

1 **RTK-dependent inducible degradation of mutant PI3K $\alpha$  drives GDC-0077 (Inavolisib)**  
2 **efficacy**

3  
4 Kyung W. Song<sup>1+</sup>, Kyle A. Edgar<sup>9+</sup>, Emily J. Hanan<sup>2</sup>, Marc Hafner<sup>4</sup>, Jason Oeh<sup>7</sup>,  
5 Mark Merchant<sup>7</sup>, Deepak Sampath<sup>7</sup>, Michelle A. Nannini<sup>7</sup>, Rebecca Hong<sup>7</sup>, Lilian Phu<sup>3</sup>, William  
6 F. Forrest<sup>4</sup>, Eric Stawiski<sup>4</sup>, Stephen Schmidt<sup>8</sup>, Nicholas Endres<sup>8</sup>, Jane Guan<sup>1</sup>, Jeffrey J. Wallin<sup>7#</sup>,  
7 Jonathan Cheong<sup>6</sup>, Emile Plise<sup>6</sup>, Gail Philips<sup>1</sup>, Laurent Salphati<sup>6</sup>, Timothy P. Heffron<sup>2</sup>, Alan  
8 Olivero<sup>2</sup>, Shiva Malek<sup>1</sup>, Steven T. Staben<sup>2</sup>, Donald S. Kirkpatrick<sup>3</sup>, Anwasha Dey<sup>1\*</sup>, Lori S.  
9 Friedman<sup>9\*</sup>

10  
11 **Affiliations:** Departments of Discovery Oncology<sup>1</sup>, Discovery Chemistry<sup>2</sup>, Microchemistry,  
12 Proteomics & Lipidomics<sup>3</sup>, Bioinformatics<sup>4</sup>, Structural Biology<sup>5</sup>, Drug Metabolism and  
13 Pharmacokinetics<sup>6</sup>, Translational Oncology<sup>7</sup>, and Biochemical and Cell Biology<sup>8</sup>, Genentech,  
14 Inc., 1 DNA Way, South San Francisco, California 94080, USA

15 + These authors contributed equally

16 \* Corresponding authors

17 Anwasha Dey, PhD and Lori S. Friedman, PhD

18 Department of Discovery Oncology

19 Genentech, Inc.

20 1 DNA Way, MS 41-1a, South San Francisco, CA 94080

21 E-mail: [dey.anwasha@gene.com](mailto:dey.anwasha@gene.com); [friedman.lori@gmail.com](mailto:friedman.lori@gmail.com)

22 <sup>9</sup>Current address: Oric Pharmaceuticals 240 E. Grand Ave, F12 South San Francisco, CA 94080,  
23 USA

24

25 **Abstract**

26 *PIK3CA* is one of the most frequently mutated oncogenes; the p110 $\alpha$  protein it encodes plays a  
27 central role in tumor cell proliferation and survival. Small molecule inhibitors targeting the PI3K  
28 p110 $\alpha$  catalytic subunit have entered clinical trials, with early-phase GDC-0077 (Inavolisib)  
29 studies showing anti-tumor activity and a manageable safety profile in patients with *PIK3CA*-  
30 mutant, hormone receptor-positive breast cancer as a single agent or in combination therapy.  
31 Despite this, preclinical studies have shown that PI3K pathway inhibition releases negative  
32 feedback and activates receptor tyrosine kinase signaling, reengaging the pathway and attenuating  
33 drug activity. Here we discover that GDC-0077 and tselisib more potently inhibit mutant PI3K  
34 pathway signaling and cell viability through unique HER2-dependent degradation. Both are more  
35 effective than other PI3K inhibitors at maintaining prolonged pathway suppression, resulting in  
36 enhanced apoptosis and greater efficacy. This unique mechanism against mutant p110 $\alpha$  reveals a  
37 new strategy for creating inhibitors that specifically target mutant tumors with selective  
38 degradation of the mutant oncoprotein and also provide a strong rationale for pursuing PI3K $\alpha$   
39 degraders in patients with HER2-positive breast cancer.

40

41 **Keywords:** GDC-0077 (Inavolisib), tselisib, breast cancer, HER2-positive, *PIK3CA*, PI3K,  
42 p110 $\alpha$

43

44 **Introduction**

45 Oncogenic mutations in the *PIK3CA* gene increase lipid kinase activity and transform cells (Isakoff  
46 et al., 2005; Kang et al., 2005; Samuels et al., 2005). The alpha isoform of PI3K is a dimer

47 composed of the p110 $\alpha$  catalytic subunit and a p85 regulatory subunit which functions to stabilize  
48 p110 $\alpha$  and reduce kinase activity (Yu et al., 1998). The binding of a phosphorylated receptor  
49 tyrosine kinase (RTK) activates p110 $\alpha$  through the release of a subset of inhibitory contacts with  
50 p85 (Burke and Williams, 2013). Common hotspot mutations in *PIK3CA* helical (*E542K*, *E545K*)  
51 and kinase (*H1047R*) domains function by perturbing local interfaces between p85 and p110 $\alpha$   
52 (Echeverria et al., 2015; Miled et al., 2007) and increasing dynamic events required for catalysis  
53 on membranes (Burke et al., 2012). Several inhibitors of PI3K have entered clinical trials, yet in  
54 patients with *PIK3CA*-mutant tumors the efficacy has been modest, in part due to a limited  
55 therapeutic index (Krop et al., 2016; Martin et al., 2017; Mayer et al., 2017; Rodon et al., 2013).  
56 Hence, we reasoned that it might be possible to improve the therapeutic index by identifying  
57 compounds with increased specificity for mutant p110 $\alpha$ .

58

## 59 **Results**

### 60 **PI3K inhibitor potency in *PIK3CA*-mutant cells**

61 A selection of PI3K inhibitors were profiled for biochemical activity and pharmacokinetic  
62 properties, including inhibitors across several chemical classes and with varying p110 isoform  
63 selectivity (Figure 1A). Taselisib and GDC-0077 showed increased mutant potency in cell viability  
64 assays in a cancer cell line panel compared with other PI3K inhibitors (including the alpha isoform-  
65 selective inhibitor, BYL719) (Figure 1B). Furthermore, taselisib (and later, GDC-0077) was a  
66 stronger inducer of cell death compared with other compounds, specifically in *PIK3CA*-mutant  
67 cancer cell lines (Figure S1A), suggesting that taselisib is more potent in *PIK3CA*-mutant cells  
68 compared with to other PI3K inhibitors. We compared PI3K inhibitor potencies in parental  
69 isogenic SW48 colon cancer cells bearing wild-type (WT) *PIK3CA* and matched isogenic lines

70 expressing *H1047R* or *E545K* hotspot mutants knocked into one allele of the *PIK3CA* locus.  
71 Taselisib potency (half maximal effective concentration [EC<sub>50</sub>]) increased 3-fold in *PIK3CA*-  
72 mutant cells versus parental WT SW48 cells, while GDC-0941 (Folkes et al., 2008) displayed  
73 comparable EC<sub>50</sub> in mutant and WT cells (Figure 1C). To assess whether this potency shift was  
74 correlated with the taselisib chemical scaffold, structurally related analogs with increased alpha-  
75 isoform specificity, GNE-102 and GNE-326 (Heffron et al., 2016), and a PI3K $\alpha$  inhibitor from an  
76 unrelated chemical class (BYL719) (Furet et al., 2013) were assessed and found to not have a  
77 potency differential in mutant versus WT isogenic cells (Figure S1B). We also confirmed that  
78 inhibition of multiple PI3K isoforms did not play a role in this increased potency. Taselisib binds  
79 equipotently to p110 $\alpha$  and p110 $\delta$  isoforms but is selective against p110 $\beta$  and p110 $\gamma$  isoforms  
80 (Figure 1A). However, combination of a p110 $\alpha$  inhibitor (GNE-102) with a p110 $\delta$  inhibitor  
81 (idelalisib) (Sadhu et al., 2003) did not impact cell potency, nor did the combination of taselisib  
82 with a p110 $\beta$  inhibitor (TGX-221) (Jackson et al., 2005) (Figures S1C and S1D). Neither cell  
83 permeability differences nor intracellular accumulation of inhibitors could explain the increased  
84 potency of taselisib in mutant cells (Figure S1E).

85

### 86 ***In vivo* efficacy of GDC-0077**

87 We next asked whether this greater potency and enhanced cell death manifested *in vivo* in  
88 mutant PI3K tumor xenografts. Indeed, we observed greater efficacy for taselisib and GDC-0077  
89 compared with a maximum tolerated dose (MTD) of BYL719 (Figure 1D). In addition, GDC-0077  
90 treatment at the MTD *in vivo* resulted in tumor regressions in multiple *PIK3CA*-mutant xenograft  
91 and patient-derived xenograft models (HCC1954, KPL4, and HCI-003 PDX) (Figures S1F).

92           Given the potency of GDC-0077 and the significant improvement in PI3K $\alpha$  isoform  
93 selectivity over both taselelisib and BYL719 (Figures 1A and 1B), we next evaluated the efficacy of  
94 GDC-0077 and the suitability for combination with standard of care in hormone receptor (HR)-  
95 positive/HER2-negative breast cancers. These included aromatase inhibitors and, more recently,  
96 CDK4/6 inhibitors such as palbociclib. We therefore assessed the effect of combining GDC-0077  
97 with these drugs to evaluate efficacy and safety. First, we measured *in vitro* growth of five  
98 *PI3KCA*-mutant HR-positive lines across different concentrations of GDC-0077 with or without  
99 E2 (to mimic aromatase inhibitors [AIs]) and with or without 0.15  $\mu$ M of palbociclib. To assess  
100 growth, endpoint cell population, as measured by CyQuant assay, was normalized to the cell  
101 population at the time of treatment using the growth-rate inhibition (GR) method (Hafner et al.,  
102 2016). A GR value of 1 meant no inhibition, 0 meant no net growth, and negative values  
103 represented cell loss. Response in *PIK3CA E545K*-mutant MCF-7 cells showed that addition of  
104 GDC-0077 induced a strong cytotoxic response in all combination treatments, as reflected by  
105 negative GR values for concentrations of 0.12 $\mu$ M and above (Hafner et al., 2016) (Figure 2A).  
106 When assessing the efficacy of the combination treatments across all five cell lines, we found that  
107 the GR values measured in the condition with palbociclib and without E2 (equivalent to AI)  
108 decreased by a median of 0.25 when adding 0.123  $\mu$ M of GDC-0077, showing a broad increase in  
109 efficacy by combining GDC-0077 with standard of care treatments (Figure 2B and S2A).

110           We then sought to validate this increased efficacy *in vivo*. Palbociclib (50 mg/kg) combined  
111 with fulvestrant (200mg/kg) only conferred tumor growth inhibition (TGI) of 71%, whereas  
112 addition of GDC-0077 (25 mg/kg) further reduced tumor burden (mean TGI of 106%, N = 12,  
113 Figure 2C), consistent with the response observed *in vitro* (Figure 2A). Weight loss was modest at  
114 8.1% in the triple combination cohort (Figure S2B), suggesting tolerability of adding GDC-0077

115 to a regimen with endocrine therapy plus CDK4/6 inhibitors. Similar efficacy results were obtained  
116 with taselisib combined with palbociclib (Figure S2C), although weight loss was higher (Figure  
117 S2C) with taselisib as expected. Also, taselisib could only be combined with palbociclib and not  
118 the triple combination. Taken together, these data suggested the possibility of combining GDC-  
119 0077 with both palbociclib and fulvestrant for superior efficacy in *PIK3CA*-mutant, *HER2*-  
120 negative tumors and providing a therapeutic window not achievable with earlier PI3K inhibitors.

