).

We expressed all three FGFR2 kinase domain mutants in T47D cells through lentiviral transduction, as well as wildtype (WT) FGFR2 and GFP as negative controls. All three mutants elicited higher kinase activity than WT FGFR2 constitutively, demonstrated by levels of p-FRS2, a direct substrate for FGFR2 (Fig.6B). MAPK and AKT signaling were also increased, as indicated by increased p-ERK and p-AKT levels, respectively (Fig.6B). The addition of FGF2 ligand further enhanced downstream signaling for all FGFR2 mutants, and the enhanced signaling was blocked by PD173074 for FGFR2 M538I and K660N, but not for N550K (Fig.6B).

FGFR2 mutants were also expressed under a tetracycline responsive promoter in T47D cells grown in low doses of doxycycline to determine the functionality at lower expression levels. At lower levels of expression, FGFR2 N550K still led to increased levels of p-ERK and p-AKT, independent of FGF2 ligand stimulation. FGFR2 M538I and K660N also resulted in higher p-ERK and p-AKT levels in the presence of FGF2 as compared to FGFR2 WT (Fig.6C). FGFR2 mutants rendered cells more sensitive to trametinib than did GFP or WT FGFR2 (Fig.6D), further supporting the finding that FGFR signaling requires the MAPK pathway in this context. Taken together, these results indicate that all three FGFR2 mutations acquired in breast cancer patients are functionally

418 active – FGFR2 N550K is constitutively active while FGFR2 M538I and K660N may be
419 more ligand-dependent at low levels of expression.
420
421 All 3 FGFR2 mutants led to modest resistance to fulvestrant (Fig.6E), which was
422 enhanced in the presence of FGF2 ligand. PD173074 resensitized cells overexpressing
423 FGFR2 M538I and FGFR2 K660N as well as WT FGFR to fulvestrant, but not cells
424 overexpressing FGFR2 N550K (Fig.6E). Consistent results were observed when cells
425 were treated with the combination of fulvestrant and palbociclib (Fig.6F). Similar results
426 were obtained in MCF7 cells (Supplemental Data Fig.S18).
427
428 FGFR2 M538I and N550K have been shown to confer resistance to multi-kinase inhibitor
429 dovitinib in BaF3 cells, and N550K is also resistant to PD173074 [30]. Because of the
430 differential responses of these FGFR2 mutants to PD173074, we tested the ability of
431 additional FGFR inhibitors to resensitize cells expressing these mutants to fulvestrant.
432 FIIN-2 and FIIN-3 are two irreversible covalent pan-FGFR inhibitors that target a
433 cysteine conserved in FGFR1-4 and have exquisite selectivity for some FGFR2 mutations
434 including M538I and K659N [31]. In addition, FGFR2 N550K was previously shown to
435 respond to the selective FGFR inhibitor AZD4547 [32].
436
437 Both FIIN-2 and FIIN-3 were more effective in inhibiting the downstream signaling (p-
438 FRS2, p-ERK and p-AKT) induced by FGFR2 N550K as compared to PD173074 and
439 AZD4547 (Fig.7A). Furthermore, FIIN compounds reduced the level of downstream
440 effectors back to baseline levels, with FIIN-3 being more potent than FIIN-2

441 (Supplemental Data Fig.S19). T47D cells stably overexpressing FGFR2 mutant were
442 exquisitely sensitive to FIIN-2 and FIIN-3 as compared to cells expressing GFP or
443 FGFR2 WT (Fig.7B). The resistance to fulvestrant induced by both WT FGFR2 and all
444 mutant FGFR2 was completely blocked by FIIN-2 (1 μM) and FIIN-3 (100 nM)
445 (Fig.7C).
446
447 While resistance to WT FGFR1/2 and FGFR2 M538I and FGFR2 K660N can be
448 reversed by multiple FGFR inhibitors, for some mutants like FGFR2 N550K, only the
449 irreversible pan-FGFR inhibitors successfully resensitized cells to fulvestrant,
450 highlighting the fact that specific resistance mutations might require different strategies
451 to overcome or preempt endocrine resistance.
452
453 **Discussion**
454 In this study, we used a genome-scale gain-of-function screen to identify potential
455 mechanisms of resistance to selective estrogen receptor degraders. We nominated several
456 different candidate resistance genes and pathways, particularly genes in the ERBB
457 pathway, FGFR pathway, insulin receptor signaling and the MAPK pathway. Consistent
458 with this finding, genomic profiling of paired pre-treatment and post-treatment tumor
459 samples from 60 patients with ER+ metastatic breast cancer who developed resistance to
460 endocrine therapy identified acquired alterations in *FGFR1*, *FGFR2*, and *FGF3/FGF4* in
461 20% of patients. Experimental studies confirmed that these alterations confer resistance
462 to endocrine therapy as well as CDK4/6 inhibitors, through activation of MAPK pathway,
463 and demonstrated that this resistance can be reversed by FGFR inhibitors. Taken

464 together, our results suggest that activating FGFR pathway alterations are a distinct
465 mechanism of acquired resistance to multiple forms of ER-directed therapy in MBC that
466 can be overcome by FGFR inhibitors.
467
468 Our genome-scale screen provided a comprehensive view into the resistance mechanisms
469 to SERDs. Similar resistance genes were nominated for fulvestrant and GDC-0810,
470 thereby confirming the two drugs have similar mechanism of action. Of note, two ESR1
471 ORFs conferred resistance specifically to GDC-0810 but not fulvestrant, possibly due to
472 GDC-0810 having a less potent effect on ER degradation than fulvestrant [21]. Among
473 the resistance mechanisms shared by fulvestrant and GDC-0810, many are frequently
474 altered in ER+ MBC, such as *CCNDs/CDK6*, *KRAS/MAPK*, *EGFR/ERBB2* and
475 *PIK3CA/AKTs/PIMs*, and agents targeting those alterations are under clinical
476 development to be combined with endocrine therapy [14, 33]. We also identified
477 potential resistance mechanisms that are not characterized to the same extent, such as G
478 protein-coupled receptors, Wnt pathway (*FZD10, RSPO1, RSPO3*) and Src family
479 kinases (*YES1, FYN, FGR*), providing clues as to the potential crosstalk between these
480 pathways and ER signaling [34-36] and suggesting that breast cancer patients harboring
481 functional alterations in these pathways may develop resistance to SERDs. Notably, some
482 of the resistance genes were also nominated in gain-of-function screens designed to
483 identify resistance mechanisms for MAPK pathway inhibitors in melanoma (*AXL, CRK,*
484 *CRKL, FGR, GPCR* genes) [15] and PI3K inhibitors in ER+ breast cancer (*AKT1, AKT2,*
485 *CRKL, FGF3, FGF10, PIM* genes) [17]. This may reveal multi-drug resistance
486 mechanisms and thus guide clinical drug combinations to overcome resistance. We have

provided the full genome-scale screen data as a resource to the community of researchers interested in resistance to ER-directed therapies as well as the biology of estrogen receptor dependencies in ER+ breast cancer.

Our ultimate goal is to identify resistance mechanisms that are clinically relevant and can be therapeutically targeted. By comparing paired pre-treatment and post-treatment tumors, our evolutionary analyses identified acquired *FGFR1* and *FGFR2*, and *FGF3/FGF4* alterations in 12 out of 60 post-treatment samples, further highlighting a potential role for the FGFR pathway in driving drug resistance and disease progression. Most notably, all four alterations in FGFR2 in our cohort were found to be acquired after the development of resistance to endocrine therapy. Our overall findings are consistent with two recent cohort studies which noted some patients with acquired *FGFR1* and *FGFR2* alterations following treatment of endocrine therapy [37, 38], and provides a mechanistic explanation for these acquisitions.

