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

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Running title: FGFR/FGF alterations confer resistance to endocrine therapy

Key words: Drug resistance, endocrine therapy, ER+ breast cancer, FGF, FGFR

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Word count (excluding abstract, references and figure legends): 6182
Total number of figures: 7

Competing Financial Interest Statement

S.A.W. is a consultant for Foundation Medicine and Eli Lilly, and a consultant and equity holder for InfiniteMD. J.C. is an employee/stockholder in Foundation Medicine, a wholly owned subsidiary of Roche. V.A.M. is an employee of Foundation Medicine/Roche, officer and board member of Revolution Medicines, and stockholder of Roche and Revolution Medicines. N.U.L is a consultant for Puma Biotechnology and Daichii, and received research funding from Pfizer, Genentech, Novartis and Seattle Genetics. E.P.W. is a consultant for InfiniteMD, Genentech, and Eli Lilly. N.W. was previously a stockholder and consultant for Foundation Medicine; has been a consultant/advisor for Novartis and Eli Lilly; and has received sponsored research support from Novartis and Puma Biotechnology. None of these entities had any role in the conceptualization, design, data collection, analysis, decision to publish, or preparation of the manuscript

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

70 dependencies in ER+ breast cancer. We demonstrate that activating FGFR/FGF
71 alterations are a mechanism of acquired resistance to ER-directed therapies and CDK4/6
72 inhibitors in ER+ metastatic breast cancer and can be overcome by combination therapy
73 targeting both the ER and the FGFR pathway. The detection of targetable, clonally
74 acquired genetic alterations in metastatic tumor biopsies highlights the value of serial
75 tumor testing to dissect mechanisms of resistance in human breast cancer and its potential
76 application in directing clinical management.
77

78 **Introduction**

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

88

89 Although various resistance mechanisms have been proposed for tamoxifen and
90 aromatase inhibitor resistance, including loss or modification in ER expression (*ESR1*
91 activating mutations and *ESR1* fusions) [3-7], and regulation of alternative signal
92 transduction pathways (PI3K/AKT/mTOR and EGFR/ERBB2/MAPK) [8-10],
93 mechanisms of resistance to SERDs remain understudied. Mechanisms of endocrine
94 resistance identified in patients include acquired mutations in the estrogen receptor itself
95 [4-7], acquired activating mutations in *ERBB2* (HER2) [11, 12], loss of function of *NF1*
96 [13], and other alterations in MAPK pathway genes [14]. Additional mechanisms remain
97 to be identified.

98

99 Gain-of-function screens have played a pivotal role in identification of resistance
100 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

120 **Results**

121 ***A Genome-Scale Gain-of-Function Screen for Resistance to Selective Estrogen***

122 ***Receptor Degraders***

123 To identify the spectrum of genes whose overexpression confers resistance to SERDs *in*

124 *vitro*, we expressed 17,255 human open reading frames (ORFs), corresponding to 10,135

125 distinct human genes, in ER+ T47D breast cancer cells in the presence of fulvestrant or

126 GDC-0810. T47D cells were infected with the pooled lentiviral ORF library hORFeome

127 [22]. Fulvestrant, GDC-0810, or vehicle control (DMSO) was added following infection

128 and selection. ORF representation was assessed by sequencing after 21 days of drug

129 exposure. Genes that confer drug resistance will be enriched under drug selection,

130 indicated by a positive log fold change (LFC) for ORF representation before and after

131 DMSO/drug selection.

132

133 Using a Z score >3 as a criterion to identify resistance candidates, we identified 64 genes

134 (93 ORFs) that conferred resistance to fulvestrant and 57 genes (83 ORFs) that conferred

135 resistance to GDC-0810 (Fig.1A and Supplemental Table.1). 37 genes (55 ORFs)

136 conferred resistance to both drugs, a degree of overlap which was anticipated given the

137 mechanistic similarities between fulvestrant and GDC-0810. The LFC and corresponding

138 Z score for each ORF in fulvestrant and GDC-0810 treatment arms were highly

139 correlated, with a correlation coefficient of 0.77 (Fig.1A).

140

141 To confirm these results, we conducted a secondary screen using a smaller pooled library

142 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

166 in MAPK pathway genes are enriched in endocrine-resistant tumors [13, 14]. We sought
167 to further examine the role of FGFR and FGF genes in resistance to SERDs in MBC.
168
169 ***Identification of acquired FGFR and FGF alterations in metastatic biopsies from***
170 ***patients with resistant ER+ MBC***
171 To examine the potential role of the FGFR and FGF alterations in the development of
172 endocrine resistance clinically, we analyzed whole exome sequencing (WES) data from
173 paired pre-treatment and post-treatment metastatic tumor biopsies or cell free DNA from
174 60 patients with ER+ metastatic breast cancer who had received at least one endocrine
175 therapy (tamoxifen, AI, SERDs) for more than 120 days between the two biopsies [23].
176
177 Amongst the 60 post-treatment samples, we found *FGFR1* amplifications in 15% (9/60),
178 *FGFR2* amplifications in 5% (3/60), *FGFR2* activating mutations in 3.3% (2/60), and
179 *FGF3/FGF4* amplifications (which are adjacent genes and therefore co-amplified) in
180 28.3% (17/60) – for a total of 40% (24/60) of the cohort with at least one alteration in one
181 of these four genes (Fig.2A). Overall, the prevalence of *FGFR1*, *FGFR2*, and
182 *FGF3/FGF4* alterations in the resistant metastatic setting seen here is increased
183 compared to what was observed in previously published cohorts of primary ER+ breast
184 cancer, such as The Cancer Genome Atlas (TCGA) [24] (Supplemental Table.3). The
185 incidence of *FGFR2* alterations (6.7%), in particular, is markedly increased compared to
186 primary treatment-naive breast cancer, in which the incidence is less than 2% in TCGA
187 (Supplemental Table.3).
188

189 To determine if this enrichment of FGFR/FGF alterations in the metastatic setting was
190 due to acquisition/selection under the selective pressure of endocrine therapy, we
191 compared the WES from the paired pre-treatment and post-treamtent samples for the 24
192 patients that exhibited FGFR/FGF alterations in their post-treatment samples. These 24
193 pairs of samples included 23 tumor biopsies and one cell-free DNA sample at the pre-
194 treatment timepoint, and 22 tumor biopsies, and two cell-free DNA samples at the post-
195 treatment timepoint. We performed an evolutionary analysis to evaluate clonal structure
196 and dynamics, including changes in mutations and copy number. The evolutionary
197 inference and clonal dynamics of mutations was based on changes in the estimated
198 fraction of tumor cells harboring each genomic alteration (the cancer cell fraction, CCF)
199 as previously shown for acquired HER2 mutations [11]. The evolutionary inference of
200 copy number changes was based on measuring differences in copy number amplitudes
201 between pre-treatment and post-treatment samples, while accounting for differences in
202 cancer cell fraction ("purity") in the sample and correcting for differences in ploidy. The
203 resultant purity-corrected values provide an estimate of "copy number above ploidy"
204 (CNAP) (see Methods).
205
206 For this analysis, we define "acquired" alterations as alterations with higher
207 representation in the post-treatment sample as compared to the pre-treatment sample. For
208 single nucleotide variants (SNVs), this means that the mutation had a substantially higher
209 CCF in the post-treatment sample compared to the pre-treatment sample (including lack
210 of detection in the pre-treatment sample despite having sufficient power to detect the
211 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