121

### 122 **Taselisib and GDC-0077 induce mutant p110 $\alpha$ degradation**

123 In order to determine the mechanistic basis for this efficacy differential in *PIK3CA*-mutant  
124 tumors, we compared the effects downstream (pAKT) and upstream (pHER3) of PI3K signaling  
125 for GDC-0077 versus BYL719 over a timecourse. In both cases we observed robust acute  
126 inhibition of pAKT treatment; however, GDC-0077 demonstrated sustained inhibition of pAKT  
127 over the 24-hour treatment time despite inducing release of negative feedback, as measured by  
128 upregulation of pHER3 (as is also observed for BYL719) (Figure 3A).

129 We reasoned that one possible mechanism of enabling of sustained inhibition of pAKT in  
130 spite of elevated pHER3 levels would be via drug-induced sequestration of PI3K away from the  
131 plasma membrane. In order to investigate this hypothesis, levels of p110 $\alpha$  protein in sub-cellular  
132 fractions were evaluated by western blot at various timepoints after taselisib treatment.  
133 Unexpectedly, we discovered time-dependent p110 $\alpha$  protein depletion from the *PIK3CA*-mutant  
134 cells following taselisib treatment, regardless of the subcellular fraction evaluated (Figure S3A).  
135 BYL719 did not significantly impact p110 $\alpha$  protein levels. Comparing whole cell lysates from  
136 *PIK3CA*-mutant and -WT breast cancer cells by western blot, taselisib treatment for 8 hours caused  
137 the dose-dependent depletion of p110 $\alpha$  protein specifically in *PIK3CA H1047R*-mutant HCC1954

138 cells. No significant change was observed for p110 $\alpha$  in *PIK3CA*-WT HDQ-P1 cells (Figure S3B).  
139 Treatment with BYL719 did not affect p110 $\alpha$  levels in any cell lines tested. Transcription of  
140 *PIK3CA* alleles was not diminished following taselesib treatment (Figure S3C), implicating post-  
141 transcriptional regulation of mutant p110 $\alpha$  protein.

142 We investigated p110 $\alpha$  protein depletion in p110 $\alpha$  mutant xenograft breast cancer models  
143 and found that membrane-associated p110 $\alpha$  was depleted 4 hours after a single oral dose of 15  
144 mg/kg taselesib in individual tumors from a p110 $\alpha$ -mutant HCC1954 xenograft model, and that  
145 p110 $\alpha$  depletion was not observed with 40 mg/kg BYL719 (Figure S3D). In the same xenograft  
146 model, a single oral dose of 50 mg/kg GDC-0077 depleted p110 $\alpha$  protein expression for up to 8  
147 hours, further confirming a similar mechanism of action for both taselesib and GDC-0077. In  
148 Phase Ia clinical trials, taselesib and GDC-0077 had antitumor activity in *PIK3CA*-mutant tumors  
149 as assessed by response rates (Juric et al., 2017). The free drug exposures achieved in the clinic  
150 were evaluated in tissue culture experiments with *PIK3CA* mutant breast cancer lines. Unlike  
151 pictilisib (GDC-0941), both taselesib (GDC-0032) and inavolisib (GDC-0077) treatment for 24  
152 hours resulted in p110 $\alpha$  degradation at clinically relevant concentrations (Figure S4A).

153 To generate direct evidence that taselesib or GDC-0077 treatment was preferentially  
154 depleting mutant p110 $\alpha$  protein within a mixed WT and mutant allelic population, we took  
155 advantage of a neo-tryptic peptide encoded by the *H1047R* mutation to assess p110 $\alpha$  protein levels  
156 by mass spectrometry. HCC1954 parental cells expressing both mutant and WT p110 $\alpha$  were  
157 treated with taselesib for 24 hours, revealing loss of the mutant specific peptide only in the taselesib-  
158 treated sample,  $p=0.00016$  (Figure 3B). There was no significant change in WT p110 $\alpha$ , while the  
159 total p110 $\alpha$  pool decreased commensurate with the initial abundance of the mutant protein (Figure  
160 S4B). Similarly, depletion of the E545K mutant protein was observed in HCC202 heterozygous

161 cells, as shown by assaying a second mutant specific neo-tryptic peptide at this locus,  $p=0.032$   
162 (Figure S4C).

163 To further explore the molecular mechanisms underlying inhibitor-induced mutant p110 $\alpha$   
164 depletion, we examined whether reduction of p110 $\alpha$  could be attributed to degradation. A  
165 proteasome inhibitor, MG132, and a ubiquitin-activating enzyme E1 inhibitor both rescued the  
166 p110 $\alpha$  degradation induced by tselisib and GDC-0077 (Figure 3C), while lysosomotropic agents  
167 failed to do so (Figure S4D). We next employed ubiquitin pull down assays to confirm that mutant  
168 p110 $\alpha$  is inducibly ubiquitinated upon tselisib treatment, and that this signal further accumulated  
169 when cells were co-treated with MG132 to prevent proteasomal degradation of ubiquitinated  
170 p110 $\alpha$  (Figure 3D). Accordingly, no ubiquitinated p110 $\alpha$  was detected in cells treated with UAE1  
171 inhibitor (Figure 3D). Importantly, tselisib also induced ubiquitination and degradation of E545K  
172 p110 $\alpha$  in the HCC202 cell model, with comparable rescue by MG132 and UAE1 inhibition (Figure  
173 3D).

174 Visualization of p110 $\alpha$  depletion on western blot was more easily discerned in HCC1954  
175 cells, which have an increased copy number of the mutant *p110 $\alpha$*  allele. Quantitative Reverse  
176 Transcription PCR (qRT-PCR) analysis confirmed higher expression of the mutant allele (Figure  
177 S4E). In order to generate a clean system to better compare differential effect of inhibitors on WT  
178 and mutant p110 $\alpha$ , we used CRISPR/cas9 to generate isogenic HCC1954 cell lines bearing  
179 either the *H1047* WT allele or the mutant *H1047R* allele, named HCC1954\_mutant and  
180 HCC1954\_WT (Figure S4E). Tselisib treatment resulted in ubiquitination and depletion of p110 $\alpha$   
181 protein only in the HCC1954\_mutant cells, and not in the matched HCC1954\_WT cells (Figure  
182 3E and Figure S4F).



183           Since the basal level of ubiquitination was significantly higher for membrane-bound  
184 mutant p110 $\alpha$  compared with WT p110 $\alpha$  (Figure S4G), we hypothesized that the mutant p110 $\alpha$   
185 protein may be inherently less stable than the WT protein, as previously noted for mutant EGFR  
186 (Greig et al., 2015). Consistent with prior literature (Yu et al., 1998), pulse-chase experiments in  
187 HCC1954\_WT isogenic cells indicated a protein half-life for WT p110 $\alpha$  of ~26.7 hours. In  
188 contrast, the H1047R mutant protein half-life was ~9.6 hours in the basal state, and was further  
189 shortened to ~4 hours upon treatment with tasisib (Figure 3F). Together these data demonstrate  
190 that mutant p110 $\alpha$  oncoprotein is inherently less stable and more vulnerable to inhibitor-mediated  
191 degradation in a ubiquitin and proteasome dependent manner.

192

### 193 **p85 $\beta$ potentiates p110 $\alpha$ degradation by recruiting p110 $\alpha$ to the membrane**

194           It has previously been shown that p110 $\alpha$ /p85 dimers are activated by growth factor-  
195 stimulated RTK signaling (Cantley et al., 1991). The mechanism of this activation involves release  
196 of inhibitory contacts between p110 and p85 once bound to phosphotyrosine residues of an RTK  
197 (Backer, 2010; Burke and Williams, 2013). We next reasoned that the ability of our small molecule  
198 degraders to accelerate the turnover of activated mutant protein might be through appropriation of  
199 the p110 $\alpha$  activation process. In support of this hypothesis, cell fractionation studies showed that  
200 tasisib-inducible ubiquitination of p110 $\alpha$  occurred preferentially in the membrane fraction  
201 (Figure 4A).

202           One major effector that recruits p110 $\alpha$  to RTK is the p85 PI3K regulatory subunit. There  
203 are three p85 isoforms in class 1A PI3K: p85 $\alpha$ , p85 $\beta$ , and p55 $\gamma$ . We first confirmed comparable  
204 expression levels of all three isoforms in HCC1954 cells (Figure S5A). By co-immunoprecipitation  
205 we also demonstrated that all three isoforms interact with mutant p110 $\alpha$  (Figure S5A). We next

206 evaluated the effect of p85 isoforms on inhibitor-induced p110 $\alpha$  degradation. Knockdown of both  
207 p85 $\alpha$  and p55 $\gamma$  showed similar levels of taselesib-induced p110 $\alpha$  degradation compared with  
208 control siRNA-treated cells. In contrast, we observed that p85 $\beta$  KD rescued taselesib-mediated  
209 p110 $\alpha$  degradation (Figure 4B). We further observed that p85 $\beta$  KD resulted in inhibition of  
210 pathway signaling, shown by reduced pAKT levels. These data suggest that p85 isoforms do not  
211 have redundant roles and that the p85 $\beta$ , but not the p85 $\alpha$  or p55 $\gamma$  isoforms is involved in mutant  
212 p110 $\alpha$  degradation. Reduced pAKT levels with p85 $\beta$  KD is most likely a result of reduced p110 $\alpha$   
213 membrane localization.

214 This observation also suggested that p85 isoforms have differential affinity for RTK  
215 interaction. To address this question, we next performed a series of co-immunoprecipitation  
216 experiments. p85 $\alpha$  or p85 $\beta$  were immunoprecipitated from the membrane fraction and  
217 immunoblotted with several antibodies, including four receptors that are highly activated in  
218 HCC1954 cells (Figure S5B). Both p85 $\alpha$  and p85 $\beta$  interacted with p110 $\alpha$ , consistent with previous  
219 results. We also observed a stronger association between p85 $\beta$  and HER2 and HER3 than observed  
220 for p85 $\alpha$  or p55 $\gamma$  protein (Figure 4C, Figure S5C). We could not detect an interaction between p85  
221 isoforms with EGFR or c-MET under these conditions.

222 To further confirm this observation, we next tested whether inhibition of HER2  
223 phosphorylation blocked p85 $\beta$  binding to HER2 and rescue p110 $\alpha$  degradation. Cells were treated  
224 with taselesib or lapatinib alone or in combination for various timepoints. These cell lysates were  
225 next immunoprecipitated using p85 $\alpha$  or p85 $\beta$  antibody and immunoblotted with HER2 and HER3  
226 antibodies. In taselesib-treated cells, strong interaction between p85 $\beta$  and HER2/3 was observed.  
227 Treatment with lapatinib blocked this interaction, confirming that activated HER2 contributes to  
228 p85 $\beta$  membrane binding (Figure S5D). These results further demonstrated that p85 $\beta$  plays an

229 important role in recruiting p110 $\alpha$  to the membrane, and that this is likely through binding to  
230 activated HER2 and HER3.