This analysis was enabled by a novel method we developed to compare the magnitude of amplification in matched pre- and post-treatment samples while considering key confounders to allow for more reliable assignment of copy gain or loss. Since matched tumor samples of the same patient are highly variable in the cancer cell fraction (purity) and often variable in ploidy (with genome duplication taking place in the metastatic setting), we computed the purity-corrected copy number above ploidy and set a relatively stringent threshold of changes in CNAP to define acquired amplification (See Methods), as cancer clones bearing amplifications with high focality and magnitude in FGFR/FGF

genes are more likely to induce dependency on FGFR pathway and result in endocrine resistance.

Our genomic analysis has some limitations and caveats. The observed alterations may not exclusively result from endocrine therapy as some patients received other therapies between the two collected biopsies. Moreover, tumors with FGFR/FGF alterations also harbor alterations in other cancer genes, which may contribute to drug resistance as well (Supplemental Data Fig.S6 and Supplemental Table.5). Despite these caveats, with the evidence from unbiased screens, genomic evidence in relevant patient samples, and confirmatory experimental models, the FGFR pathway clearly emerges as a clinically important resistance mechanism for SERDs and CDK4/6 inhibitors.

Strategies to target the FGFR pathway in breast cancer patients with FGFR alterations are currently being assessed in clinical trials. FGFR inhibitors currently under clinical development in breast cancer include non-selective tyrosine kinase inhibitors (dovitinib and lucitanib), FGFR1-3 selective inhibitors (AZD4547 and BGJ398), and others, although clinical trials have achieved mixed results to date [39-44]. The combination of FGFR inhibitors and endocrine therapy is also being clinically investigated. For example, the combination of dovitinib and fulvestrant showed promising clinical activity [39]. As FGFR pathway activation also results in resistance to CDK4/6 inhibitors, a triple combination with the addition of CDK4/6 inhibitors may also be considered. One challenge for the use of FGFR inhibitors is to identify reliable biomarkers. Our results suggest focal and high level amplifications, clonal activating mutations or high

533 expression levels of FGFR and FGF genes, particularly in the metastatic setting, may be
534 used to guide the clinical use of FGFR inhibitors. Activating alterations in *FGFR2*, which
535 are rare in primary treatment naïve breast cancer but appear to be clonally acquired in a
536 subset of patients with resistant ER+ MBC, may be a particularly good biomarker for the
537 development of FGFR inhibitors.
538
539 Our work also highlights that the effective clinical use of FGFR inhibitors needs to
540 consider the variable drug sensitivity of different FGFR2 mutations, which were acquired
541 in some patients following endocrine therapy. The two irreversible pan-FGFR kinase
542 inhibitors, FIIN-2 and FIIN-3, had superior efficacy in targeting all FGFR2 mutants
543 including N550K when compared to other FGFR inhibitors, although *in vivo* efficacy,
544 off-target effects and toxicity of FIIN compounds still warrant further investigation.
545
546 Alterations in FGFR1 and FGFR2 activated the MAPK pathway, and MEK inhibition
547 was able to overcome the resistance conferred by FGFR pathway to some degree. We
548 previously demonstrated acquired ERBB2 mutations resulted in elevated MAPK
549 transcriptional signature [11]. Furthermore, increased frequency of alterations in MAPK
550 pathway genes was found in tumors post hormonal therapy, including *EGFR, ERBB2* and
551 *NF1* [14]. The fact that multiple mechanisms of resistance to ER-directed therapies
552 and/or CDK4/6 inhibitors activate the MAPK pathway suggests that this may be an
553 important node of resistance in ER+ MBC. Thus, combining endocrine therapy and
554 CDK4/6 inhibitors with agents that target MAPK pathway, such as MEK inhibitors
555 and/or SHP2 inhibitors [45, 46], may be a unifying strategy to overcome or prevent

556 resistance resulting from multiple genetic aberrations that lead to resistance in ER+
557 MBC.
558
559 In summary, the integration of a functional genomic screen and genomic analysis of pre-
560 and post-treatment biopsies revealed the FGFR pathway as an important resistance
561 mechanism for endocrine therapy and CDK4/6 inhibitors in ER+ breast cancer. With the
562 increasing use of SERDs and CDK4/6 inhibitors in the clinic, we anticipate that the
563 prevalence of FGFR/FGF alterations might increase in the future. Targeting the FGFR
564 pathway with FGFR inhibitors or agents that target downstream MAPK signaling may
565 improve clinical outcomes in patients with aberrations in FGFR/FGF genes. Furthermore,
566 our study highlights the need to sequence metastatic biopsy or blood biopsies at the time
567 of resistance to identify patients with these alterations who may benefit from targeting the
568 FGFR pathway.
569
570 **Methods**
571 ***Cell culture***
572 293T, T47D and MCF7 cells were purchased from American Type Culture Collection
573 (ATCC) and were cultured as described in the Supplemental Methods.
574
575 ***Genome-Scale Gain-of-Function Screen***
576 The pooled lentiviral ORF library hORFeome [22] consists of 17,255 barcoded human
577 open reading frames (ORFs), corresponding to 10,135 distinct human genes with at least
578 99% nucleotide and protein match. These ORFs were cloned into pLX317 vector and

579 pooled together for transfection into 293T cells to make pooled lentivirus (with 2nd
580 generation packaging plasmids). In 6-well plates, pooled lentivirus was infected in cells
581 to achieve ~50% infection rate and ensure ~1000 infected cells per ORF for 17255.
582 Media was supplemented with 4 μg/mL polybrene (Thermo Fisher Scientific #
583 TR1003G) to boost transfection efficiency. After infection, cells were pooled and
584 selected with 1.5 μg/mL puromycin for 5 days. Upon completion of selection, cells were
585 plated for three different drug conditions: DMSO, 100 nM fulvestrant, 1 μM GDC-0810.
586 There were three replicates for each condition screened. A subset of cells was saved for
587 sequencing as early time point (ETP) samples to confirm ORF representation. The dose
588 for each drug was chosen for the two drugs to achieve potent anti-proliferation effect that
589 could be rescued with ESR1 mutant Y537N and Y537S. Infected cells were passaged
590 upon confluency and maintained in DMSO or drugs for 21 days to allow sufficient time
591 for cells carrying resistance to be enriched from the population. At the end of the time
592 course, cells were harvested for isolating genomic DNA as late time point samples (LTP).
593 All genomic DNA samples were amplified with PCR primers flanking the ORF region
594 and sequenced. The ORF representation at the final harvesting (LTP) is compared to the
595 representation of ORFs in cells collected before drug addition (ETP). Cells carrying
596 ORFs that are driving resistance will grow and gradually enrich the population and
597 therefore, will be over-represented in the sequencing data for the final passage compared
598 to the early time point. An ORF with significant enrichment (a Z score >3) is defined as a
599 resistance candidate gene. A secondary validation screen was performed as described in
600 the Supplemental Methods.
601

Patients and Tumor Samples

Prior to any study procedures, all patients provided written informed consent for research biopsies and whole exome sequencing of tumor and normal DNA, as approved by the Dana-Farber/Harvard Cancer Center Institutional Review Board (DF/HCC Protocol 05-246). Metastatic core biopsies were obtained from patients and samples were immediately snap frozen in OCT and stored in -80°C. Archival FFPE blocks of primary tumor samples were also obtained. A blood sample was obtained during the course of treatment, and whole blood was stored at -80°C until DNA extraction from peripheral blood mononuclear cells (for germline DNA) was performed. In a few instances, cell free DNA was obtained from plasma for circulating tumor DNA analysis, as previously described[47].