258 *CCND1* amplification, suggesting that this acquisition can occur as an independent
259 genomic event. Similarly, 2 out of the 4 cases with acquired *FGF3/FGF4* did not have
260 co-acquisition of *FGF19* amplification. The relationship of acquisitions of *FGF3*, *FGF4*,
261 *FGF19*, and *CCND1* are depicted in Supplemental Data Fig.S5 and further details about
262 these amplicons are described in Supplemental Methods. Further exploration of the full
263 genomic contexts and other concurrent genetic alterations for the 12 patients with
264 acquired FGFR/FGF alterations are shown in Supplemental Data Fig.S6 and
265 Supplemental Table.5-6.
266
267 Figure 3 depicts clinical vignettes for six of the patients with acquired *FGFR1*, *FGFR2*,
268 and/or *FGF3/FGF4* alterations in their post-treatment biopsies. All patients were treated
269 with ER-directed therapy before acquiring FGF or FGFR alterations, including tamoxifen
270 (3 patients), AIs (6 patients), and fulvestrant (3 patients). Vignettes for the other six
271 patients with acquired *FGFR1*, *FGFR2*, and/or *FGF3/FGF4* alterations in their post-
272 treatment biopsies are shown in Supplemental Data Fig.S7. Detailed clinicopathological
273 features and therapy details for all 12 patients are found in Supplemental Table.7.
274
275 In addition to these 12 patients in our cohort, we identified several additional patients
276 with acquired *FGFR1* and *FGFR2* activating mutations following the development of
277 resistance to endocrine therapy (Supplemental Data Fig.S8 and Supplemental Table.8).
278 FM patient 1 acquired a clonal *FGFR1* N546K mutation (a known activating mutation
279 paralogous to *FGFR2* N550K) following treatment with an AI. FM patient 2 acquired a
280 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

303 We next overexpressed FGFR1, FGFR2, or GFP in T47D cells through lentiviral
304 transduction and examined the impact on susceptibility to SERDs. Overexpression of
305 FGFR1 or FGFR2 alone did not affect sensitivity to fulvestrant or GDC-0810. However,
306 with the addition of FGF2 ligand, both FGFR1 and FGFR2 rendered cells highly resistant
307 to the two SERDs (Fig. 4C and Supplemental Data Fig. S11). In comparison, FGF2
308 ligand alone reduced sensitivity to SERDs in control cells expressing GFP to a much
309 lesser extent than in the FGFR1 or FGFR2 expressing cells, suggesting the potent
310 resistance phenotype requires both FGF ligand and receptor. This requirement for the
311 presence of both FGF ligand and receptor for maximal resistance phenotype may also
312 explain why only FGFs but not FGFR1 or FGFR2 scored in the resistance screen (Fig.1
313 A-B). The resistance phenotype resulting from FGFR1 and FGFR2 overexpression was
314 completely reversed by the addition of PD173074 (Fig. 4C). Similar results were
315 obtained in MCF7 cells (Supplemental Data Fig.S12 A-B).
316
317 FGFR1 and FGFR2 overexpression (in the presence of FGF2 ligand) induced more
318 potent phosphorylation of AKT and ERK than the GFP control (Fig.4D). These results
319 are consistent with previous findings that FGFR1 activation led to MAPK activation and
320 fulvestrant resistance [28]. Activation of downstream effectors p-ERK and p-AKT by
321 FGFR1/2 overexpression was reversed to baseline levels with PD173074 (Fig. 4D and
322 Supplemental Data Fig. S12C). Examination of a larger number of kinases using kinase
323 antibody arrays demonstrated that AKT, ERK and RSK (downstream effector of ERK)
324 were the only kinases of those tested to exhibit increased phosphorylation following
325 FGFR1/2 overexpression and FGF2 stimulation (Supplemental Data Fig.S13).

326 Collectively, these findings suggest that FGFR1 and FGFR2 cause SERD resistance
327 through the activation of MAPK and/or PI3K/AKT pathways.
328
329 We examined the sensitivity of cells overexpressing FGFR1 or FGFR2 to several
330 inhibitors of downstream effectors: the MEK inhibitor trametinib, the AKT inhibitor
331 AZD5363, and the mTOR inhibitor everolimus. FGFR1 or FGFR2 overexpression in the
332 presence of FGF2 led to hypersensitivity to trametinib (Supplemental Data Fig.S14A). In
333 contrast, FGFR1 or FGFR2 overexpression in the presence of FGF2 reduced sensitivity
334 to AKT and mTOR inhibitors (Supplemental Data Fig.S14A).
335
336 We attempted to reverse FGFR-induced resistance to fulvestrant by inhibiting the MAPK
337 pathway. Treatment of FGFR1 overexpressing cells with trametinib partially resensitized
338 cells to fulvestrant, and treatment of FGFR2 overexpressing cells with trametinib fully
339 resensitized the cells to fulvestrant (Fig.4E-F). Treatment with the mTOR inhibitor
340 everolimus also partially blocked resistance conferred by FGFR1 or FGFR2
341 overexpression (Supplemental Data Fig.S14B). We also performed long-term colony
342 formation assays and observed similar results (Supplemental Data Fig.S14C).
343
344 Together, these results suggest that the MAPK pathway is the primary downstream
345 effector of FGFR activation resulting in endocrine resistance. This is consistent with the
346 pathway analysis of resistance genes in our initial overexpression screen, which
347 demonstrated enrichment in MAPK pathway genes (Figure 1D), as well as our prior
348 findings of MAPK activation through acquired HER2 mutations in endocrine

349 resistance—adding further support to the idea that the MAPK pathway may be a common
350 node of endocrine resistance [11, 14].
351
352 ***FGFR activation confers cross-resistance to CDK4/6 inhibitors***
353 Since the combination of endocrine therapy and CDK4/6 inhibitors is now a standard of
354 care treatment for patients with ER+ metastatic breast cancer, we also examined the
355 effect of FGFR signaling on sensitivity to the combination of fulvestrant and the CDK4/6
356 inhibitor palbociclib. In T47D cells, FGFR1 and FGFR2 overexpression in the presence
357 of FGF2 also conferred resistance to combination treatment of fulvestrant and palbociclib
358 (Fig. 5A). The resistance phenotype was again abrogated by PD173074 (Fig. 5A).
359 Resistance to fulvestrant and palbociclib was also partially reversed by trametinib (Fig.
360 5B and Supplemental Data Fig. S14D), further providing the support for the role of
361 MAPK pathway activation in FGFR-mediated drug resistance. The reversal of resistance
362 by trametinib was accompanied by reduced ERK phosphorylation (Fig. 5C). Similar
363 results were achieved in MCF7 cells, although everolimus was more effective than
364 trametinib in reversing the resistance phenotype by FGFR1 or FGFR2 overexpression
365 (Supplemental Data Fig.S15 and Fig.S16).
366
367 In the presence of fulvestrant and palbociclib, FGFR1 or FGFR2 overexpression was
368 accompanied by increased p-Rb and CCND1 levels, both of which were partially
369 reversed by trametinib (Fig.5C). CCND1 knockdown in cells overexpressing GFP,
370 FGFR1 or FGFR2 impaired cell proliferation similarly across all three cell lines without
371 affecting the IC50 of fulvestrant (Supplemental Data Fig.S17). This suggests that the