231 To further confirm the role of p85 $\beta$  in p110 $\alpha$  degradation, we next tested the effects of p85  
232 knockdown on K63 and K48 polyubiquitin chain formation on p110 $\alpha$ . Upon knockdown of p85 $\alpha$   
233 or p85 $\beta$  and following inhibitor treatment, K63 or K48 ubiquitin conjugated proteins were  
234 immunoprecipitated using linkage specific antibodies and immunoblotted with p110 $\alpha$ . In both  
235 control- and p85 $\alpha$ -depleted cells, tasisib treatment induced both K63 and K48 linked  
236 ubiquitination on p110 $\alpha$ . However, upon p85 $\beta$  depletion, ubiquitination of p110 $\alpha$  was no longer  
237 detected (Figure 4D). These data further confirm that the p85 $\beta$  regulatory subunit plays an  
238 important role in tasisib-induced mutant p110 $\alpha$  degradation, by recruiting p110 $\alpha$  to the  
239 membrane where ubiquitination occurs.

240

#### 241 **Tasisib- and GDC-0077-induced mutant p110 $\alpha$ degradation is dependent on RTK activity**

242 To further understand whether inhibitor-induced mutant p110 $\alpha$  degradation is dependent  
243 on its recruitment to activated RTK, we next investigated the efficacy of the two clinically relevant  
244 molecules, GDC-0077 and BYL719 (Alpelisib). Unexpectedly, we observed a significant  
245 difference in the sensitivity of *HER2*-amplified (~20-fold difference between the mean IC<sup>50</sup> values)  
246 versus *HER2*-negative cell lines (~6-fold difference between two inhibitors) to GDC-0077 versus  
247 BYL719. The inhibitors were not differentiated in WT cell lines regardless of *HER2* status (Figure  
248 5A). These data imply that RTK activity may define the sensitivity of a cell line to inducible  
249 degradation of mutant-p110 $\alpha$ . To confirm this finding, a panel of over 50 cell lines harboring  
250 *PIK3CA* hot spot mutations were analyzed (Figure 5B). Most of these were heterozygous, carrying  
251 both WT and mutant *PIK3CA* alleles at differing frequencies. For easier visualization of mutant

252 p110 $\alpha$  depletion, we analyzed the cell lines with higher copy numbers of mutant alleles that also  
253 represented each hot spot mutation across various tumors (Figure 5B, colored in blue). In a cell  
254 proliferation assay, these cell lines have varying GDC-0077 sensitivity. All of the selected  
255 representative cell lines responded to GDC-0077, as measured by inhibition of pAKT. Not all cell  
256 lines showed visible p110 $\alpha$  degradation; those that showed mutant p110 $\alpha$  degradation were  
257 HCC2185, HCC1954, MDAMB453, and KPL4 (Figure 5B). It was particularly striking that all  
258 *HER2*-amplified cell lines showed p110 $\alpha$  degradation. In contrast, the *HER2*-negative cell lines  
259 were resistant to degradation except HCC2185. To further understand this observation, we tested  
260 if activating RTK by addition of growth factors would induce p110 $\alpha$  degradation. A subset of  
261 *HER2*-negative cell lines, each harboring WT or one of three p110 $\alpha$  hot spot mutations, were  
262 treated with GDC-0077 alone or in combination with growth factors. All responded to growth  
263 factors, as shown by induction of HER3 phosphorylation. Consistent with our data, p110 $\alpha$  levels  
264 did not decrease in *HER2*-negative cell lines treated with GDC-0077 alone. However, the  
265 combination of growth factors and GDC-0077 induced p110 $\alpha$  degradation in all three p110 $\alpha$ -  
266 mutant lines. WT p110 $\alpha$  protein expression was not affected by addition of growth factors (Figure  
267 5C). Immunoprecipitation and pull down of p110 $\alpha$  revealed that p110 $\alpha$  degradation was markedly  
268 delayed when RTK interaction with the p110 $\alpha$ /p85 complex was disrupted, with limited inducible  
269 degradation observed at the 8 hr timepoint (Figure 5D, upper panel). Conversely, inhibition of  
270 RTK phosphorylation by lapatinib partially rescued tasisib-mediated p110 $\alpha$  degradation in  
271 HCC1954 cells (Figure 5D, lower panel). Similar to tasisib, GDC-0077-induced mutant p110 $\alpha$   
272 degradation was rescued by lapatinib treatment (Figure 5E). In support of these results, we  
273 observed that in multiple patient derived xenograft (PDX) models, high basal pHER2 and pHER3  
274 expression level correlated with better tasisib-mediated p110 $\alpha$  degradation, with weaker

275 degradation observed in tumors with low levels of pRTK (Figure S6A). In addition, within this  
276 group of PDX models, the degree of degradation appeared to correlate with basal pRTK  
277 expression. Taken together, our data support a model where small molecule-induced p110 $\alpha$  mutant  
278 degradation depends on RTK activity in *PIK3CA* mutant cancers.

279

280 **p110 $\alpha$ -mutant degrading inhibitors provide more sustained benefit in HER2-positive versus**  
281 **HER2-negative p110 $\alpha$ -mutant cancers**

282         Given that RTK reactivation could potentially limit efficacy, we also aimed to understand  
283 activity in HER2-positive breast cancers. Degradation of mutant p110 $\alpha$  blocked the feedback  
284 induced pathway reactivation and resulted in enhanced potency of tasisib and GDC-0077 in  
285 cellular assays and an increase in apoptosis in mutant cells. Our data thus far predict that drugs  
286 which induce degradation of mutant p110 $\alpha$  may show more sustained benefit over non-degraders  
287 by preventing pathway reactivation. We speculated that the extent of negative feedback depends  
288 on the amount of RTK expression, implying that inhibitor-mediated feedback pathway reactivation  
289 would be weak in *HER2*-negative cells. Also, based on our findings, low RTK activity may result  
290 in inefficient mutant p110 $\alpha$  degradation. Pathway reactivation was not observed in *HER2*-negative  
291 cells, in contrast to *HER2*-amplified cells, irrespective of the inhibitors used. This suggests that in  
292 *HER2*-negative mutant cells, the degrader mechanism of action may not provide additional benefit  
293 over drugs with a non-degrader mechanism (model in Figure 6A). This was further confirmed in  
294 a panel of *PIK3CA* mutant cell lines. In *HER2*-amplified cells, GDC-0077 resulted in sustained  
295 pathway inhibition, while BYL719 activity was attenuated, as evidenced by rebound of pAKT  
296 levels (Figure S6B). In contrast, in a panel of *HER2*-negative lines, there was no difference in  
297 pathway inhibition by both inhibitors, GDC-0077 or BYL719.

298           Based on these data, we posited that we could best leverage the degradation potential of  
299 GDC-0077 in *HER2*-amplified cancers. In *HER2*-driven breast cancers, *HER2*-targeted therapy is  
300 the standard of care. However, *HER2*-amplified tumors with p110 $\alpha$  mutations are less responsive  
301 to *HER2*-targeted therapy. Therefore, combination treatment of the PI3K pathway inhibitors and  
302 *HER2* targeted therapy should result in enhanced efficacy. Indeed, combination of *HER2*  
303 inhibitors, trastuzumab and pertuzumab, in combination with taselisib in a *HER2*-positive mutant  
304 p110 $\alpha$  KPL-4 xenograft model showed better response compared with single drugs alone (Figure  
305 6B). Furthermore, combining ado-trastuzumab emtansine (TDM-1) with GDC-0077 showed a  
306 synergistic effect in the KPL-4 xenograft model as well (Figure 6C).

307           Given that most p110 $\alpha$ -mutant cells are heterozygous, selective mutant p110 $\alpha$  degradation  
308 would imply a possible scenario where WT p110 $\alpha$  protein is still present and can reactivate the  
309 same pathway and dampen inhibitor activity (Figure S6C). To test this possibility, we compared  
310 levels of feedback reactivation in an engineered homozygous versus heterozygous line. In a  
311 HCC1954 WT homozygous isogenic line, the pathway was reactivated by both GDC-0077  
312 (degrader) and BYL719 (non-degrader), as expected (Figure S6D). In a HCC1954 mutant  
313 homozygous isogenic line, BYL719, but not GDC-0077 treatment reactivated pathway signaling.  
314 Importantly, when compared, the phenotype of a heterozygous parental line behaved similar to  
315 that of a p110 $\alpha$  mutant homozygous line, suggesting that in heterozygous cells, mutant p110 $\alpha$   
316 functions as the main driver of cell signaling (Figure S6D). Furthermore, cell proliferation assays  
317 mirrored the inhibitor efficacy as well. GDC-0077 showed the same efficacy between homozygous  
318 and heterozygous mutant lines but showed reduced efficacy in WT isogenic lines (Figure S6D),  
319 while no shift in IC<sub>50</sub> was observed between all lines following BYL719 treatment. To further  
320 confirm these results, the same experiment was performed in *HER2*-negative lines. Consistent with

321 our hypothesis for the role of HER2 in pathway reactivation, feedback pathway reactivation was  
322 not detected even in a homozygous WT line upon treatment with GDC-0077 or BYL719. In cell  
323 viability assays, neither inhibitor showed a shift in efficacy between isogenic lines (Figure S6E).  
324 Taken together, our data demonstrate that degradation potential of GDC-0077 would be most  
325 leveraged in HER2-positive breast cancers for more sustained pathway inhibition.

326

## 327 **Discussion**

328 In summary, we have discovered that the mutant p110 $\alpha$  oncoprotein has unique  
329 characteristics compared with WT p110 $\alpha$ : a shorter half-life, ubiquitination in the membrane  
330 fraction, and proteasome-mediated turnover. Furthermore, the mutant oncoprotein is susceptible  
331 to increased proteasome-mediated degradation upon binding particular PI3K inhibitors such as  
332 tasisib and GDC-0077. Our results suggest that RTK activity plays a key role in regulating  
333 p110 $\alpha$  degradation by recruiting p110 $\alpha$  to the membrane. The mutant oncoprotein may be  
334 particularly vulnerable to additional local conformational changes that impact membrane binding,  
335 and tasisib and GDC-0077 may be enhancing this effect to accelerate proteasome-mediated  
336 degradation. This discovery reveals a new mechanism of action to exploit in *PIK3CA*-mutant  
337 tumors, opening an exciting path to increasing drug efficacy for the predominant oncogene in  
338 cancer. With a combined mutant-p110 $\alpha$  degrader mechanism and exquisite p110 $\alpha$  isoform  
339 selectivity, the PI3K $\alpha$ -selective inhibitor and mutant PI3K $\alpha$  degrader GDC-0077 may provide  
340 opportunities for previously inaccessible combination therapy with greater clinical responses.  
341 Moreover, this suggests that it may be possible to exploit endogenous mechanisms and the intrinsic  
342 instability of mutant oncoproteins more broadly to develop tumor-selective therapeutic agents.  
343 Based on these mechanistic discoveries, it may be possible to create efficacious compounds that

344 specifically deplete the mutant p110 $\alpha$  protein without blocking WT PI3K signaling, similar to  
345 engineered mouse models of cancer in which removal of the mutant *PIK3CA* oncogene induces  
346 regressions in the presence of WT PI3K (Cheng et al., 2016; Engelman et al., 2008). For the over  
347 2 million cancer patients diagnosed annually with *PIK3CA*-mutant tumors, this discovery opens  
348 the possibility of a future therapeutic agent that solely targets tumor cells bearing mutant *p110 $\alpha$*   
349 without the systemic adverse effects of inhibiting WT signaling.