WES and data analysis

DNA was extracted from primary tumors, metastatic tumors, plasma, and peripheral blood mononuclear cells (for germline DNA) from all patients and whole exome sequencing was performed, as detailed in the Supplemental Methods. Sequencing data were analyzed using tools to identify somatic point mutations and small insertions/deletions (indels), and copy number changes using established algorithms (see Supplemental Methods).

To better measure segment-specific copy-number, we subtracted the genome ploidy for each sample to compute copy number above ploidy (CNAP). CNAP of at least 3 are considered as amplifications (AMP), CNAP below 3 are considered low amplification

and ignored in our analysis). CNAP of at least 6 are considered high amplifications (HighAMP), and CNAP of at least 9 and fewer than 100 genes [48] is considered very high focal amplification (FocalAMP).

The evolutionary classification of amplifications accounts for the magnitude of the observed copy-number difference between the pre-treatment and the post-treatment samples. If the difference between the CNAP of the post-treatment and the CNAP of the pre-treatment is smaller than 50%, the amplification is defined as "Shared". If the CNAP of the post-treatment is larger than the CNAP by more than 50% and the lower pre-treatment CNAP is not at "FocalAMP" level, the evolutionary classification is "Acquired". If CNAP of the post-treatment is smaller by at least 50%, comparing to the pre-treatment sample and the lower post-treatment CNAP is not at "FocalAMP" level, the evolutionary classification is "Loss". Otherwise, the evolutionary classification of amplifications is defined as "Indeterminate".

Generation of plasmids and engineered cells

T47D or MCF7 cells were infected with lentivirus to derive stable cell lines overexpressing wildtype (WT) or mutant ORFs. All WT ORFs were obtained from the Broad Institute. Mutant ORFs (FGFR2 M538I, N550K and K660N) were made using QuickChange II site-directed mutagenesis kit (Agilent Technologies #200523). Most stable cells lines express ORFs in pLX317 vector and were selected with puromycin (Life Technologies #A1113803). Stable cell lines expressing CCND1 and PIM1 in pLX304 vector were selected with blasticidin (Life Technologies #A1113903).

For inducible cell lines, WT or Mutant FGFR2 IIIb ORFs were cloned into pInducer20 Tetracycline-inducible lentiviral vector (Addgene #44012) using gateway cloning technology (Invitrogen #11791019). Lentivirus was then generated and infected to establish Tet-inducible cells lines cultured in 10% Tet-system approved FBS (Clontech #631106) and 500 µg/mL G418 (Life Technologies #10131035). Doxycycline (Clontech #631311) was used to induce ORF expression.

Kill curves and CellTiter-Glo viability assay

Cells were plated in 96-well tissue culture ViewPlate (Perkin Elmer # 6005181) on Day 1 and treated with drug on Day 2. Media with or without drugs was refreshed on Day 5. On Day 8, cells were equilibrated to room temperature, media was removed, and cells were lysed in a mixture of 50 µL media and 50 µL CellTiter-Glo 2.0 reagent (Promega # G9243) per well. Plates were then incubated on an orbital shaker for 2 mins. Following another 10 mins of incubation at room temperature to stabilize signal, luminescence was recorded to measure cell viability on Infinite M200 Pro microplate reader (Tecan).

Colony formation assay

2,000-30,000 cells were plated in 6-well plates on Day 1 and treated on Day 2. Media was refreshed every 3-4 days until crystal violet staining. On the day of staining, cells were fixed with ice-cold 100% methanol for 10 minutes and then incubated with 0.5% crystal violet solution (Sigma Aldrich #C6158) in 25% methanol at room temperature for 10 minutes.

Western blotting

Western blotting was performed as described in supplemental methods.

Statistical analysis

Statistical analyses related to drug response curve were performed with student t-test in Graphpad Prism.

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701

702 **Author contributions**

703 P.M., O.C. and N.W. conceived and designed the study; P.M. K.K. and J.K. performed
704 experiments; O.C. and J.B. performed the computational analyses; P.E., S.A.W., A.G.W.,
705 and N.W. performed clinical data abstraction and annotation; J.C., V.M., and M.C.
706 provided the clinical and genomic data for the 3 patients in the Foundation Medicine
707 cohort; F.P and D.R. helped with the design and execution of the ORF screen; E.P.W.,
708 N.U.L., and N.W. oversaw patient enrollment and sample collection on the metastatic
709 biopsy protocol; P.M., O.C. and N.W. wrote the manuscript with input from all authors;
710 N.W. supervised the study.