372 proliferation advantage provided by active FGFR signaling is partially dependent on
373 CCND1. This is consistent with prior results suggesting that CCND1 was involved in
374 FGF2-mediated drug resistance [29].
375
376 Clinical evidence also supports the finding that FGFR alterations can cause resistance to
377 CDK4/6 inhibitors. Following the acquisition of *FGFR2* N550K (along with *FGFR2*
378 amplification), Pt 0300350 did not respond to the combination of letrozole and
379 palbociclib (Fig.3), suggesting that *FGFR2* alterations may lead to intrinsic resistance to
380 the combination of endocrine therapy and CDK4/6 inhibitors. Another patient with an
381 *FGFR2* N550K mutation (FM Patient 2) also did not respond to the combination of
382 fulvestrant and palbociclib (Supplemental Data Fig.S8). Collectively, this suggests
383 targeting the FGFR pathway may also be a viable strategy to overcome FGFR/FGF-
384 mediated resistance to SERDs and CDK4/6 inhibitors.
385
386 ***FGFR2 mutations found in patients are activating and can be targeted with***
387 ***irreversible FGFR inhibitors***
388 We identified 3 acquired mutations in the kinase domain of FGFR2 in patients who
389 developed resistance to endocrine therapy. Two of these, FGFR2 N550K and K660N, are
390 known activating FGFR2 mutations that have been previously identified in breast cancer
391 [25, 27]. FGFR2 N550K is part of the molecular brake at the kinase hinge region, which
392 allows the receptor to adopt an active conformation more easily (Fig.6A). N550K is a
393 recurring hotspot mutation reported to confer resistance to several FGFR inhibitors
394 including PD173074 and dovitinib [30]. FGFR2 K660N is located in a conserved region

395 in the tyrosine kinase domain and has been confirmed to increase kinase activity [27, 30].
396 The third mutation, FGFR2 M538I, has not been previously reported in breast cancer.
397 Based on its location, M538I appears to stabilize the active kinase confirmation by
398 strengthening the hydrophobic spine of the FGFR2 kinase [30] (Fig.6A).
399
400 We expressed all three FGFR2 kinase domain mutants in T47D cells through lentiviral
401 transduction, as well as wildtype (WT) FGFR2 and GFP as negative controls. All three
402 mutants elicited higher kinase activity than WT FGFR2 constitutively, demonstrated by
403 levels of p-FRS2, a direct substrate for FGFR2 (Fig.6B). MAPK and AKT signaling were
404 also increased, as indicated by increased p-ERK and p-AKT levels, respectively (Fig.6B).
405 The addition of FGF2 ligand further enhanced downstream signaling for all FGFR2
406 mutants, and the enhanced signaling was blocked by PD173074 for FGFR2 M538I and
407 K660N, but not for N550K (Fig.6B).
408
409 FGFR2 mutants were also expressed under a tetracycline responsive promoter in T47D
410 cells grown in low doses of doxycycline to determine the functionality at lower
411 expression levels. At lower levels of expression, FGFR2 N550K still led to increased
412 levels of p-ERK and p-AKT, independent of FGF2 ligand stimulation. FGFR2 M538I
413 and K660N also resulted in higher p-ERK and p-AKT levels in the presence of FGF2 as
414 compared to FGFR2 WT (Fig.6C). FGFR2 mutants rendered cells more sensitive to
415 trametinib than did GFP or WT FGFR2 (Fig.6D), further supporting the finding that
416 FGFR signaling requires the MAPK pathway in this context. Taken together, these results
417 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

487 provided the full genome-scale screen data as a resource to the community of researchers
488 interested in resistance to ER-directed therapies as well as the biology of estrogen
489 receptor dependencies in ER+ breast cancer.
490
491 Our ultimate goal is to identify resistance mechanisms that are clinically relevant and can
492 be therapeutically targeted. By comparing paired pre-treatment and post-treatment
493 tumors, our evolutionary analyses identified acquired *FGFR1* and *FGFR2*, and
494 *FGF3/FGF4* alterations in 12 out of 60 post-treatment samples, further highlighting a
495 potential role for the FGFR pathway in driving drug resistance and disease progression.
496 Most notably, all four alterations in FGFR2 in our cohort were found to be acquired after
497 the development of resistance to endocrine therapy. Our overall findings are consistent
498 with two recent cohort studies which noted some patients with acquired *FGFR1* and
499 *FGFR2* alterations following treatment of endocrine therapy [37, 38], and provides a
500 mechanistic explanation for these acquisitions.
501
502 This analysis was enabled by a novel method we developed to compare the magnitude of
503 amplification in matched pre- and post-treatment samples while considering key
504 confounders to allow for more reliable assignment of copy gain or loss. Since matched
505 tumor samples of the same patient are highly variable in the cancer cell fraction (purity)
506 and often variable in ploidy (with genome duplication taking place in the metastatic
507 setting), we computed the purity-corrected copy number above ploidy and set a relatively
508 stringent threshold of changes in CNAP to define acquired amplification (See Methods),
509 as cancer clones bearing amplifications with high focality and magnitude in FGFR/FGF

510 genes are more likely to induce dependency on FGFR pathway and result in endocrine
511 resistance.
512
513 Our genomic analysis has some limitations and caveats. The observed alterations may not
514 exclusively result from endocrine therapy as some patients received other therapies
515 between the two collected biopsies. Moreover, tumors with FGFR/FGF alterations also
516 harbor alterations in other cancer genes, which may contribute to drug resistance as well
517 (Supplemental Data Fig.S6 and Supplemental Table.5). Despite these caveats, with the
518 evidence from unbiased screens, genomic evidence in relevant patient samples, and
519 confirmatory experimental models, the FGFR pathway clearly emerges as a clinically
520 important resistance mechanism for SERDs and CDK4/6 inhibitors.
521
522 Strategies to target the FGFR pathway in breast cancer patients with FGFR alterations are
523 currently being assessed in clinical trials. FGFR inhibitors currently under clinical
524 development in breast cancer include non-selective tyrosine kinase inhibitors (dovitinib
525 and lucitanib), FGFR1-3 selective inhibitors (AZD4547 and BGJ398), and others,
526 although clinical trials have achieved mixed results to date [39-44]. The combination of
527 FGFR inhibitors and endocrine therapy is also being clinically investigated. For example,
528 the combination of dovitinib and fulvestrant showed promising clinical activity [39]. As
529 FGFR pathway activation also results in resistance to CDK4/6 inhibitors, a triple
530 combination with the addition of CDK4/6 inhibitors may also be considered. One
531 challenge for the use of FGFR inhibitors is to identify reliable biomarkers. Our results
532 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