350 With respect to the clinical relevance of our findings, a first-in-human, open-label, Phase  
351 I/IB dose escalation study of oral daily GDC-0077 alone and in combination with endocrine and  
352 targeted therapies for *PIK3CA*-mutant solid tumors is ongoing. The single-agent portion of the  
353 study showed that GDC-0077 had a manageable safety profile with a maximum tolerated dose of  
354 9 mg once daily (supported by a linear pharmacokinetic profile), with promising anti-tumor  
355 activity (Juric et al., 2019). When combined at this dose with letrozole with and without palbociclib  
356 (Jhaveri et al., 2019), or when combined with fulvestrant the safety profile was also manageable  
357 with promising anti-tumor activity (Kalinsky et al., 2020). No drug-drug interactions were  
358 observed in the letrozole  $\pm$  palbociclib portion of the study (Jhaveri et al., 2019). Phase III trials  
359 are now ongoing to assess efficacy and safety in a randomized, controlled manner in locally  
360 advanced or metastatic HR-positive/HER2-negative breast cancer in combination with palbociclib  
361 and fulvestrant (NCT04191499). To further leverage the degradation potential of GDC-0077 in  
362 HER2-positive breast cancers, this work has also provided the rationale for a first-in-human, open-  
363 label, Phase I/IB dose escalation study of oral daily GDC-0077 in combination with standards of  
364 care (trastuzumab and pertuzumab) is also being enabled.

365

366 **Methods**



367 **Chemical reagents**

368 The proteasome inhibitor MG132 (474790) was obtained from Calbiochem EMD Millipore  
369 (Billerica, MA). Chloroquine (14774) was obtained from Cell Signaling (Danvers, MA).  
370 Ammonium Chloride NH<sub>4</sub>Cl (254134) was obtained from Sigma-Aldrich (St. Louis, MO). PI3K  
371 inhibitors and ubiquitin-activating E1 inhibitor (Chen et al., 2011) were provided by the chemistry  
372 department at Genentech, Inc. (South San Francisco, CA).

373

374 **Antibody reagents**

375 Antibodies to p110 $\alpha$  (4249), cleaved poly (ADP-ribose) polymerase (PARP) (9541),  
376 phosphorylated (phospho)-Akt Ser473 (4060), pS6 S235/236 (2111), anti-ubiquitin (3936), p110 $\delta$   
377 (34050), HER3 (12708), HER2 (2242), pHER2 Y1221/Y1222 (2243), and pHER3 Y128 (4791)  
378 were obtained from Cell Signaling (Danvers, MA). The antibody to  $\beta$ -actin (A5441) was from  
379 Sigma-Aldrich. Antibodies to Ras (ab52939), p85 $\alpha$  (ab133595), and p85 $\beta$  (ab28356) were  
380 obtained from Abcam (Cambridge, MA). Ubiquitin reagent TUBE1 (UM101) was obtained from  
381 LifeSensors, Inc. (Malvern, PA). The p55 $\gamma$  antibody (MAB6638) was obtained from R&D  
382 Systems (Minneapolis MN). Antibodies to GAPDH (MAB374) and p110 $\beta$  (04-400) were obtained  
383 from EMD Millipore.

384

385 **pPRAS40 ELISA assay**

386 SW48 isogenic cells were plated in 384-well tissue-culture treated assay plates (Cat. No. 781091;  
387 Greiner Bio-One; Monroe, NC) and incubated overnight at 37°C and 5% CO<sub>2</sub>. The three isogenic  
388 SW48 lines (WT, E545K, and H1047R) were plated and assayed in parallel. The following day,  
389 test compounds were serially diluted in DMSO and added to cells (final DMSO concentration of

390 0.5%). Cells were then incubated with drugs for 24 hours at 37°C and 5% CO<sub>2</sub>. After 24 hours,  
391 cells were lysed and phosphorylated proline-rich AKT substrate of 40 kDa (pPRAS40) levels were  
392 measured using the Meso Scale Discovery (MSD<sup>®</sup>) custom pPRAS40 384w Assay Kit (Cat. No.  
393 L21CA-1; Rockville, MD). Cell lysates were added to assay plates pre-coated with antibodies  
394 against pPRAS40 and allowed to bind to the capture antibodies overnight at 4°C. The detection  
395 antibody (anti-total pPRAS40, labeled with an electrochemiluminescent SULFO-TAG<sup>™</sup>) was  
396 added to the bound lysate and incubated for 1 hour at room temperature. The MSD<sup>®</sup> Read Buffer  
397 was added such that when voltage was applied to the plate electrodes, the labels bound to the  
398 electrode surface emitted light. The MSD<sup>®</sup> Sector Instrument measured the intensity of the light  
399 and quantitatively measured the amount of pPRAS40 in the sample. Percent inhibition of  
400 pPRAS40 per concentration of compounds was calculated relative to untreated controls. The EC<sub>50</sub>  
401 values were calculated using the 4-parameter logistic nonlinear regression dose-response model.  
402 Reported EC<sub>50</sub> values indicate an average value from three independent experiments. Standard  
403 deviations are reported as ± the reported EC<sub>50</sub> values for each cell line. The EC<sub>50</sub> in WT cells was  
404 divided by EC<sub>50</sub> in mutants to derive the fold-increase in potency in mutant cells.

405

#### 406 **Viability assay CellTiter-Glo<sup>®</sup>**

407 Cells were seeded (1000–2000 cells/well) in 384-well plates for 16 hours. On day two, nine serial  
408 1:3 compound dilutions were made in DMSO in a 96-well plate. The compounds were then further  
409 diluted into growth media using a Rapidplate robot (Zymark Corp., Hopkinton, MA). The diluted  
410 compounds were then added to quadruplicate wells in the 384-well cell plate and incubated at 37°C  
411 and 5% CO<sub>2</sub>. After 4 days, relative numbers of viable cells were measured by luminescence using  
412 CellTiter-Glo<sup>®</sup> (Promega) according to the manufacturer's instructions and read on a Wallac

413 Multilabel Reader (PerkinElmer, Foster City). The EC<sup>50</sup> calculations were carried out using Prism  
414 6.0 software (GraphPad, San Diego). The GR calculations and figures were performed using R  
415 scripts based on Hafner et al. (Hafner et al., 2016).

416

#### 417 **Nucleosome ELISA**

418 MDA-MB-453, HCC202, and Cal85-1 cells were plated in 96-well tissue-culture treated assay  
419 plates (Corning; Cat. No. 3904; Corning, NY) and incubated overnight at 37°C and 5% CO<sub>2</sub>. The  
420 following day, PI3K inhibitors were serially diluted in DMSO and added to cells (final DMSO  
421 concentration of 0.5%). Cells were then incubated with drugs for 72 hours at 37°C and 5% CO<sub>2</sub>.  
422 After 72 hours, cells were lysed and centrifuged at 200 g for 10 min. Histone-associated DNA-  
423 fragment levels were analyzed using the Cell Death Detection ELISAPLUS (Roche; Cat. No.  
424 11920685001; Basel Switzerland). 20 μL of supernatant was added to each well of the streptavidin  
425 capture plate followed by 80 μl of anti-histone-biotin, and DNA-peroxidase immunoreagent.  
426 Plates were incubated at room temperature for 2 hours shaking (300 RPM). Contents of the plate  
427 were removed followed by three washes with Incubation buffer. 100 μL of ABTS solutions was  
428 added to each well and plates were incubated for 10–20 min, after which 100 μL of ABTS Stop  
429 Solution was added to each well. Absorbance was measured at 405 nm and 490 nm of each plate.  
430 Fold increases were generated by assessing the increased of absorbance (A<sub>405 nm</sub>–A<sub>490 nm</sub>) of  
431 wells from cells treated with compounds, normalized to those treated with DMSO alone.

432

#### 433 **Cell lines and cell culture**

434 Cell lines were obtained from the ATCC. All cell lines underwent authentication by Short Tandem  
435 Repeat profiling, SNP fingerprinting, and mycoplasma testing (Yu et al., 2015). The isogenic colon

436 cancer cell lines SW48 human PIK3CA (H1047R/+) (HD103-005) and SW48 human PIK3CA  
437 (E545K/+) (HD103-001) and SW48 parental line were obtained from Horizon Discovery Ltd.  
438 (Cambridge, UK). Cell lines were grown under standard tissue-culture conditions in RPMI media  
439 with 10% fetal bovine serum (Gibco, 10082-147), 100 U/mL penicillin- streptomycin (Gibco,  
440 15140-122), 2 mmol/L L-glutamine (Gibco, 15030-081). *PIK3CA* mutation status and frequencies  
441 of all cell lines are summarized in **Figure 5B**. Cells were treated with compounds for the indicated  
442 periods of time. For rescue experiments, 1  $\mu$ M final concentration of proteasome inhibitor MG132  
443 (EMD Millipore; Cat. No. 474790-5MG; Darmstadt, Germany), 2  $\mu$ M HER2 inhibitor lapatinib  
444 (Selleckchem; Cat. No. GW-572016), or 2  $\mu$ M Ubiquitin activating enzyme E1 inhibitor  
445 (synthesized at Genentech), were added before cell harvest.

446

#### 447 **CRISPR engineering of HCC1954 cells**

448 HCC1954 breast cancer cells were engineered using CRISPR to knock out either the *PIK3CA* WT  
449 allele or the mutant alleles, to create an isogenic pair of cell lines designated as HCC1954\_mutant  
450 and HCC1954\_WT. gRNAs were designed using the CRISPRtool (<http://crispr.mit.edu>) to  
451 minimize potential off-target effects and cloned into pCas-Guide-EF1a-GFP vector (Blueheron  
452 Biotech). To generate the HCC1954\_mutant line bearing p110 $\alpha$  H1047R as homozygous mutant,  
453 two CRISPR-Cas9 constructs were designed. One was designed to specifically target the wild type  
454 allele in exon 21 (gRNA H1047R-2, ATGAATGATGCACATCATGG) and the second designed  
455 to target the intron of both WT and mutant alleles (gRNA H1047R-7,  
456 ACATTTGAGCAAAGACCTGA). To generate the HCC1954\_WT line, two CRISPR-Cas9  
457 constructs were designed with one gRNA targeting the mutant allele in exon 21 (H1047R-5,  
458 ATGAATGATGCACGTCATGG) and the second gRNA targeting the intron of both WT and

459 mutant alleles (H1047R-8, TATTAAACTCCTGACATGCC). Plasmids for each targeting pair  
460 were co-transfected using Turbofectin (Thermofisher). After 48 hours cells were put under  
461 selection with 1 ug/mL puromycin. Puromycin resistant cells were further selected by collecting  
462 GFP expressing cells by flow cytometry, and clones were expanded in standard cell culture  
463 conditions to create stable lines. Targeting efficiency of the CRISPR-induced allelic knockouts  
464 was assessed by PCR flanking the target sites (forward: TGCTGTGAAGGAAAATGGAA,  
465 (reverse: TGCAGTGTGGAATCCAGAGTGAGC), and clones further validated with qRT-PCR  
466 and DNA sequencing.