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Figure 1

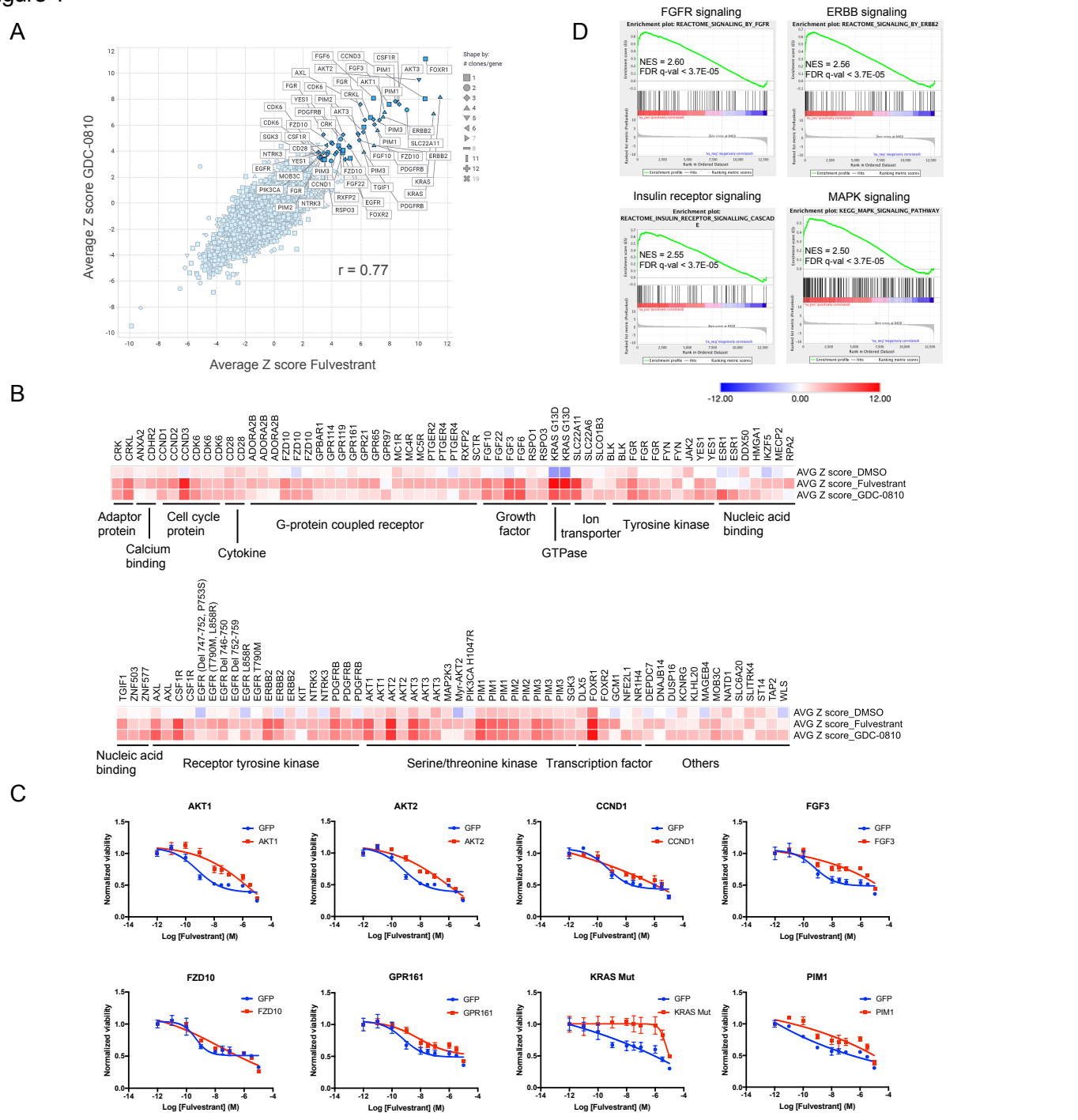


Figure 1. A genome-scale gain-of-function screen identified resistance genes to fulvestrant and GDC-0810. 17,255 human open reading frames (ORFs), corresponding to 10,135 distinct genes, were expressed in ER+ T47D breast cancer cells in the presence of fulvestrant or GDC-0810. Fulvestrant, GDC-0810, or vehicle control (DMSO) was added following infection and selection. ORF representation was assessed by sequencing after 21 days of drug exposure. Genes that confer drug resistance will be enriched under drug selection, indicated by a positive log fold change (LFC) for ORF representation before and after DMSO/drug selection. A, The average Z score for LFC of each ORF was plotted for both the fulvestrant (X-axis) and GDC-0810 (Y-axis) arms. The average Z score was calculated from three replicates in each condition. The ORFs with a Z score > 3 in both drug arms are highlighted and labeled with gene ID. The shape of each data point represents the total number of ORFs for that gene in the library. B, Heatmap of top ORF hits with a Z score > 3 in fulvestrant or GDC-0810 arm. The Z score in the DMSO arm is also presented. ORF hits are grouped by their molecular function according to Uniprot annotation. Information on the complete list of ORFs can be found in Supplemental Table.1. C, Individual ORFs were overexpressed in T47D cells and validated to confer resistance to fulvestrant by drug response curves. *KRAS* G12D ORF was used for overexpression in T47D cells while other selected ORFs are wildtype. Cell viability was measured by CellTiter-Glo and all the data points were normalized to growth under DMSO condition. D, Gene set enrichment analysis was performed for the gene list ranked by LFC in the fulvestrant arm. For genes with multiple ORFs, the ORF with highest LFC was selected. 1000 permutations were performed for the analysis. NES, normalized enrichment score. The full list of nominated pathways is shown in Supplemental Table.2.

Figure 2

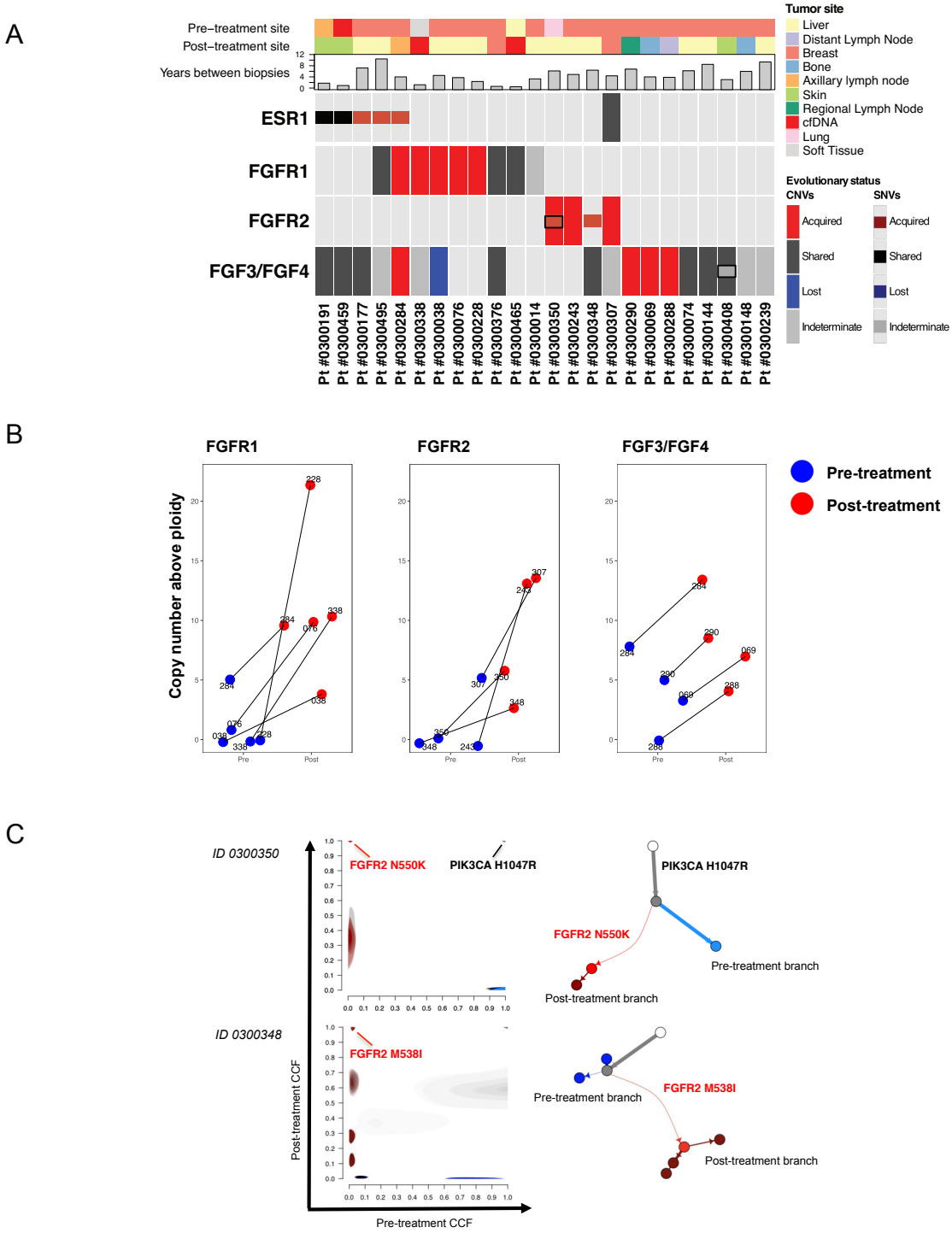


Figure 2. Identification of acquired FGFR and FGF alterations in metastatic biopsies from patients with resistant ER+ MBC. A, Evolutionary status of *ESR1*, *FGFR1*, *FGFR2*, and *FGF3/FGF4* alterations is presented by comparing the pre-treatment and post-treatment mutational status for each patient (red = acquired, blue = lost, black = shared, grey = indeterminate). Clinical and pathology tracks depict the site of biopsy for both matched samples, and the duration between the pre-treatment and post-treatment biopsies. B, Copy number alterations for *FGFR1*, *FGFR2* and *FGF3/FGF4* in pre- and post-treatment tumor samples are shown with copy number above ploidy (CNAP) depicted to illustrate the magnitude of the acquired amplification in each case. To better measure segment-specific copy number, we subtracted the genome ploidy for each sample to compute CNAP. The purity and ploidy for tumor samples are shown in Supplemental Table.4. C, Clonal evolution analysis showing the overall clonal structure and acquisition for FGFR2 mutations observed in two patients. In the pre-treatment biopsies, *FGFR2* M538I (*ID 0300348*) and *FGFR2* N550K (*ID 0300350*) were with cancer cell fraction (CCF) of 2% (single read) and 0% (unobserved), respectively, while being observed as clonal mutations in the post-treatment sample with a CCF of 1. The phylogenetic relationships among clones are reconstructed for each patient starting from the normal cell (white circle) connected to the ancestral cancer cells (grey trunk). The phylogenetic divergence to the pre-treatment clones (and subclones) is depicted with blue edges, and phylogenetic divergence to the metastatic clones (and subclones) is in red. Selected mutations in cancer genes are marked on the corresponding branches of the cancer phylogeny.