602 ***Patients and Tumor Samples***

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

613

614 ***WES and data analysis***

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

621

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

625 and ignored in our analysis). CNAP of at least 6 are considered high amplifications
626 (HighAMP), and CNAP of at least 9 and fewer than 100 genes [48] is considered very
627 high focal amplification (FocalAMP).
628
629 The evolutionary classification of amplifications accounts for the magnitude of the
630 observed copy-number difference between the pre-treatment and the post-treatment
631 samples. If the difference between the CNAP of the post-treatment and the CNAP of the
632 pre-treatment is smaller than 50%, the amplification is defined as "Shared". If the CNAP
633 of the post-treatment is larger than the CNAP by more than 50% and the lower pre-
634 treatment CNAP is not at "FocalAMP" level, the evolutionary classification is
635 "Acquired". If CNAP of the post-treatment is smaller by at least 50%, comparing to the
636 pre-treatment sample and the lower post-treatment CNAP is not at "FocalAMP" level, the
637 evolutionary classification is "Loss". Otherwise, the evolutionary classification of
638 amplifications is defined as "Indeterminate".
639
640 ***Generation of plasmids and engineered cells***
641 T47D or MCF7 cells were infected with lentivirus to derive stable cell lines
642 overexpressing wildtype (WT) or mutant ORFs. All WT ORFs were obtained from the
643 Broad Institute. Mutant ORFs (FGFR2 M538I, N550K and K660N) were made using
644 QuickChange II site-directed mutagenesis kit (Agilent Technologies #200523). Most
645 stable cells lines express ORFs in pLX317 vector and were selected with puromycin (Life
646 Technologies #A1113803). Stable cell lines expressing CCND1 and PIM1 in pLX304
647 vector were selected with blasticidin (Life Technologies #A1113903).

648

649 For inducible cell lines, WT or Mutant FGFR2 IIIb ORFs were cloned into pInducer20
650 Tetracycline-inducible lentiviral vector (Addgene #44012) using gateway cloning
651 technology (Invitrogen #11791019). Lentivirus was then generated and infected to
652 establish Tet-inducible cells lines cultured in 10% Tet-system approved FBS (Clontech
653 #631106) and 500 µg/mL G418 (Life Technologies #10131035). Doxycycline (Clontech
654 #631311) was used to induce ORF expression.

655

656 ***Kill curves and CellTiter-Glo viability assay***

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

664

665 ***Colony formation assay***

666 2,000-30,000 cells were plated in 6-well plates on Day 1 and treated on Day 2. Media
667 was refreshed every 3-4 days until crystal violet staining. On the day of staining, cells
668 were fixed with ice-cold 100% methanol for 10 minutes and then incubated with 0.5%
669 crystal violet solution (Sigma Aldrich #C6158) in 25% methanol at room temperature for
670 10 minutes.

671

672 ***Western blotting***

673 Western blotting was performed as described in supplemental methods.

674

675 ***Statistical analysis***

676 Statistical analyses related to drug response curve were performed with student t-test in

677 Graphpad Prism.

678

679 **Acknowledgements**

680 We thank Qaren Quartey, Christian Kapstad and Gabriela Johnson for technical

681 assistance; Karla Helvie, Laura Dellostritto, Lori Marini, Nelly Oliver, Shreevidya

682 Periyasamy, Colin Mackichan, Max Lloyd, and Mahmoud Charif for assistance with

683 patient sample collection and annotation; and Flora Luo, Tinghu Zhang and Nathanael

684 Gray for providing reagents. We thank Jorge Gómez Tejeda Zañudo for helpful

685 discussions and comments on the manuscript. We are grateful to all the patients who

686 volunteered for our tumor biopsy protocol and generously provided the tissue analyzed in

687 this study.

688

689 **Grant support**

690 This work was supported by the Department of Defense W81XWH-13-1-0032 (N.W.),

691 AACR Landon Foundation 13-60-27-WAGL (N.W.), NCI Breast Cancer SPORE at

692 DF/HCC #P50CA168504 (N.W., N.U.L and E.P.W), Susan G. Komen CCR15333343

693 (N.W.), The V Foundation (N.W.), The Breast Cancer Alliance (N.W.), The Cancer

694 Couch Foundation (N.W.), Twisted Pink (N.W.), Hope Scarves (N.W.), Breast Cancer
695 Research Foundation (N.U.L. and E.P.W.), ACT NOW (to Dana-Farber Cancer Institute
696 Breast Oncology Program), Fashion Footwear Association of New York (to Dana-Farber
697 Cancer Institute Breast Oncology Program), Friends of Dana-Farber Cancer Institute (to
698 N.U.L.), Stand Up to Cancer (N.W.), National Science Foundation (N.W.), SU2C-TVF
699 Convergence Scholarship (P.M.), and The American Association for Cancer Research
700 Basic Science Fellowship (P.M.).
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