467

#### 468 **siRNA transfection**

469 Transfection of siRNA was carried out using Lipofectamine RANiMAX reagent (Thermofisher),  
470 72 hours in advance of drug treatment.

471

#### 472 **Western blots**

473 Protein concentration was determined using the Pierce BCA Protein Assay Kit. For immunoblots,  
474 equal protein amounts were loaded and then separated by electrophoresis through NuPAGE Novex  
475 Bis-Tris 4–12% gradient gels (Invitrogen). Proteins were transferred onto Nitrocellulose  
476 membranes using the iBlot system and protocol from InVitrogen (Carlsbad, CA).

477

#### 478 **Subcellular fractionation**

479 Cells were washed once with phosphate-buffered saline, before scraping into 0.8 ml/dish  
480 hypotonic lysis buffer (HLB: 25 mM Tris–HCl pH7.5, 10 mM NaCl, 1 mM EDTA, protease and  
481 phosphatase inhibitors). The cells were lysed by 30 strokes in a Dounce homogenizer, subjected

482 to centrifugation at 1500 g (3000 RPM) for 5 min to pellet nuclei and unbroken cells, followed by  
483 centrifugation of the supernatant at 100,000 g (44,000 RPM) in TLA55 rotor for 40 min. The  
484 supernatant (800  $\mu$ L) was collected (S100 fraction) and the pellet resuspended in 200ul HLB plus  
485 1% NP40 (P100 fraction). The resuspended pellet was centrifuged 5 min in high speed in  
486 microfuge and supernatant collected.

487

#### 488 **Immunoprecipitation and pulldown**

489 Cells were lysed in 20 mM TrisHCL pH 7.5, 137 mM NaCl, 1 mM EDTA, 1% NP40, 10% glycerol  
490 plus protease and phosphatase inhibitors. For p110 $\alpha$  immunoprecipitation, lysates were incubated  
491 with p110 $\alpha$  antibody (Cell Signaling, 4249) overnight. 50  $\mu$ L of proteinA agarose beads were  
492 added to each sample and incubated additional 2 hours. For ubiquitinated protein pulldown  
493 experiment, cells were lysed in lysis buffer containing 200  $\mu$ g/mL TUBE1 (Lifesensors UM101).  
494 Lysates were isolated and added 50  $\mu$ L of glutathione agarose beads (Sigma, G4705). The samples  
495 were incubated overnight and captured ubiquitinated protein was eluted in SDS reducing sample  
496 buffer.

497

#### 498 **RNA isolation and *PIK3CA* allele-specific quantitative RT-PCR**

499 Total RNA was isolated from cells using RNeasy Plus Mini Kit (Qiagen) following the protocol  
500 described in the kit. First-strand cDNA synthesis and RT-qPCR was carried out using One step  
501 RT QPCR reagent (Roche). Resulting signal was detected on Applied Biosystems Real Time PCR  
502 System. Primers and allele specific probes were:

503 *PIK3CA* H1047R-forward: GGCTTTGGAGTATTTTCATGAAACA

504 *PIK3CA* H1047R-reverse: GAAGATCCAATCCATTTTTGTTGTC

505 *PIK3CA* H1047R WT-probe: ATGATGCACATCATGGT  
506 *PIK3CA* H1047R Mut-probe: TGATGCACGTCATGGT  
507 *PIK3CA* E545K-forward: GCAATTTCTACACGAGATCCTCTCT  
508 *PIK3CA* E545K-reverse: CATTTTAGCACTTACCTGTGACTCCAT  
509 *PIK3CA* E545K WT-probe: TGAAATCACTGAGCAGGAG  
510 *PIK3CA* E545K Mut-probe: TGAAATCACTAAGCAGGA

511

### 512 **Intracellular drug concentration**

513 Taselisib (1  $\mu$ M in RPMI medium) was added to *PIK3CA*-mutant and -WT cancer cell lines in  
514 triplicate and incubated under standard culture conditions (37°C, 5% CO<sub>2</sub>) for 18 hours. Medium  
515 containing taselisib was aspirated and cells were washed twice with 1 mL of ice-cold Hank's  
516 balanced salt solution (HBSS). Cells were lysed by sonication for 1 min in HBSS followed by the  
517 addition of an equal volume of acetonitrile containing analytical internal standard (propranolol,  
518 100 nM). The supernatant was analyzed in positive mode on a SCIEX API-4000 LC/MS/MS  
519 system with the transitions of 461/418 and 260/116 for taselisib and propranolol, respectively. The  
520 LC separations were achieved using a LUNA C18 column (4  $\mu$ m, 50 x 2.1 mm) from Phenomenex,  
521 Inc. (Torrance, CA) and Agilent 1100 series pumps consisting of mobile phase A (water containing  
522 0.1% formic acid) and mobile phase B (acetonitrile containing 0.1% formic acid). The flow rate  
523 through the system was 1 mL/min. The initial condition was set at 5% B, which was ramped to  
524 95% B over 40 sec. After allowing the system to hold at 95% B for 20 sec, the gradient was  
525 changed back to the initial condition of 5% B and was allowed to equilibrate for 54 sec before the  
526 next injection. A parallel replicate plate containing *PIK3CA*-mutant and -WT cancer cells was  
527 reserved for the determination of protein concentrations. Cells were washed with HBSS buffer and

528 then lysed with 0.5 mL of ProteaPrep cell lysis solution (Protea Biosciences, Morgantown, WV).  
529 After 30 min, the solution was neutralized with 0.5 mL HBSS buffer and 100  $\mu$ L of the lysed cells  
530 were used to determine the protein concentration by the standard Bradford protein assay (Thermo  
531 Scientific, Rockford, IL), with bovine serum albumin as a standard.

532

### 533 **PI3K ADP-Glo assays for Ki measurement**

534 As described in WO 2017/001645 A1, the PI3K lipid kinase reaction was performed in the  
535 presence of PIP2:3PS lipid substrate (Promega #V1792) and ATP. Following the termination of  
536 the kinase reaction, turnover of ATP to ADP by the phosphorylation of the lipid substrate was  
537 detected using the Promega ADP-Glo™ (Promega #V1792) assay. Reactions were carried out  
538 using the following conditions for each PI3K isoform: PI3Kalpha (Millipore #14-602-K) kinase  
539 concentration 0.2 nM, ATP 40  $\mu$ M, PIP2:3PS 50  $\mu$ M; PI3Kbeta (Promega #V1751) kinase  
540 concentration 0.6 nM, ATP 40  $\mu$ M, PIP2:3PS 50  $\mu$ M; PI3K delta (Millipore #14-604-K), kinase  
541 concentration 0.25 nM, ATP 40  $\mu$ M, PIP2:3PS 50  $\mu$ M; PI3K gamma (Millipore #14-558-K),  
542 kinase concentration 0.4 nM, ATP 25  $\mu$ M, PIP2:3PS 50  $\mu$ M. After 120 min of reaction time, the  
543 kinase reaction was terminated. After the reaction, any ATP remaining was depleted leaving only  
544 ADP. Then the Kinase Detection Reagent was added to convert ADP to ATP, which was used in  
545 a coupled luciferin/luciferase reaction. The luminescent output was measured and was correlated  
546 with kinase activity. All reactions were carried out at room temperature. For each PI3K isoform a  
547 3  $\mu$ L mixture (1:1) of enzyme/lipid substrate solution was added to a 384 well white assay plate  
548 (Perkin Elmer #6007299) containing 50  $\mu$ L of test compound or DMSO only for untreated  
549 controls. The reaction was started by the addition of 2  $\mu$ L ATP/MgCl<sub>2</sub>. The kinase reaction buffer  
550 contained 50 mM HEPES, 50 mM NaCl<sub>2</sub>, 3 mM MgCl<sub>2</sub>, 0.01% BSA, 1% DMSO, and enzyme and



551 substrate concentrations. The reaction was stopped by the addition of 10  $\mu$ L ADP-Glo reagent.  
552 Plates were read in a Perkin Elmer Envision system using luminescence mode. A 10-point dose  
553 response curve was generated for each test compound.  $K_i$  values for each compound were  
554 determined using the Morrison Equation.

555

#### 556 **Kinetic solubility**

557 Compounds were dissolved in DMSO to a concentration of 10 mM. These solutions were diluted  
558 into PBS buffer (pH 7.2, composed with NaCl, KCl,  $\text{Na}_2\text{HPO}_4$ , and  $\text{KH}_2\text{PO}_4$ ) to a final compound  
559 concentration of 100  $\mu$ M, DMSO concentration of 2%, at pH 7.4. The samples were shaken for 24  
560 hours at room temperature followed by filtration. LC/CLND was used to determine compound  
561 concentration in the filtrate, with the concentration calculated by a caffeine calibration curve and  
562 the samples nitrogen content. An internal standard compound was spiked into each sample for  
563 accurate quantification.

564

#### 565 **Plasma protein binding**

566 As described in literature (Heffron et al., 2016), the extent of protein binding was determined in  
567 vitro, in CD-1 mouse, Sprague–Dawley rat, and human plasma (Bioreclamation, Inc., Hicksville,  
568 NY) by equilibrium dialysis using the RED Device (Thermo Fisher Scientific, Rockford, IL).  
569 Compounds were added to pooled plasma ( $n \geq 3$ ) at a total concentration of 5  $\mu$ M. Plasma samples  
570 were equilibrated with phosphate-buffered saline (pH 7.4) at 37°C in 90% humidity and 5%  $\text{CO}_2$   
571 for 4 hours. Following dialysis, compound concentration in plasma and buffer were measured by  
572 LCMS/MS. The percent of unbound compound in plasma was determined by dividing the

573 concentration measured in the post-dialysis buffer by that measured in the post-dialysis plasma  
574 and multiplying by 100.

575

### 576 **MDCK permeability**

577 Experiments were carried out as previously described (Irvine et al., 1999).

578

### 579 **Drug half-life *in vivo***

580 Experiments to determine half-life in mice were carried out as previously described (Furet et al.,  
581 2013; Pang et al., 2014; Salphati et al., 2011).

582

### 583 **Pulse-chase**

584 Cells were starved in methionine/cysteine deficient media overnight. Cells were pulse labeled with  
585 <sup>35</sup>S cysteine and methionine (Pro-mix 1-[<sup>35</sup>S], Amersham) in RPMI lacking cysteine and  
586 methionine for 6 hours, followed by three washes with RPMI containing no label. Cells were then  
587 incubated in normal media containing methionine and cysteine, either with or without compounds.  
588 Lysates were collected at various time points up to 48 hours. Radiolabeled p110 $\alpha$  was  
589 immunoprecipitated and run on SDS-PAGE. Images were acquired on the Typhoon Scanner (GE  
590 Healthcare-Amersham), and signals quantified and normalized using ImageQuant TL software.  
591 Data was fit to a single exponential decay function using Prism (GraphPad, San Diego, CA) with  
592  $Y=(Y_0-NS)*\exp(-K*X) + NS$  and constraints  $Y_0=100, NS=0$ .