Figure 3

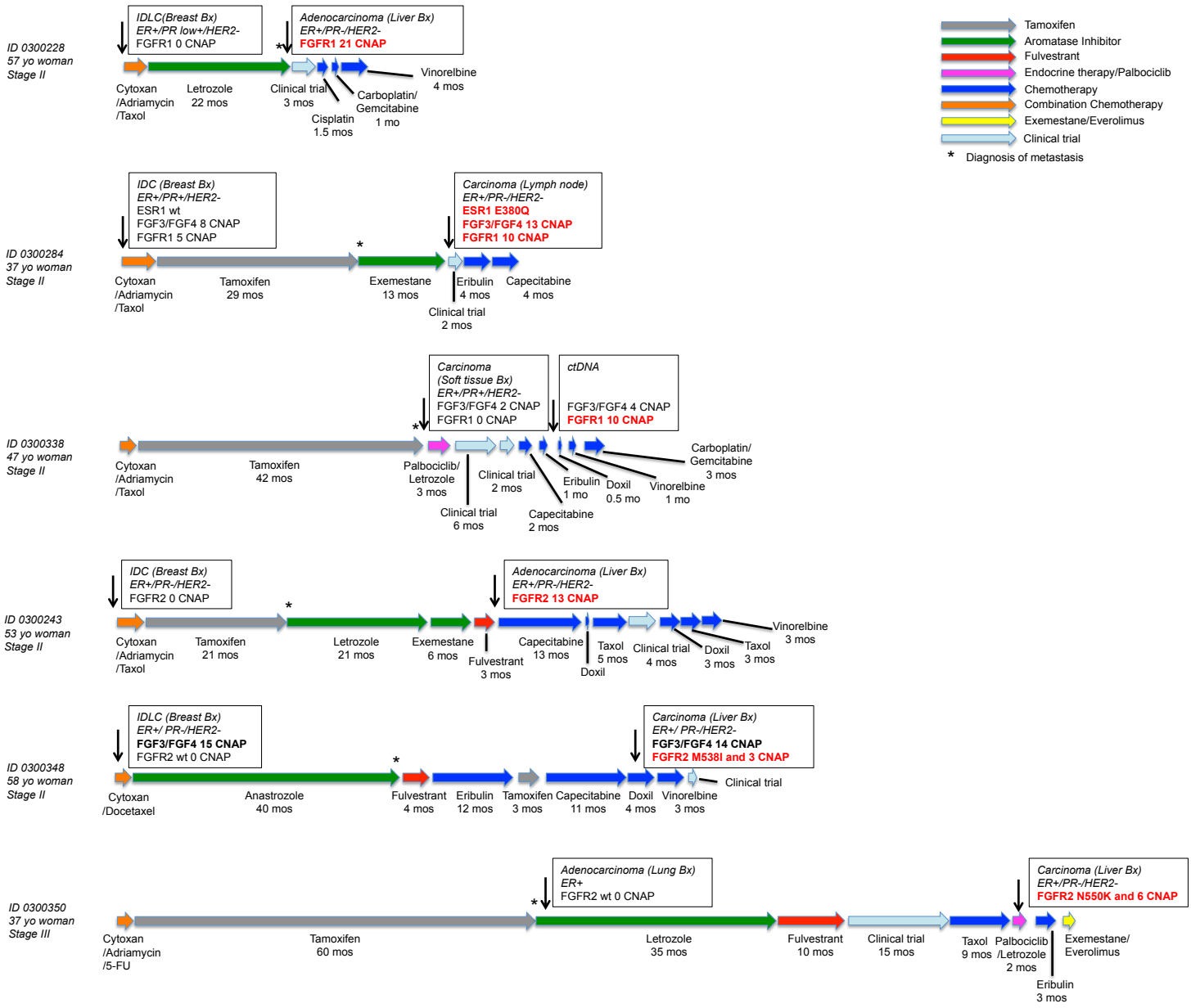


Figure 3. Clinical vignettes of patients who acquired FGFR/FGF alterations following endocrine therapy. The clinical vignettes for selected patients with acquired alterations in *FGFR1*, *FGFR2*, and/or *FGF3/FGF4* illustrate detailed information on age and stage of disease at diagnosis, therapies patients received, duration of response to each therapy, and time of biopsies collected during the clinical course. For each biopsy, available information on biopsy type, tissue site, receptor status and selected genomic alterations detected by whole exome sequencing is shown. In each case, the asterisk indicates the time that metastatic disease was diagnosed. The complete clinicopathologic information for each patient is provided in Supplemental Table.7. IDC: invasive ductal carcinoma, IDLC: invasive ductal-lobular carcinoma; CNAP: copy number above ploidy; yo: years old; Bx: biopsy; PR: progesterone receptor; wt: wildtype.