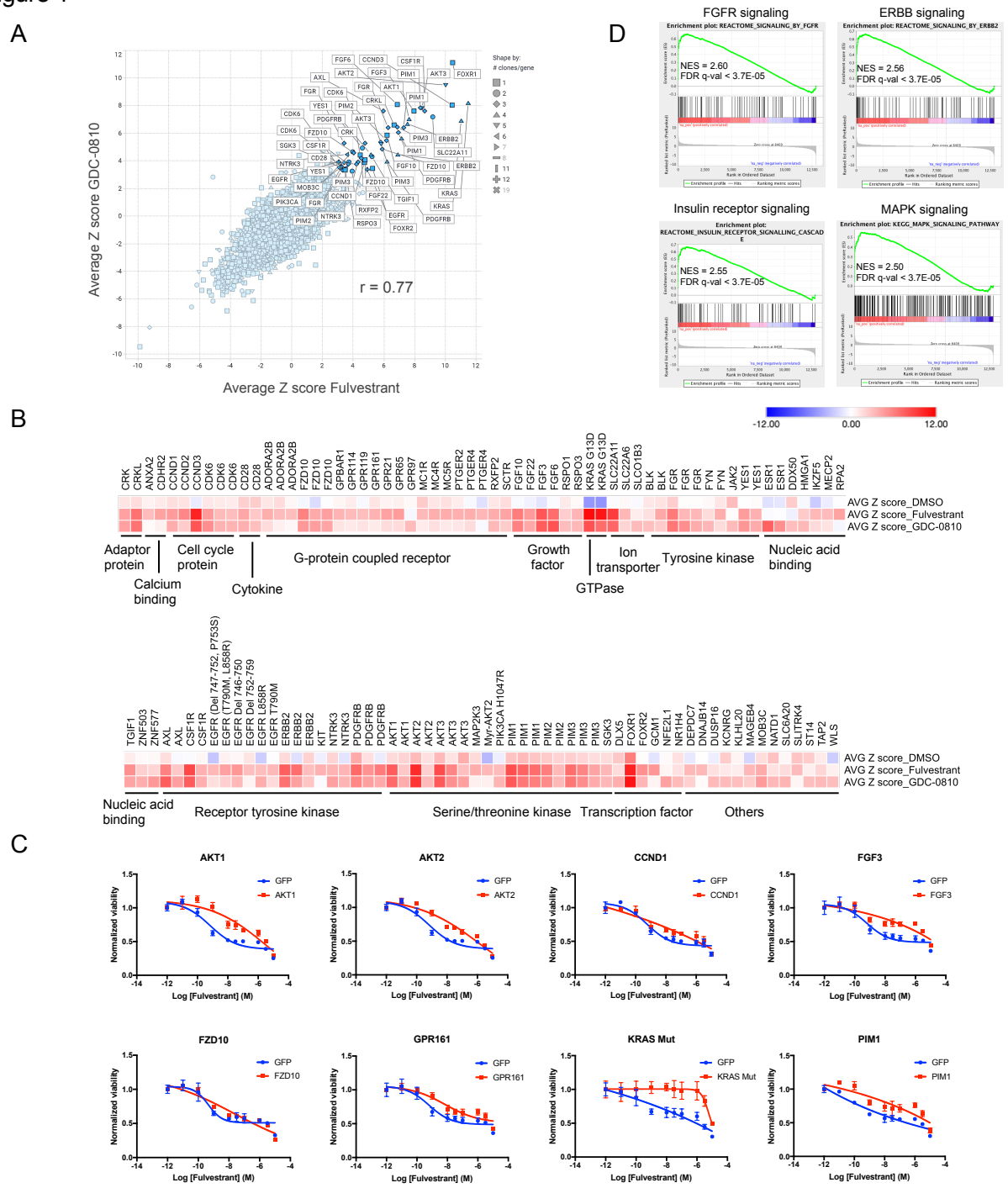


Figure 1. A genome-scale gain-of-function screen identified resistance genes to fulvestrant and GDC-0810. 17,255 human open reading frames (ORFs), corresponding to 10,135 distinct genes, were expressed in ER+ T47D breast cancer cells in the presence of fulvestrant or GDC-0810. Fulvestrant, GDC-0810, 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

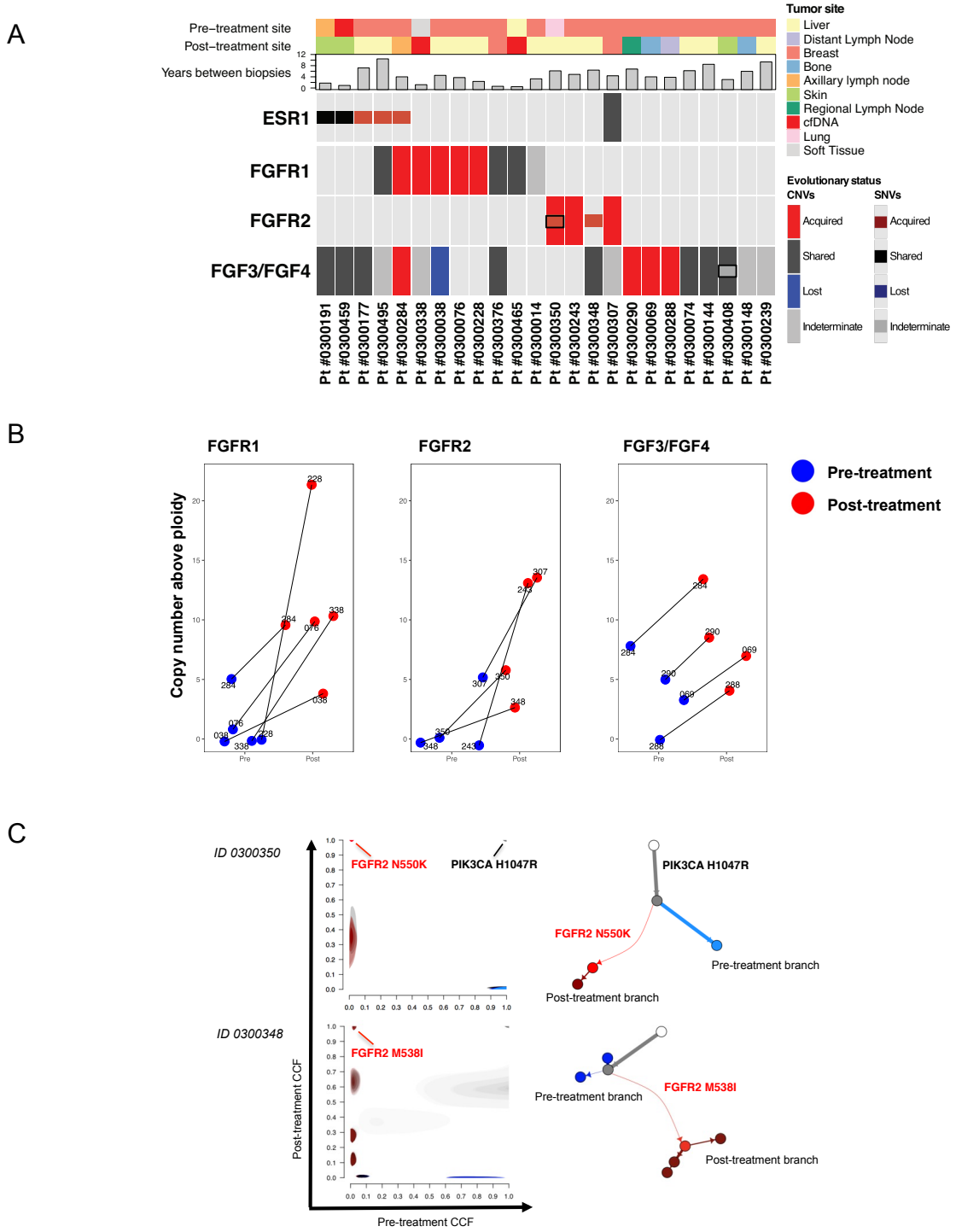


Figure 2. Identification of acquired FGFR and FGF alterations in metastatic biopsies from patients with resistant ER+ MBC. A, Evolutionary status of *ESR1*, *FGFR1*, *FGFR2*, and *FGF3/FGF4* alterations is presented by comparing the pre-treatment and post-treatment mutational status for each patient (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