593

### 594 **Animal studies**

595 The in vivo efficacy of PI3K inhibitors was tested in three breast cancer xenograft models that  
596 harbor *PIK3CA* mutations, HCC1954 (HER2-positive, *PIK3CA* H1047R), WHIM20 (ER-  
597 positive/HER2-negative, *PIK3CA* H1047R) and HCI-003 (ER-positive/HER2-negative, *PIK3CA*  
598 E545K). All in vivo studies were approved by Genentech and Institutional Animal Care and Use  
599 Committee (IACUC) and adhered to the NIH Guidelines for the Care and Use of Laboratory  
600 Animals. HCC1954 tumor cells ( $5 \times 10^6$ ) were inoculated in the 2/3 mammary fat pads of female  
601 NCR nude mice (Taconic Farms, Hudson NY) while WHIM20 and HCI-003 tumors ( $50 \text{mm}^3$ ) were  
602 engrafted in 2/3 mammary fat pads in female NOD-SCID gamma mice (Jackson Laboratories, Bar  
603 Harbor, Maine). Tumor volumes were measured using Ultra Cal-IV calipers (Model 54-10-111;  
604 Fred V.Fowler Co.; Newton, MA). The following formula was used in Excel, version 11.2 to  
605 calculate tumor volume: Tumor Volume ( $\text{mm}^3$ ) = (Length x Width<sup>2</sup>) x 0.5. Mice for efficacy  
606 studies were distributed into 8–10 mice/group with a mean tumor volume of 200 to 250  $\text{mm}^3$  at  
607 the initiation of dosing. A linear mixed effect (LME) modeling approach was used to analyze the  
608 repeated measurement of tumor volumes from the same animals over time (Pinheiro et al., 2017).  
609 Cubic regression splines were used to fit a non-linear profile to the time courses of  $\log^2$  tumor  
610 volume at each dose level. These non-linear profiles were then related to dose within the mixed  
611 model. Tumor growth inhibition as a percentage of vehicle control (%TGI) was calculated as the  
612 percentage of the area under the fitted curve (AUC) for the respective dose group per day in  
613 relation to the vehicle, using the following formula: %TGI=100 x (1 - AUC<sub>dose</sub>/AUC<sub>vehicle</sub>).  
614 The PI3K inhibitors taselisib, GDC-0941, GDC-0077, and BYL719 were formulated in  
615 Methylcellulose Tween (MCT) vehicle consisting of 0.5% (w/v) methylcellulose, 0.2% (w/v)  
616 polysorbate 80 (Tween-80) and dosed orally by gavage daily. Tumor sizes and mouse body  
617 weights were recorded twice weekly, and mice with tumor volume exceeding 2000  $\text{mm}^3$  or body

618 weight loss of 20% of starting weight were promptly euthanized. For pharmacodynamic (PD) and  
619 mechanistic studies, mice were dosed once by oral gavage and tumors harvested at 4 hours post-  
620 dose. Following drug treatment, tumors were harvested and snap-frozen in liquid nitrogen,  
621 dissociated in lysis buffer containing 10  $\mu$ M Tris pH 7.4, 100  $\mu$ M NaCl, 1  $\mu$ M EDTA, 1  $\mu$ M  
622 EGTA, 1  $\mu$ M NaF, 20  $\mu$ M Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 10% glycerol, 0.1% SDS,  
623 and 0.5% deoxycholate supplemented with a phosphatase and protease inhibitor cocktail (Sigma,  
624 St. Louis, MO). Protein concentrations were determined in whole cell lysates using the BCA  
625 Protein Assay Kit (Pierce; Rockford, IL). Membrane fractions were isolated from xenograft tumors  
626 as described above, and assessed by western blot.

627

## 628 **Mass spectrometry**

629 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed on  
630 p110 $\alpha$  protein immunoprecipitated from three cell lines: HCC1954, HCC202 and HDQ-P1. Each  
631 cell line was treated for 24 hours with either DMSO (vehicle) or taselisib (500 nM). Each  
632 experiment was performed beginning with 4–6 mg protein lysate per cell line/treatment (total of 6  
633 samples/experiment) for n=4 biological replicates. One gel region per sample, corresponding to  
634 the expected migration was excised based on the migration of purified p110 $\alpha$  protein in an adjacent  
635 lane. Gel pieces were diced into  $\sim$ 1 mm<sup>3</sup> pieces and subjected to in-gel digestion as follows. Gel  
636 pieces were de-stained with 50 mM ammonium bicarbonate/50% acetonitrile and dehydrated with  
637 100% acetonitrile prior to reduction and alkylation using 50 mM dithiothreitol (30 min, 50°C) and  
638 50 mM iodoacetamide (20 min, room temperature), respectively. Gel pieces were again  
639 dehydrated, allowed to reswell in a 20 ng/ $\mu$ L trypsin in 50 mM ammonium bicarbonate/5%  
640 acetonitrile digestion buffer on ice for 2 hours, and then transferred to a 37°C oven for overnight

641 incubation. Digested peptides were transferred to microcentrifuge tubes and gel pieces were  
642 extracted twice, once with 50% acetonitrile/0.5% trifluoroacetic acid, and a second round with  
643 100% acetonitrile. Extracts were combined with digested peptides and speed-vac dried to  
644 completion. Samples were reconstituted in 5% formic acid/0.1% heptafluorobutyric acid/0.01%  
645 hydrogen peroxide 30 min prior to LC-MS/MS analysis. Samples were analyzed by LC-MS/MS  
646 with duplicate injection (with the exception of the first replicate where samples were injected once)  
647 on a Thermo LTQ Orbitrap Elite coupled to a Waters nanoAcquity UPLC. Peptides were loaded  
648 onto a 0.1mm X 100mm Waters Symmetry C18 column packed with 1.7  $\mu$ m BEH-130 resin and  
649 separated via a two-stage linear gradient where solvent B (98% acetonitrile, 2% water) was ramped  
650 from 5% to 25% over 20 min and then from 25% to 50% over 2 min. Data were acquired in data  
651 dependent mode with Orbitrap full MS scans collected at 60,000 resolution and the top 15 most  
652 intense precursors selected for CID MS/MS fragmentation in the ion trap. MS2 spectra were  
653 searched using Mascot, both against a concatenated target-decoy Uniprot database of human  
654 proteins as well as against a small database containing wild-type, E545K and H1047R mutant  
655 p110 $\alpha$  sequences in order to identify mutant peptides. Peptide spectral matches for the Uniprot  
656 search were rough filtered using linear discriminant analysis to 10% false discovery rate, then  
657 confirmed via manual inspection. Extracted ion chromatograms and peak area integration for  
658 p110 $\alpha$  peptides were generated with 10 ppm mass tolerances using in-house software (MSPlorer).  
659 Peak area data for each of 14 peptides (see [Figure 3D](#) and [Figures S4C and S4D](#)) were normalized  
660 on a per-replicate basis to the most abundant peak area among the six samples. In cases where  
661 duplicate injections (technical replicates) were available, normalized data for the two replicates  
662 were averaged to generate a single normalized peak area per peptide-condition-experiment for the  
663 protein sequence plots ([Figure S4C](#)). For statistical analysis, unnormalized peak areas across the

664 four biological replicates were consolidated in R using linear mixed effects modelling (lme4  
665 package) to determine the relative ratio and measures of uncertainty (from which can be derived  
666 p-values, confidence intervals, etc) for the comparison of DMSO (vehicle) versus taselesib (500  
667 nM) treatments per cell line for each of total p110 $\alpha$ , WT p110 $\alpha$ , and mutant p110 $\alpha$  (Bates et al.,  
668 2015). Total p110 $\alpha$  was determined based on the data generated from the following peptides:  
669 EATLITIK (residues 39-46; 444.77481 m/z), DLNSPHSR (155-162; 463.22945 m/z),  
670 LCVLEYQGK (241-249), VCGCDEYFLEK (254-264; 710.30246 m/z), VPCSNPR (376-382),  
671 EAGFSYSHAGLSNR (503-516; 748.35281 m/z), YEQYLDNLLVR (641-651; 713.37540 m/z),  
672 FGLLLESYCR (684-693; 629.32042 m/z), and LINLTDILK (712-720; 521.83039 m/z). For cells  
673 bearing the H1047R mutation (i.e. HCC-1954), WT p110 $\alpha$  was determined based on the  
674 QMNDAHGGWTTK (1042-1054; 749.82824 m/z) peptide and mutant p110 $\alpha$  based on  
675 QMNDAR (1042-1047; 375.66360 m/z) and HGGWTTK (1048-1054; 393.6983 m/z) peptides.  
676 For cells bearing the E545K mutation (i.e. HCC-202), WT p110 $\alpha$  was determined based on the  
677 DPLSEITEQEK (538-548; 644.81917 m/z) peptide and mutant p110 $\alpha$  based on DPLSEITK (538-  
678 545; 451.74627 m/z) peptides. Peptides from these two mutant loci were not considered when  
679 determining total p110 $\alpha$ . Whenever applicable, cysteine residues within p110 $\alpha$  peptides were  
680 analysed in their carbamidomethylated form (+57.0215 Da) and methionine residues quantified  
681 based on their singly oxidized (Met630 sulfoxide) form (+15.9949 Da). Quantified peptide peak  
682 areas observed after treatment with taselesib or DMSO were analysed to estimate differential  
683 abundances and accompanying measures of uncertainty for total, WT, and mutant, respectively,  
684 across the two treatments. These three fractional abundances were combined to estimate the  
685 relative proportions (and associated 95% confidence intervals) of WT versus mutant in each  
686 mutant-containing cell line in both the DMSO and taselesib treated conditions by applying the

687 conservation of mass principle, as follows. Suppose “p” and “1-p” denote the fractions of WT and  
688 mutant p110 $\alpha$ , respectively, in a DMSO-treated cell culture. Comparing peptides quantified after  
689 treatment with taselisib or DMSO from the total, WT-only, and mutant-only categories detailed  
690 above, define the three fractions:  $f_{total}$  = fraction of total p110 $\alpha$  remaining after taselisib, relative  
691 to DMSO  $f_{WT}$  = fraction of WT p110 $\alpha$  remaining after taselisib, relative to DMSO  $f_{mut}$  =  
692 fraction of mutant p110 $\alpha$  remaining after taselisib, relative to DMSO. The conservation of mass  
693 principle requires that  $f_{total} = f_{WT} * p + f_{mut} * (1-p)$ . Solving for “p” (the fraction of WT  
694 p110 $\alpha$  in DMSO-treated cells) yields an estimate of  $p = (f_{total} - f_{mutant}) / (f_{WT} - f_{mutant})$   
695 ) where log-scale estimates of the three fractions  $\{f_{total}, f_{WT}, f_{mutant}\}$  are obtained from the  
696 linear mixed effects model. All other quantities of interest (with estimated standard errors,  
697 confidence intervals, p-values, etc.) likewise can be estimated as functions of these three fractions  
698 and their measures of uncertainty, which are also obtained from the linear mixed effects model.  
699 Data are plotted as relative intensity values normalized to 1.0 and error bars represent the 95%  
700 confidence intervals for each measurement based on the linear effects model.

701

702 **Declaration of interests:** All Genentech authors are employees and shareholders at Roche.

703

#### 704 **Acknowledgements**

705 Support for third-party editing assistance for this manuscript, furnished by Daniel Clyde, PhD, of  
706 Health Interactions, was provided by F. Hoffmann-La Roche Ltd.