Figure 4

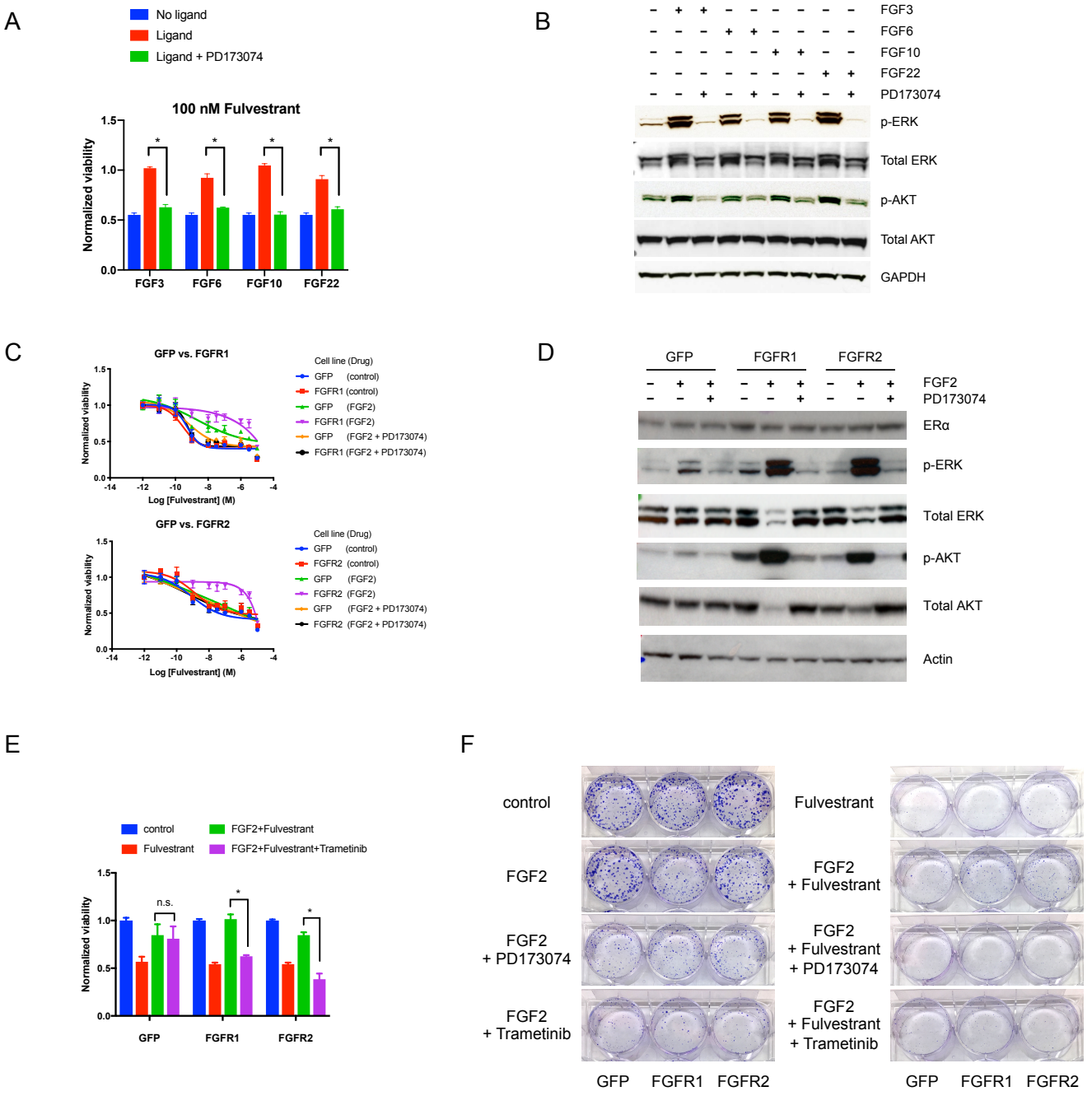
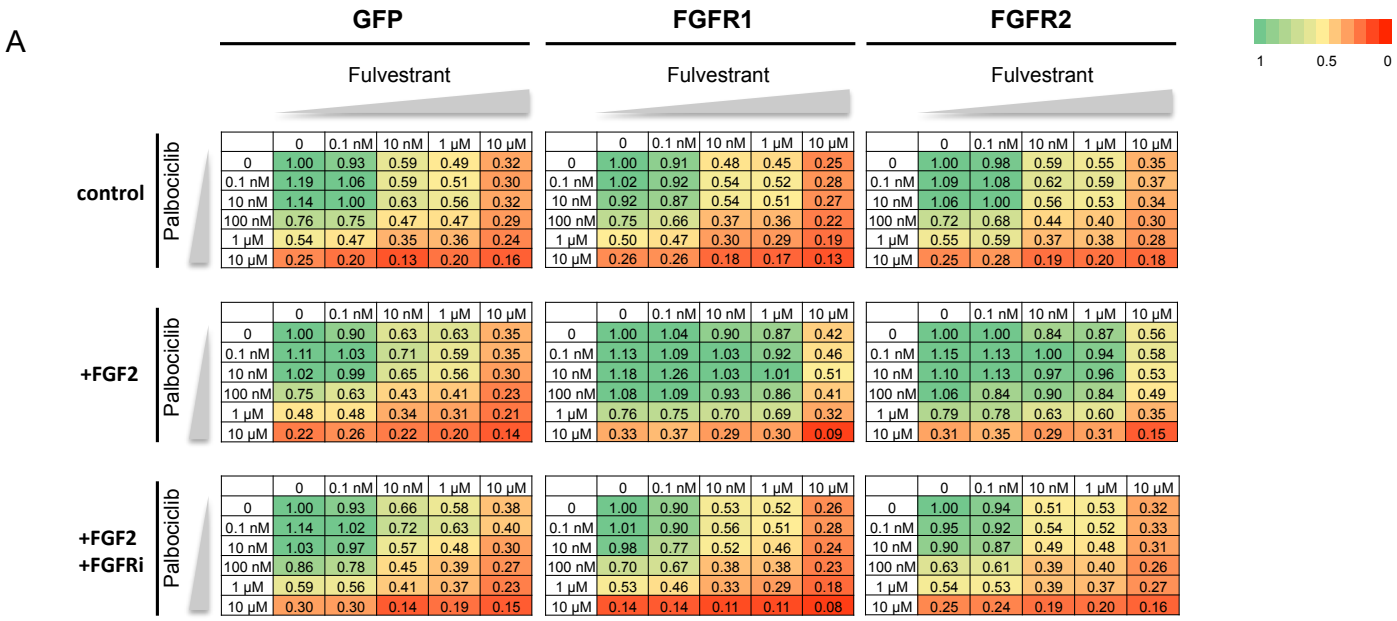


Figure 4. Active FGFR signaling leads to resistance to SERDs through activation of MAPK pathway. A, FGF ligands lead to resistance to fulvestrant, which was blocked by FGFR inhibitor PD173074. Recombinant FGF ligands were added into media every three days at the concentration of 100 ng/mL with or without 1 μM PD173014. T47D cells were treated with heparin (1 μg/mL) that facilitates the binding between FGF ligand and receptor, and sensitivity to 100 nM fulvestrant over six days was normalized to DMSO control. * p-value < 0.01. B, FGF ligands increased ERK and AKT phosphorylation, which was blocked by PD173074. T47D cells were treated as indicated for one hour before protein harvest for western blot. C, FGFR1 or FGFR2 overexpression leads to resistance to fulvestrant, which was blocked by PD173074. GFP, FGFR1 or FGFR2 was overexpressed in T47D cells to establish stable T47D_GFP, T47D_FGFR1 and T47D_FGFR2 cells. The fulvestrant sensitivity of various cell lines were determined in the presence or absence of 10 ng/mL FGF2 and 1 μM PD173074 over six days of drug treatment. D, FGFR1 and FGFR2 induced phosphorylation of ERK and AKT in the presence of FGF2, which was blocked by PD173074. Cells were treated with indicated conditions for one hour before protein harvest. E and F, Trametinib abrogated the resistance to fulvestrant conferred by FGFR1 or FGFR2. Cells were treated with different conditions as indicated: 10 ng/mL FGF2; 100 nM fulvestrant; 500 nM trametinib. CellTiter-Glo assay was performed to measure cell viability after six days for all dose response curves (E). 2,000 cells were plated on Day 1 and treated on Day 2, and colony formation assay was performed after three weeks of drug treatment (F). * p-value < 0.01, n.s. not significant.

Figure 5

A

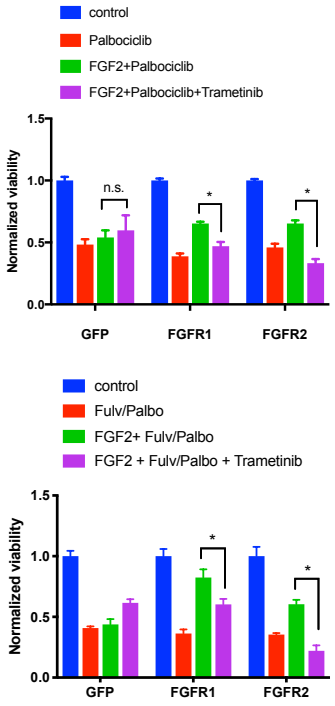


control / Palbociclib	GFP: 0	0.1 nM	10 nM	1 μM	10 μM	FGFR1: 0	0.1 nM	10 nM	1 μM	10 μM	FGFR2: 0	0.1 nM	10 nM	1 μM	10 μM
0	1.00	0.93	0.59	0.49	0.32	1.00	0.91	0.48	0.45	0.25	1.00	0.98	0.59	0.55	0.35
0.1 nM	1.19	1.06	0.59	0.51	0.30	1.02	0.92	0.54	0.52	0.28	1.09	1.08	0.62	0.59	0.37
10 nM	1.14	1.00	0.63	0.56	0.32	0.92	0.87	0.54	0.51	0.27	1.06	1.00	0.56	0.53	0.34
100 nM	0.76	0.75	0.47	0.47	0.29	0.75	0.66	0.37	0.36	0.22	0.72	0.68	0.44	0.40	0.30
1 μM	0.54	0.47	0.35	0.36	0.24	0.50	0.47	0.30	0.29	0.19	0.55	0.59	0.37	0.38	0.28
10 μM	0.25	0.20	0.13	0.20	0.16	0.26	0.26	0.18	0.17	0.13	0.25	0.28	0.19	0.20	0.18