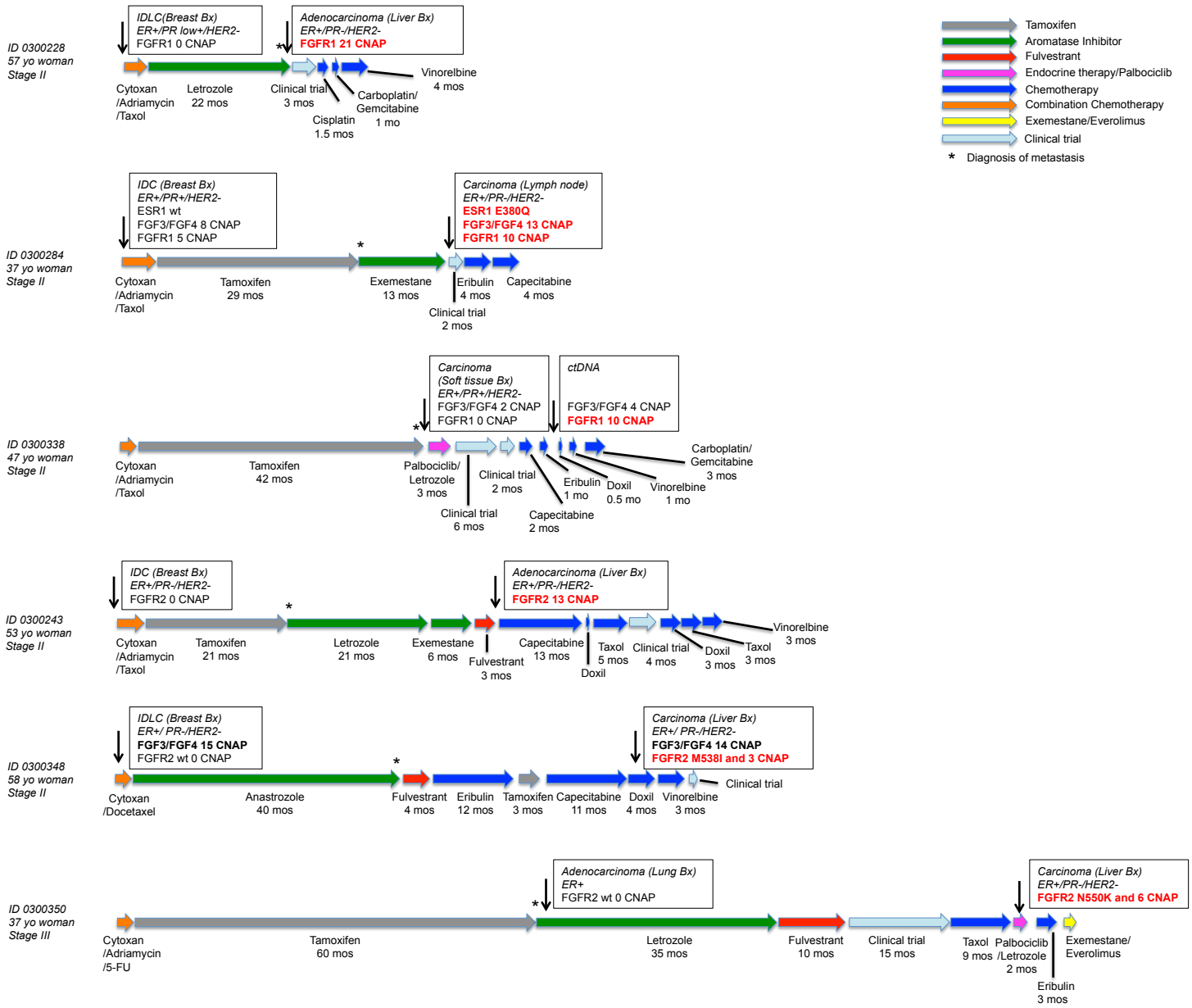


Figure 3. Clinical vignettes of patients who acquired FGFR/FGF alterations following endocrine therapy. The clinical vignettes for selected patients with acquired alterations in *FGFR1*, *FGFR2*, and/or *FGF3/FGF4* illustrate detailed information on age and stage of disease at diagnosis, therapies patients 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

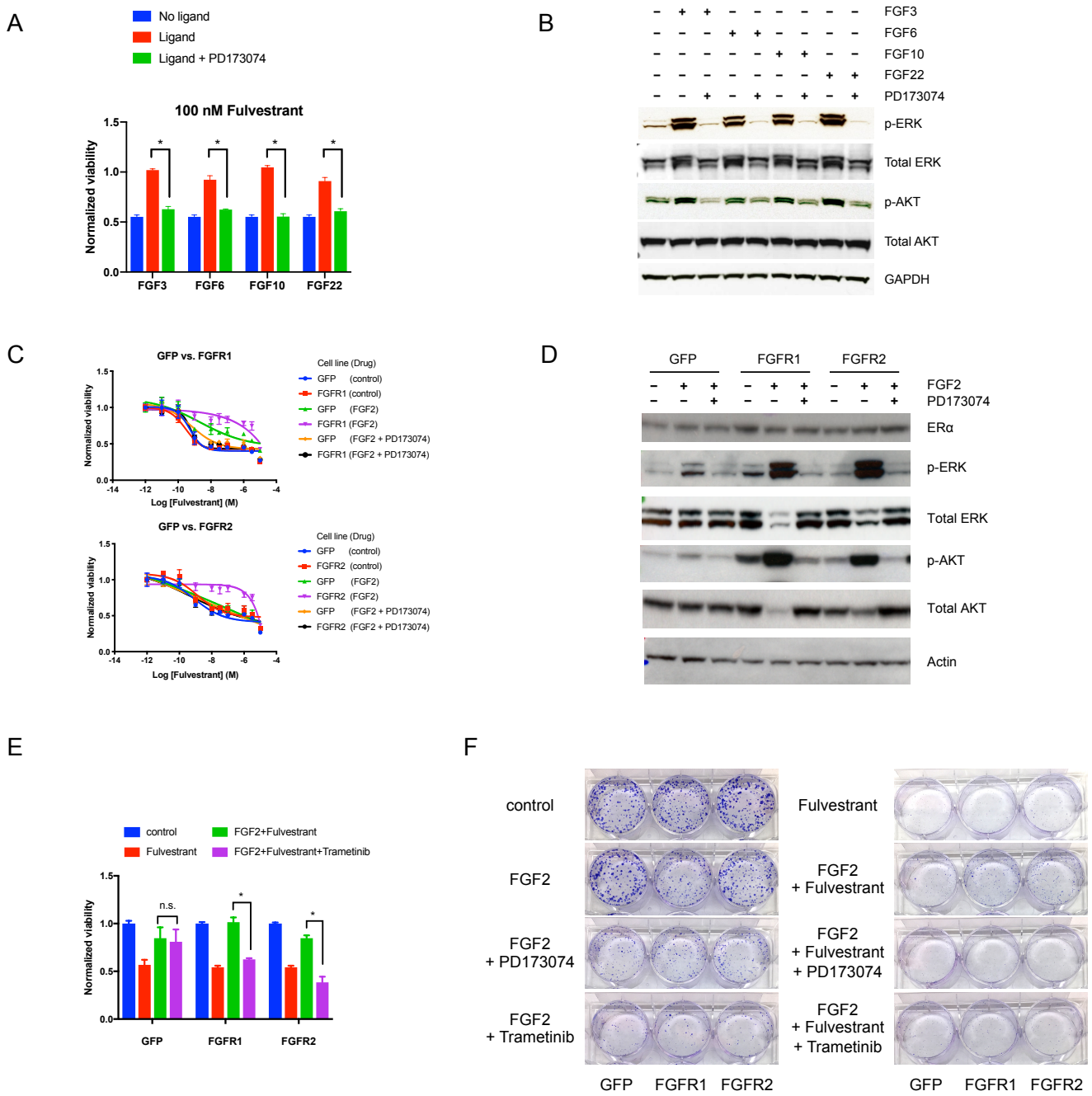
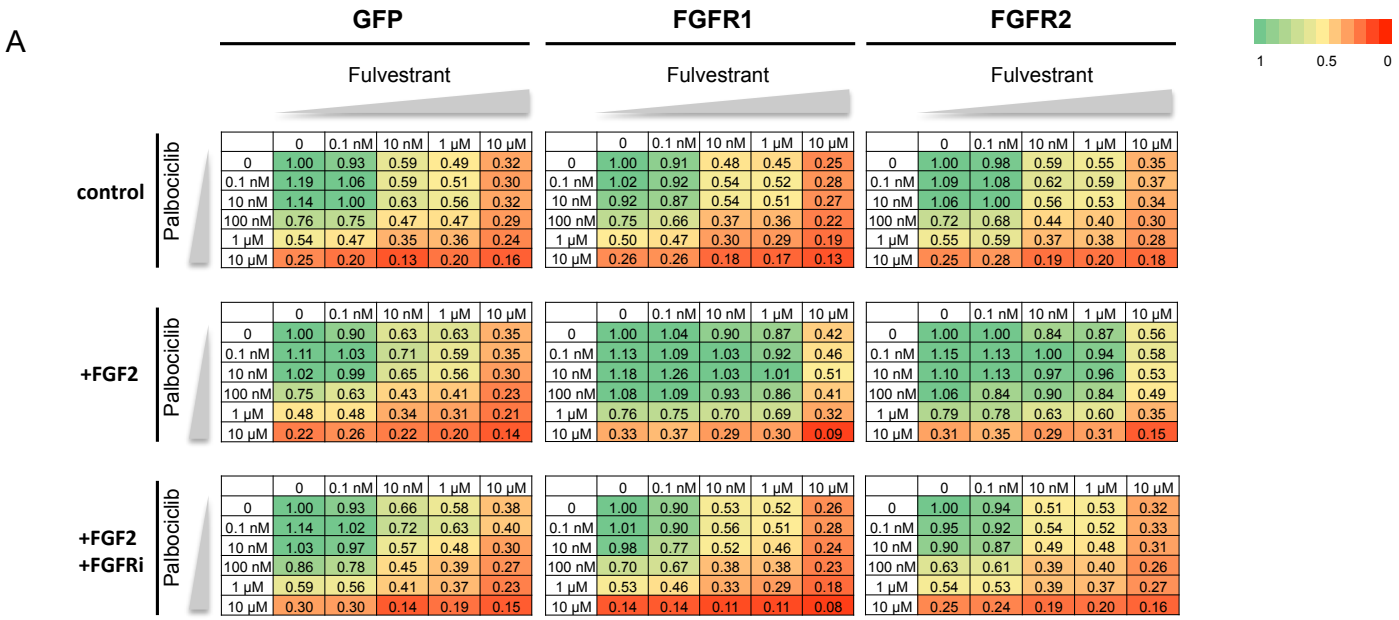