707

#### 708 **References**

709 Backer, J.M. (2010). The regulation of class IA PI 3-kinases by inter-subunit interactions. *Curr*  
710 *Top Microbiol Immunol* 346, 87-114.

711 Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting Linear Mixed-Effects Models  
712 Using lme4. *Journal of Statistical Software* 67, 48.

713 Burke, J.E., Perisic, O., Masson, G.R., Vadas, O., and Williams, R.L. (2012). Oncogenic mutations  
714 mimic and enhance dynamic events in the natural activation of phosphoinositide 3-kinase  
715 p110alpha (PIK3CA). *Proc Natl Acad Sci U S A* 109, 15259-15264.

716 Burke, J.E., and Williams, R.L. (2013). Dynamic steps in receptor tyrosine kinase mediated  
717 activation of class IA phosphoinositide 3-kinases (PI3K) captured by H/D exchange (HDX-MS).  
718 *Adv Biol Regul* 53, 97-110.

719 Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff,  
720 S. (1991). Oncogenes and signal transduction. *Cell* 64, 281-302.

721 Chen, J.J., Tsu, C.A., Gavin, J.M., Milhollen, M.A., Bruzzese, F.J., Mallender, W.D., Sintchak,  
722 M.D., Bump, N.J., Yang, X., Ma, J., *et al.* (2011). Mechanistic studies of substrate-assisted  
723 inhibition of ubiquitin-activating enzyme by adenosine sulfamate analogues. *J Biol Chem* 286,  
724 40867-40877.

725 Cheng, H., Liu, P., Ohlson, C., Xu, E., Symonds, L., Isabella, A., Muller, W.J., Lin, N.U., Krop,  
726 I.E., Roberts, T.M., *et al.* (2016). PIK3CA(H1047R)- and Her2-initiated mammary tumors escape  
727 PI3K dependency by compensatory activation of MEK-ERK signaling. *Oncogene* 35, 2961-2970.

728 Echeverria, I., Liu, Y., Gabelli, S.B., and Amzel, L.M. (2015). Oncogenic mutations weaken the  
729 interactions that stabilize the p110alpha-p85alpha heterodimer in phosphatidylinositol 3-kinase  
730 alpha. *FEBS J* 282, 3528-3542.

731 Engelman, J.A., Chen, L., Tan, X., Crosby, K., Guimaraes, A.R., Upadhyay, R., Maira, M.,  
732 McNamara, K., Perera, S.A., Song, Y., *et al.* (2008). Effective use of PI3K and MEK inhibitors to  
733 treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. *Nat Med* 14, 1351-1356.

734 Folkes, A.J., Ahmadi, K., Alderton, W.K., Alix, S., Baker, S.J., Box, G., Chuckowree, I.S., Clarke,  
735 P.A., Depledge, P., Eccles, S.A., *et al.* (2008). The identification of 2-(1H-indazol-4-yl)-6-(4-  
736 methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin-4-yl-1H-pyrimidine (GDC-0941)  
737 as a potent, selective, orally bioavailable inhibitor of class I PI3 kinase for the treatment of cancer.  
738 *J Med Chem* 51, 5522-5532.

739 Furet, P., Guagnano, V., Fairhurst, R.A., Imbach-Weese, P., Bruce, I., Knapp, M., Fritsch, C.,  
740 Blasco, F., Blanz, J., Aichholz, R., *et al.* (2013). Discovery of NVP-BYL719 a potent and selective



741 phosphatidylinositol-3 kinase alpha inhibitor selected for clinical evaluation. *Bioorg Med Chem*  
742 *Lett* 23, 3741-3748.

743 Greig, M.J., Niessen, S., Weinrich, S.L., Feng, J.L., Shi, M., and Johnson, T.O. (2015). Effects of  
744 Activating Mutations on EGFR Cellular Protein Turnover and Amino Acid Recycling Determined  
745 Using SILAC Mass Spectrometry. *Int J Cell Biol* 2015, 798936.

746 Hafner, M., Niepel, M., Chung, M., and Sorger, P.K. (2016). Growth rate inhibition metrics correct  
747 for confounders in measuring sensitivity to cancer drugs. *Nat Methods* 13, 521-527.

748 Heffron, T.P., Heald, R.A., Ndubaku, C., Wei, B., Augustin, M., Do, S., Edgar, K., Eigenbrot, C.,  
749 Friedman, L., Gancia, E., *et al.* (2016). The Rational Design of Selective Benzoxazepin Inhibitors  
750 of the alpha-Isoform of Phosphoinositide 3-Kinase Culminating in the Identification of (S)-2-((2-  
751 (1-Isopropyl-1H-1,2,4-triazol-5-yl)-5,6-dihydrobenzo[f]imidazo[1,2-d][1,4]oxazepin-9-  
752 yl)oxy)propanamide (GDC-0326). *J Med Chem* 59, 985-1002.

753 Irvine, J.D., Takahashi, L., Lockhart, K., Cheong, J., Tolan, J.W., Selick, H.E., and Grove, J.R.  
754 (1999). MDCK (Madin-Darby canine kidney) cells: A tool for membrane permeability screening.  
755 *J Pharm Sci* 88, 28-33.

756 Isakoff, S.J., Engelman, J.A., Irie, H.Y., Luo, J., Brachmann, S.M., Pearlman, R.V., Cantley, L.C.,  
757 and Brugge, J.S. (2005). Breast cancer-associated PIK3CA mutations are oncogenic in mammary  
758 epithelial cells. *Cancer Res* 65, 10992-11000.

759 Jackson, S.P., Schoenwaelder, S.M., Goncalves, I., Nesbitt, W.S., Yap, C.L., Wright, C.E.,  
760 Kenche, V., Anderson, K.E., Dopheide, S.M., Yuan, Y., *et al.* (2005). PI 3-kinase p110beta: a new  
761 target for antithrombotic therapy. *Nat Med* 11, 507-514.

762 Jhaveri, K., Kalinsky, K., Bedard, P.L., Cervantes, A., Saura, C., Krop, I.E., Hamilton, E., Schmid,  
763 P., Varga, A., Turner, N., *et al.* (2019). Phase Ib dose escalation study evaluating the mutant  
764 selective PI3K-alpha inhibitor GDC-0077 in combination with letrozole with and without  
765 palbociclib in patients with PIK3CA-mutant HR+/HER2- breast cancer. In San Antonio Breast  
766 Cancer Symposium (San Antonio).

767 Juric, D., Kalinsky, K., and Oliveira, M. (2019). A first-in-human phase Ia dose escalation study  
768 of GDC-0077, a p110a-selective and mutant-degrading PI3K inhibitor, in patients with PIK3CA-  
769 mutant solid tumors. In San Antonio Breast Cancer Symposium (San Antonio, TX, USA).

770 Juric, D., Krop, I., Ramanathan, R.K., Wilson, T.R., Ware, J.A., Sanabria Bohorquez, S.M.,  
771 Savage, H.M., Sampath, D., Salphati, L., Lin, R.S., *et al.* (2017). Phase I Dose-Escalation Study

772 of Taselisib, an Oral PI3K Inhibitor, in Patients with Advanced Solid Tumors. *Cancer Discov* 7,  
773 704-715.

774 Kalinsky, K., Juric, D., Bedard, P.L., Oliveira, M., Cervantes, A., Hamilton, E., Krop, I.E., Turner,  
775 N., Schmid, P., Varga, A., *et al.* (2020). A phase I/Ib study evaluating GDC-0077 plus fulvestrant  
776 in patients with PIK3CA-mutant, hormone receptor-positive/HER2-negative breast cancer In  
777 AACR Annual Meeting.

778 Kang, S., Bader, A.G., and Vogt, P.K. (2005). Phosphatidylinositol 3-kinase mutations identified  
779 in human cancer are oncogenic. *Proc Natl Acad Sci U S A* 102, 802-807.

780 Krop, I.E., Mayer, I.A., Ganju, V., Dickler, M., Johnston, S., Morales, S., Yardley, D.A., Melichar,  
781 B., Forero-Torres, A., Lee, S.C., *et al.* (2016). Pictilisib for oestrogen receptor-positive, aromatase  
782 inhibitor-resistant, advanced or metastatic breast cancer (FERGI): a randomised, double-blind,  
783 placebo-controlled, phase 2 trial. *Lancet Oncol* 17, 811-821.

784 Martin, M., Chan, A., Dirix, L., O'Shaughnessy, J., Hegg, R., Manikhas, A., Shtivelband, M.,  
785 Krivorotko, P., Batista Lopez, N., Campone, M., *et al.* (2017). A randomized adaptive phase II/III  
786 study of buparlisib, a pan-class I PI3K inhibitor, combined with paclitaxel for the treatment of  
787 HER2- advanced breast cancer (BELLE-4). *Ann Oncol* 28, 313-320.

788 Mayer, I.A., Abramson, V.G., Formisano, L., Balko, J.M., Estrada, M.V., Sanders, M.E., Juric,  
789 D., Solit, D., Berger, M.F., Won, H.H., *et al.* (2017). A Phase Ib Study of Alpelisib (BYL719), a  
790 PI3K $\alpha$ -Specific Inhibitor, with Letrozole in ER+/HER2- Metastatic Breast Cancer. *Clin*  
791 *Cancer Res* 23, 26-34.

792 Miled, N., Yan, Y., Hon, W.C., Perisic, O., Zvelebil, M., Inbar, Y., Schneidman-Duhovny, D.,  
793 Wolfson, H.J., Backer, J.M., and Williams, R.L. (2007). Mechanism of two classes of cancer  
794 mutations in the phosphoinositide 3-kinase catalytic subunit. *Science* 317, 239-242.

795 Pang, J., Baumgardner, M., Cheong, J., Edgar, K., Heffron, T.P., and Le, H. (2014). Preclinical  
796 Evaluation of the  $\beta$  Isoform-sparing PI3K inhibitor GDC-0032 and prediction of its human  
797 pharmacokinetics. *Drug Metabolism Reviews* 45.

798 Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and Team, t.R.C. (2017). Linear and Nonlinear  
799 Mixed Effects Models. R package version 3.1-131.

800 Rodon, J., Dienstmann, R., Serra, V., and Tabernero, J. (2013). Development of PI3K inhibitors:  
801 lessons learned from early clinical trials. *Nat Rev Clin Oncol* 10, 143-153.

802 Sadhu, C., Masinovsky, B., Dick, K., Sowell, C.G., and Staunton, D.E. (2003). Essential role of  
803 phosphoinositide 3-kinase delta in neutrophil directional movement. *J Immunol* *170*, 2647-2654.

804 Salphati, L., Pang, J., Plise, E.G., Chou, B., Halladay, J.S., Olivero, A.G., Rudewicz, P.J., Tian,  
805 Q., Wong, S., and Zhang, X. (2011). Preclinical pharmacokinetics of the novel PI3K inhibitor  
806 GDC-0941 and prediction of its pharmacokinetics and efficacy in human. *Xenobiotica* *41*, 1088-  
807 1099.

808 Samuels, Y., Diaz, L.A., Jr., Schmidt-Kittler, O., Cummins, J.M., Delong, L., Cheong, I., Rago,  
809 C., Huso, D.L., Lengauer, C., Kinzler, K.W., *et al.* (2005). Mutant PIK3CA promotes cell growth  
810 and invasion of human cancer cells. *Cancer Cell* *7*, 561-573.