+FGF2 / Palbociclib	GFP: 0	0.1 nM	10 nM	1 μM	10 μM	FGFR1: 0	0.1 nM	10 nM	1 μM	10 μM	FGFR2: 0	0.1 nM	10 nM	1 μM	10 μM
0	1.00	0.90	0.63	0.63	0.35	1.00	1.04	0.90	0.87	0.42	1.00	1.00	0.84	0.87	0.56
0.1 nM	1.11	1.03	0.71	0.59	0.35	1.13	1.09	1.03	0.92	0.46	1.15	1.13	1.00	0.94	0.58
10 nM	1.02	0.99	0.65	0.56	0.30	1.18	1.26	1.03	1.01	0.51	1.10	1.13	0.97	0.96	0.53
100 nM	0.75	0.63	0.43	0.41	0.23	1.08	1.09	0.93	0.86	0.41	1.06	0.84	0.90	0.84	0.49
1 μM	0.48	0.48	0.34	0.31	0.21	0.76	0.75	0.70	0.69	0.32	0.79	0.78	0.63	0.60	0.35
10 μM	0.22	0.26	0.22	0.20	0.14	0.33	0.37	0.29	0.30	0.09	0.31	0.35	0.29	0.31	0.15

+FGF2 +FGFRi / Palbociclib	GFP: 0	0.1 nM	10 nM	1 μM	10 μM	FGFR1: 0	0.1 nM	10 nM	1 μM	10 μM	FGFR2: 0	0.1 nM	10 nM	1 μM	10 μM
0	1.00	0.93	0.66	0.58	0.38	1.00	0.90	0.53	0.52	0.26	1.00	0.94	0.51	0.53	0.32
0.1 nM	1.14	1.02	0.72	0.63	0.40	1.01	0.90	0.56	0.51	0.28	0.95	0.92	0.54	0.52	0.33
10 nM	1.03	0.97	0.57	0.48	0.30	0.98	0.77	0.52	0.46	0.24	0.90	0.87	0.49	0.48	0.31
100 nM	0.86	0.78	0.45	0.39	0.27	0.70	0.67	0.38	0.38	0.23	0.63	0.61	0.39	0.40	0.26
1 μM	0.59	0.56	0.41	0.37	0.23	0.53	0.46	0.33	0.29	0.18	0.54	0.53	0.39	0.37	0.27
10 μM	0.30	0.30	0.14	0.19	0.15	0.14	0.14	0.11	0.11	0.08	0.25	0.24	0.19	0.20	0.16

B



C

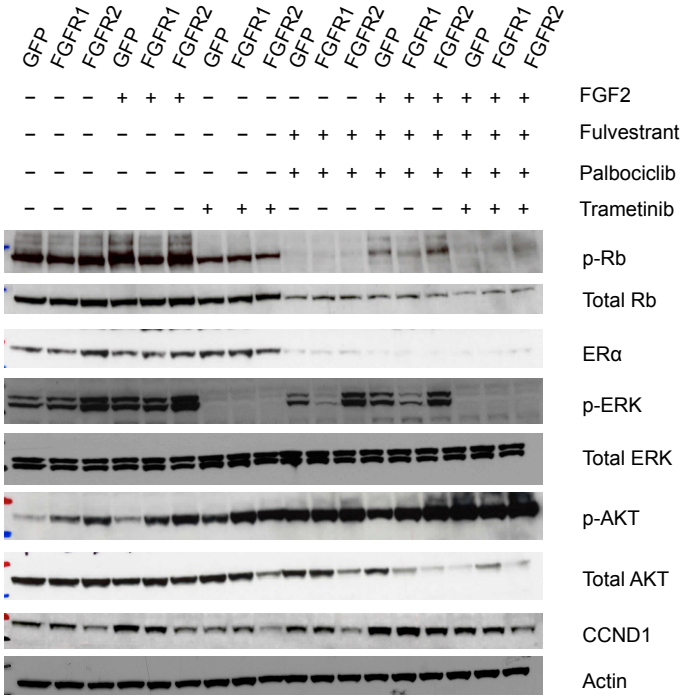


Figure 5. **Active FGFR signaling leads to resistance to the combination of SERDs and CDK4/6 inhibitors.** A, T47D_GFP, T47D_FGFR1 and T47D_FGFR2 cells were treated with the combination of fulvestrant and palbociclib at various doses for six days. The percentage of cell survival shown was normalized to DMSO control for each condition. The combination treatment was carried out under three conditions: 1, control; 2, with 10 ng/mL FGF2; 3, with 10 ng/mL FGF2 and 1 μM PD173074 (FGFRi), with media refreshed every three days. B, MEK inhibitor trametinib abrogated the resistance to palbociclib alone (Top panel) or the combination of fulvestrant and palbociclib (Bottom panel) conferred by FGFR1 or FGFR2. Cells were treated with different conditions as indicated for six days before CellTiter-Glo assay. Concentration of drugs used: 10 ng/mL FGF2; 100 nM fulvestrant (Fulv); 1 μM palbociclib (Palbo); 500 nM trametinib. * p-value < 0.01. n.s. not significant. C, Trametinib blocked ERK phosphorylation and reduced CCND1 and p-Rb levels. Cells were treated as indicated daily for two days before protein harvest and western blot.