Figure 4. Active FGFR signaling leads to resistance to SERDs through activation of MAPK pathway. A, FGF ligands lead to resistance to fulvestrant, which was blocked by FGFR inhibitor PD173074. Recombinant FGF ligands were added into media every three days at the concentration of 100 ng/mL with or without 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



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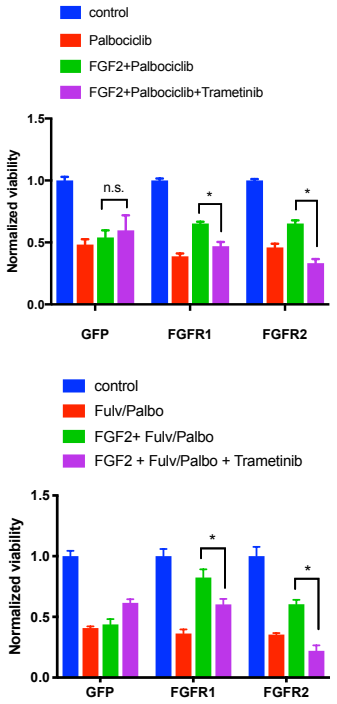

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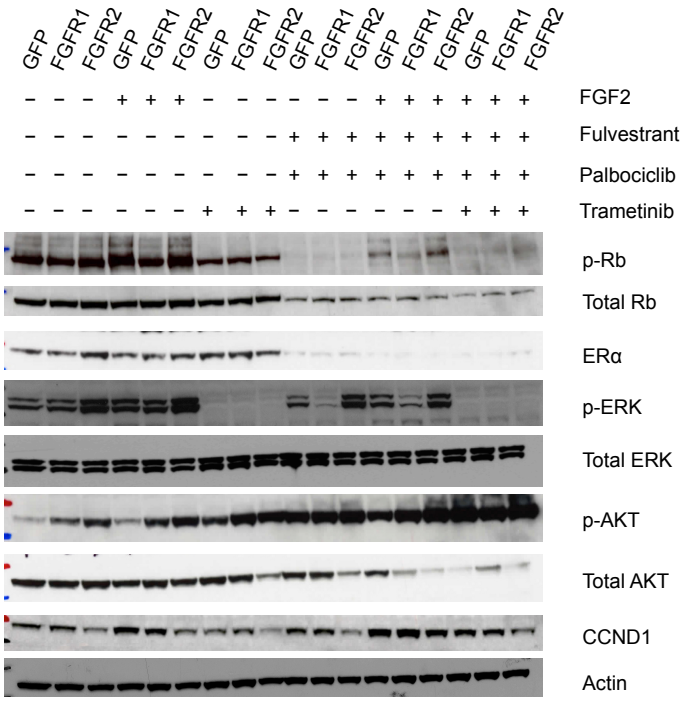

Figure 5. **Active FGFR signaling leads to resistance to the combination of SERDs and CDK4/6 inhibitors.** A, T47D_GFP, T47D_FGFR1 and T47D_FGFR2 cells were treated with the combination of fulvestrant and palbociclib at various doses for six days. The percentage of cell survival shown was normalized 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

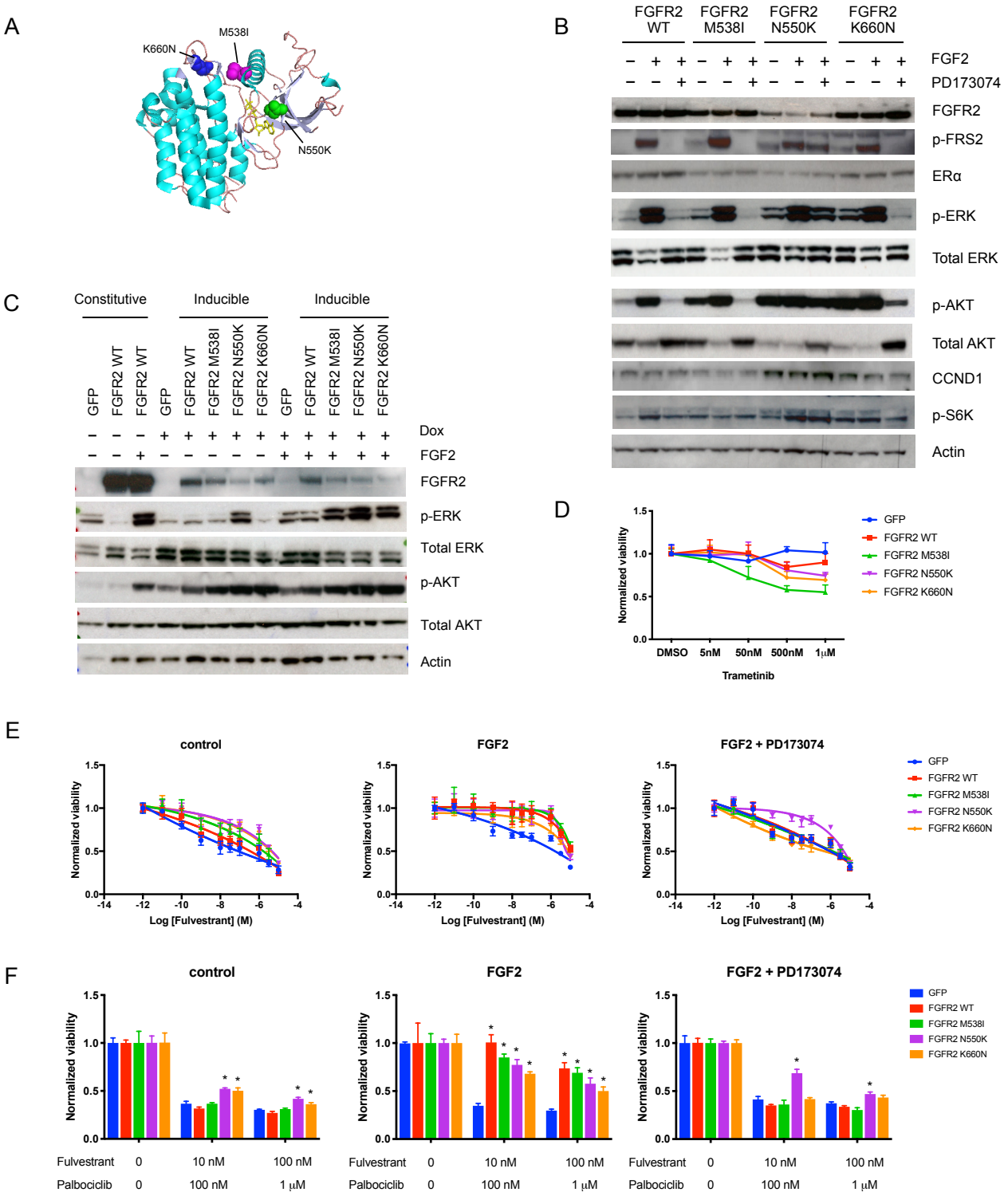

Figure 6. FGFR2 M538I, N5550K and K660N were activating mutations and conferred resistance to fulvestrant and/or palbociclib. A, Crystal structure of activated FGFR2 protein with mutations shown. FGFR2 is in complex with ATP analog (in yellow) and substrate peptide (PDB ID: 2PVF). B, Stable cell lines 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

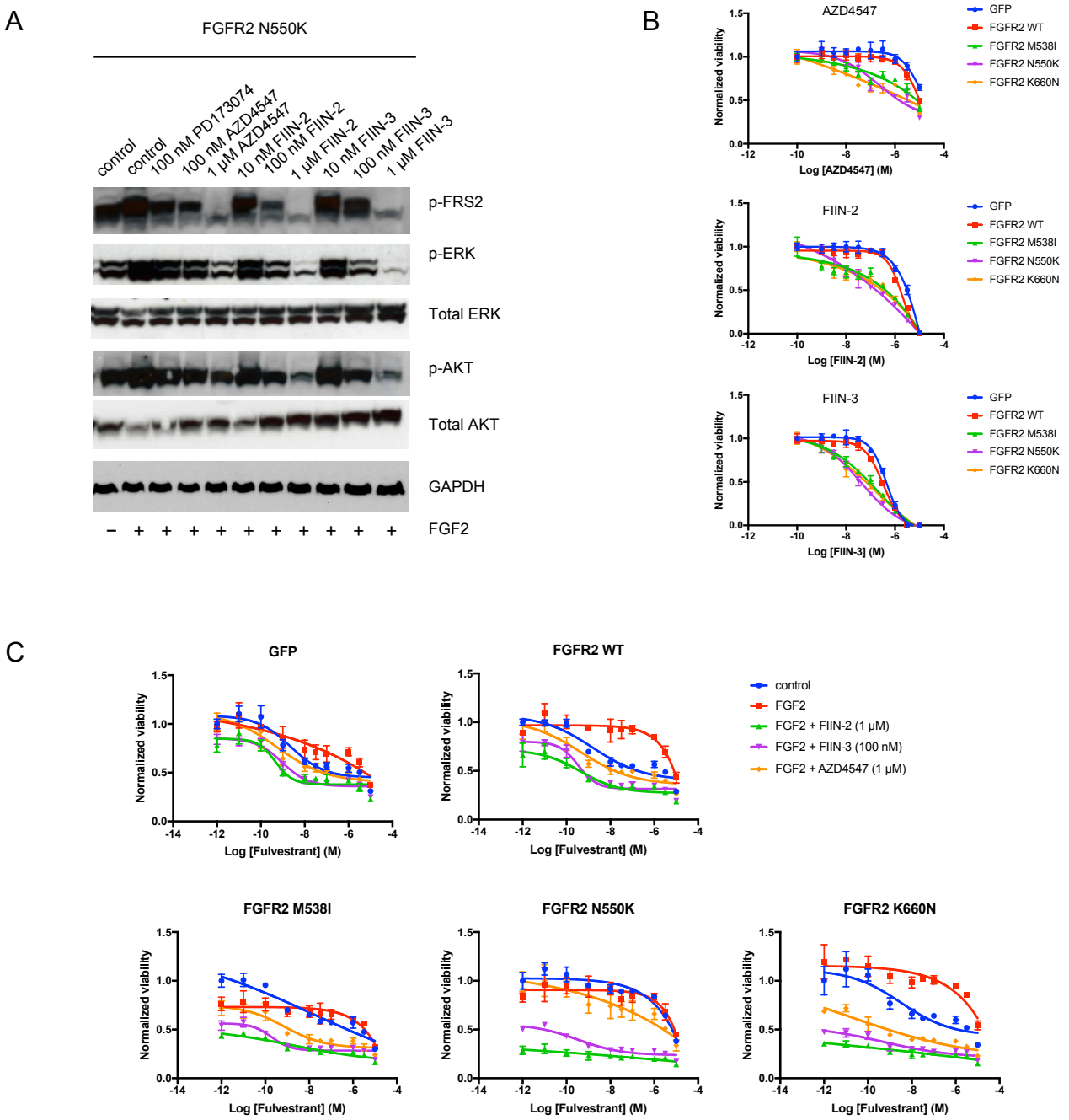

Figure 7. Activating FGFR2 mutations were targeted by irreversible kinase inhibitors FIIN-2 and FIIN-3. A, T47D cells overexpressing FGFR2 N550K cells were treated as indicated for three days and retreated for three hours before protein harvest and western blot. B, Stable cell lines overexpressing FGFR2 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.