Figure 6

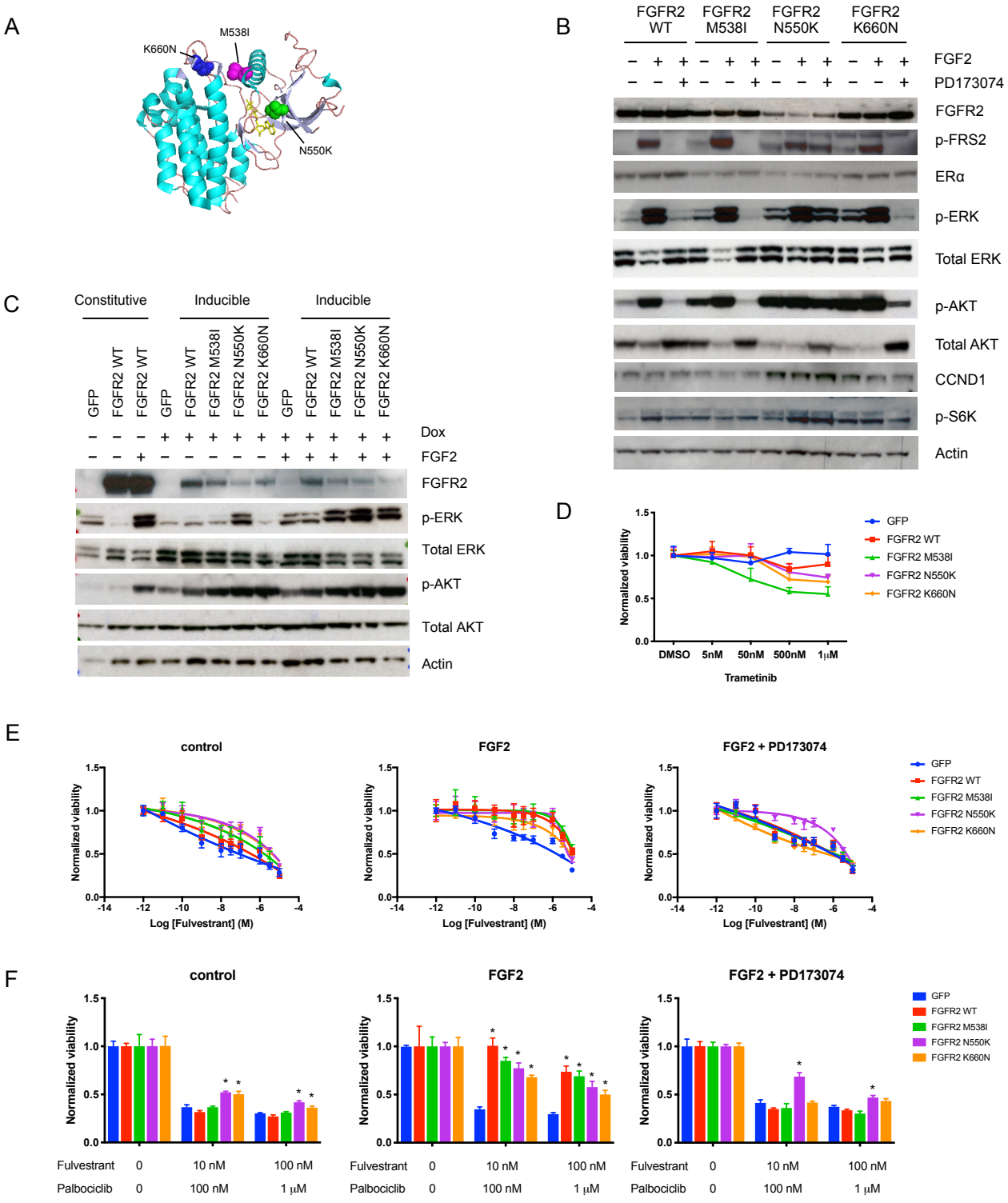


Figure 6. FGFR2 M538I, N5550K and K660N were activating mutations and conferred resistance to fulvestrant and/or palbociclib. A, Crystal structure of activated FGFR2 protein with mutations shown. FGFR2 is in complex with ATP analog (in yellow) and substrate peptide (PDB ID: 2PVF). B, Stable cell lines overexpressing FGFR2 wildtype (WT), M538I, N550K, and K660N were treated with 10 ng/mL FGF2 and/or 1 μM PD173014 for one hour before protein harvest. C, Tetracycline-inducible cell lines that express FGFR2 WT, M538I, N550K and K660N were established and treated with 100 ng/mL doxycycline (Dox) to induce gene expression. Before protein harvest, cells were treated with doxycycline 24 hours before FGF2 stimulation for 3 hours followed by another 3 hours of FGF2 stimulation. Cells were treated with heparin (1 μg/mL) that facilitates the binding between FGF2 and FGFR2. Protein lysates from T47D cells expressing GFP and T47D cells with constitutive overexpression of FGFR2 were used as control. D, Trametinib sensitivity of T47D cell lines expressing GFP or different FGFR2 constructs was examined. E and F, Stable cell lines constitutively overexpressing GFP or FGFR2 constructs (as previously described) were examined for sensitivity to fulvestrant (E) or combination of fulvestrant and palbociclib (F) with or without the treatment of FGF2 and/or PD173074. * p-value < 0.01, calculated as compared to GFP group in all conditions.

Figure 7

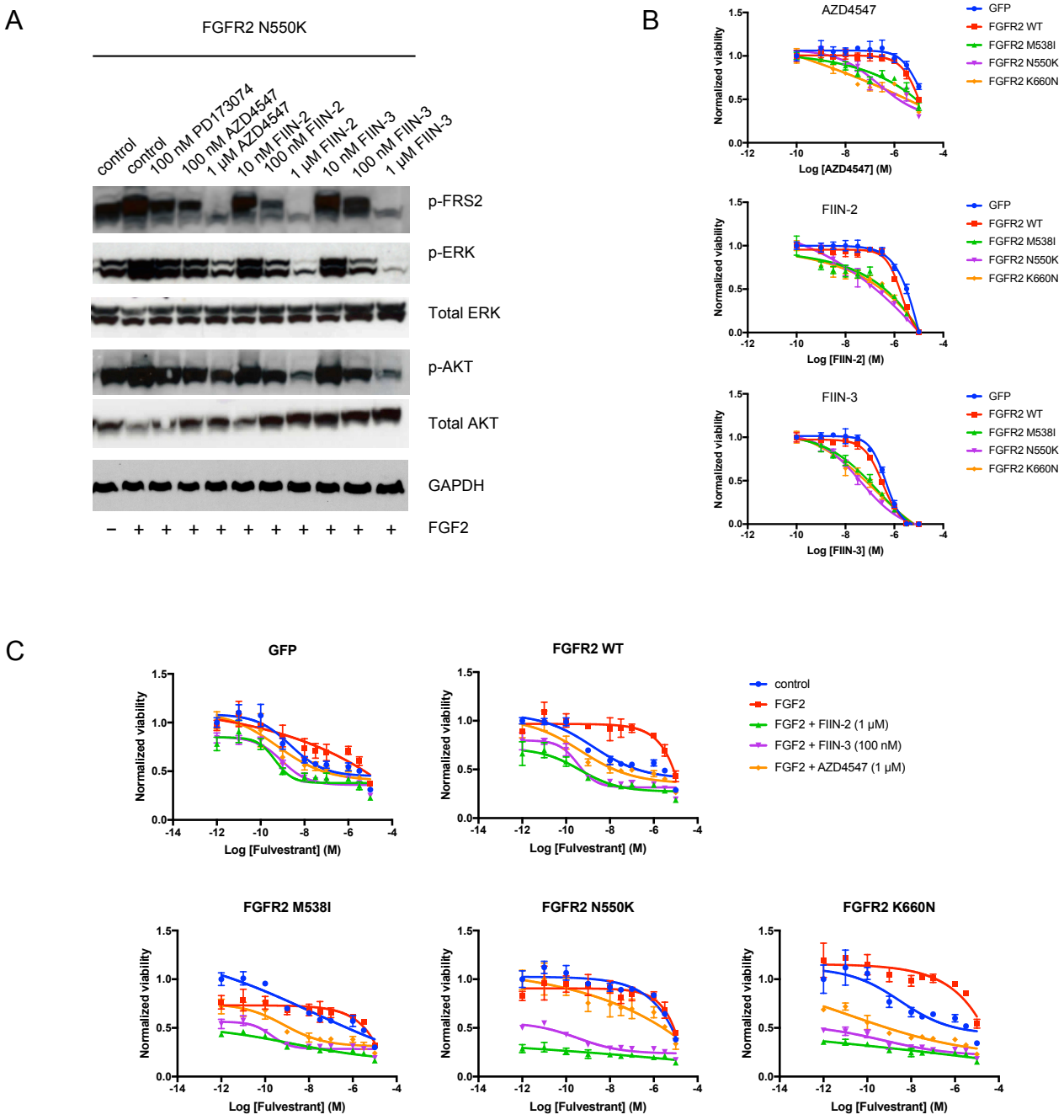


Figure 7. Activating FGFR2 mutations were targeted by irreversible kinase inhibitors FIIN-2 and FIIN-3. A, T47D cells overexpressing FGFR2 N550K cells were treated as indicated for three days and retreated for three hours before protein harvest and western blot. B, Stable cell lines overexpressing FGFR2 mutants (M538I, N550K and K660N) exhibited higher sensitivity to FGFR inhibitors AZD4547, FIIN-2 and FIIN-3, as compared to T47D cells overexpressing GFP or wildtype FGFR2. C, All stable cells lines expressing GFP or FGFR2 constructs were treated with fulvestrant under the following conditions: control, 10 ng/mL FGF2, 10 ng/mL FGF2 with 1 μM AZD4547, 10 ng/mL FGF2 with 1 μM FIIN-2, or 10 ng/mL FGF2 with 100 nM FIIN-3. Drug response curves were determined by CellTiter-Glo.