811 Yu, J., Zhang, Y., McIlroy, J., Rordorf-Nikolic, T., Orr, G.A., and Backer, J.M. (1998). Regulation  
812 of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha  
813 catalytic subunit by the p85 regulatory subunit. *Mol Cell Biol* *18*, 1379-1387.

814 Yu, M., Selvaraj, S.K., Liang-Chu, M.M., Aghajani, S., Busse, M., Yuan, J., Lee, G., Peale, F.,  
815 Klijn, C., Bourgon, R., *et al.* (2015). A resource for cell line authentication, annotation and quality  
816 control. *Nature* *520*, 307-311.

817

818 **Figure Legends**

819 **Figure 1**

820 **GDC-0032 and GDC-0077 have increased potency in *PIK3CA*-mutant cancer cells**

821 (A) Chemical structures and physicochemical properties of PI3K inhibitors.

822 <sup>a</sup>Inhibition of ATP-hydrolysis by PI3K isoforms in a biochemical assay, ADP production measured  
823 by ADP-Glo™. <sup>b</sup>Plasma protein binding determined by equilibrium dialysis.

824 <sup>c</sup> Permeability measured using Madin-Darby canine kidney (MDCK) epithelial cells; A = apical,  
825 B = basolateral. B→A / A→B used to estimate efflux potential.<sup>d</sup> Mouse PO dose as MCT  
826 suspension.

827 (B) Cell viability IC<sub>50</sub> values determined by quantifying ATP from all tumor lines at 5 days post-  
828 treatment.

829 (C) PI3K inhibitor potency in SW48 isogenic *PIK3CA*-mutant and -wild-type parental cells in a  
830 4-day viability assay. Error bars are standard deviation of quadruplicates

831 (D) *In vivo* efficacy of taselisib, GDC-0077, and BYL719 in HCC1954 *PIK3CA* H1047R breast  
832 cancer xenograft model.

833

834 **Figure 2**

835 **Activity of PI3K $\alpha$  inhibitors in combination with palbociclib in HR-positive breast cancer**  
836 **cells**

837 (A) Dose response curve of MCF-7 cells treated with GDC-0077 either alone (blue), without E2  
838 to mimic aromatase inhibitor (dark blue), with palbociclib at 0.15 $\mu$ M (purple), or with palbociclib  
839 at 0.15  $\mu$ M (purple) and without E2 (dark purple). Negative values indicate a cytotoxic response.  
840 y-axis shows normalized growth inhibition (GR value).

841 **(B)** Growth response of 5 HR-positive breast cancer cells with *PIK3CA* mutations to treatment  
842 with either palbociclib at 0.15  $\mu$ M (red), E2 withdrawal (gray), GDC-0077 at 0.123  $\mu$ M (blue),  
843 Palbociclib at 0.15 $\mu$ M and E2 withdrawal (dark red), or the triple combination (dark purple). y-  
844 axis shows normalized growth inhibition (GR value).

845 **(C)** Efficacy of palbociclib, fulvestrant and GDC-0077 as single agent or in combination in MCF7  
846 xenografts. Each group contains 12 animals at the beginning of the experiment.

847

### 848 **Figure 3**

849 **Taselisib depletes mutant p110 $\alpha$  protein through ubiquitin and proteasome mechanism in a**  
850 **dose and time dependent manner**

851 **(A)** Western blots of the inhibitor response in PI3K signaling (pHER3 and pAKT) in *PIK3CA*-  
852 mutant (HCC1954, HCC202, and MDA-MB-453) cell lines treated with 1  $\mu$ M BYL719 or 0.5  $\mu$ M  
853 GDC-0077 for indicated time points.

854 **(B)** Mass spectrometry of HCC1954 cells treated for 24 hours with 500 nM tselisib. A neo-tryptic  
855 peptide generated from *PIK3CA* H1047R was used to assess mutant protein levels compared to  
856 wild-type protein in the same lysate.

857 **(C)** Rescue of tselisib- or GDC-0077-mediated p110 $\alpha$  degradation in HCC1954 cells by either a  
858 proteasome inhibitor (MG132) or a Ubiquitin Activating Enzyme E1 (UAE1) inhibitor.

859 **(D)** HCC1954 *PIK3CA* H1047R cells were treated 8 hours or HCC202 *PIK3CA* E545K cells were  
860 treated for 18 hours with either DMSO or 1  $\mu$ M tselisib  $\pm$  MG132 or  $\pm$  UAE1 inhibitor. Protein  
861 lysates were run on western blot and probed with antibodies to p110 $\alpha$ , pAKT, and b-actin, or  
862 ubiquitinated proteins were pulled-down with TUBE1 reagent and then blotted with anti-p110 $\alpha$   
863 antibody.

864 (E) HCC1954 cells engineered to be isogenic for *PIK3CA*-mutant or -wild-type were treated with  
865 1  $\mu$ M taselisib for up to 48 hrs followed by western blotting. Experimental replicates n=3 were  
866 used to quantify p110 $\alpha$ .

867 (F) Pulse-chase of isogenic cell lines, HCC1954\_mutant and HCC1954\_wild-type. PI3K inhibitor  
868 taselisib at 1  $\mu$ M was added during the chase. Pull-down with p110 $\alpha$  antibody was followed by  
869 autoradiography, and data fit to a single exponential decay function.

870

#### 871 **Figure 4**

872 **Taselisib-mediated degradation of mutant p110 $\alpha$ ha occurs preferentially at the plasma**  
873 **membrane**

874 (A) Subcellular fractionation of isogenic HCC1954\_mutant and HCC1954\_wild-type cells. Pull  
875 down of ubiquitinated protein was followed by western blotting with anti-p110 $\alpha$ .

876 (B) HCC1954\_mutant cells were transfected with p85 $\alpha$ , p85 $\beta$  or p55 $\gamma$  isoform specific siRNA  
877 followed by 1  $\mu$ M taselisib treatment.

878 (C) HCC1954\_mutant cell line was treated with 1  $\mu$ M taselisib for up to 24 hours. Cell lysates  
879 were precipitated with p85 $\gamma$  or p85 $\beta$  antibody, followed by immunoblot with antibodies indicated  
880 to the left.

881 (D) HCC1954\_mutant cells were transfected with p85 $\alpha$  or p85 $\beta$  siRNA followed by taselisib  
882 treatment. Cells were harvested at various time points and fractionated. K63 or K48 linked  
883 ubiquitin conjugated protein was pulled down from membrane fraction and analyzed by  
884 immunoblotting with p110 $\alpha$  antibody.

885

#### 886 **Figure 5**

887 **Taselisib- and GDC-0077-induced mutant p110 $\alpha$  degradation is dependent on RTK activity**

888 **(A)** Cell viability IC<sub>50</sub> values determined by quantifying ATP from breast tumor lines: HER2-  
889 positive *PIK3CA*-mutant (n=6), HER2-positive *PIK3CA*-wild-type (n=4), HER2-negative  
890 *PIK3CA*-mutant (n=10), and HER2-negative *PIK3CA*-wild-type (n=20) at 5 days post-treatment.

891 **(B)** Bar plot showing *PIK3CA*-mutant frequency among tumor lines harboring *PIK3CA* hotspot  
892 mutations. ATP based cell viability assay in selected cell line (HCC2185, SW948, EFM-19,  
893 HCC1954, T-47D, NCI-1048, VP303, MDAMB453 and KPL4). western blot of the p110 $\alpha$  protein  
894 levels and pAKT signaling in same cell lines treated with GDC-0077 for indicated concentrations  
895 for 24 hours.

896 **(C)** HER2-negative *PIK3CA*-wild-type or -mutant cells cultured in standard media with 10% FBS  
897 and treated with GDC-0077 alone or addition of growth factors (EGF and neuregulin).

898 **(D)** HCC1954 *PIK3CA* H1047R cells treated with tselisib alone or combination of tselisib and  
899 lapatinib. Cell lysates were precipitated with p110 $\alpha$  antibody, followed by western blot with HER3  
900 antibody.

901 **(E)** Cell lysates following treatment with tselisib or GDC-0077 alone or combination with  
902 lapatinib for indicated time points followed by western blot analysis with indicated antibodies.

903

904 **Figure 6:**

905 **p110 $\alpha$ -mutant degrading inhibitors provide more benefit in HER2-positive versus HER2-**  
906 **negative p110 $\alpha$ -mutant cancers**

907 **(A)** Mechanistic model of drug-induced p110 $\alpha$  degradation in HER2-positive and HER2-negative  
908 *PIK3CA*-mutant cells.

909 (B) Tumor growth curve from KPL-4 (HER2+, PIK3CA H1047R) xenograft treated with vehicle,  
910 taselisib, trastuzumab plus pertuzumab, or the indicated drug combination.

911 (C) Tumor growth curve from KPL-4 (HER2+, PIK3CA H1047R) xenograft treated with vehicle,  
912 GDC-0077, TDM-1 or the indicated drug combination.

913

## 914 **Figure S1**

### 915 **Taselisib and GDC-0077 have increased potency in *PIK3CA*-mutant cancer cells**

916 (A) PI3K inhibitors assessed for apoptosis induction in mutant and wild-type breast cancer cell  
917 lines in 72 hour Nucleosome ELISA. Error bars are standard deviation of triplicates.

918 (B) Cell potency in 4-day CellTiter-Glo® viability assay for taselisib and PI3K $\alpha$  inhibitors  
919 BYL719, GNE-102, GNE-326 in SW48 isogenic H1047R and wild-type cells. Error bars are  
920 standard deviation of triplicates.

921 (C) Combination of PI3K $\alpha$  inhibitor GNE-102 with PI3K $\delta$  inhibitor idelalisib in 4-day viability  
922 assay in HCC1954 PIK3CA H1047R mutant cells. Error bars are standard deviation of  
923 quadruplicates.

924 (D) Combination of taselisib with PI3K $\beta$  inhibitor TGX-221 in HCC1954 PIK3CA H1047R  
925 mutant cells in a 4-day viability assay. Error bars are standard deviation of quadruplicates.

926 (E) Intracellular drug concentrations of taselisib in cancer cell lines treated for 18 hours with 1  $\mu$ M  
927 taselisib. Results of LC/MS/MS for triplicate wells are shown. Error bars are standard deviation  
928 of triplicates.

929 (F) *In vivo* efficacy of GDC-0077 in PIK3CA H1047R breast cancer xenograft HCC1954 and  
930 KPL4 models, and breast cancer HCI003 PDX (patient-derived xenograft) model. GDC-0077  
931 dosed orally and daily (QD) in MCT (0.5 % methycellulose/0.2% Tween-80) vehicle.



932 **Figure S2:**

933 **Activity of GDC-0077 in combination with Palbociclib and/ or Fulvestrant**

934 (A) Dose response curve of MCF-7 cells treated with GDC-0077 alone (blue), without E2 to mimic  
935 aromatase inhibitor (dark blue).

936 (B) Weight loss for MCF-7 xenograft experiment.

937 (C) Efficacy and weight loss for MDA-MB-453 xenograft experiment.

938

939 **Figure S3**

940 **Taselisib and GDC-0077 induce mutant p110 $\alpha$  and not WT p110a degradation**

941 (A) Subcellular fractionation of HCC1954 PIK3CA H1047R mutant cells treated with PI3K  
942 inhibitors taselisib or BYL719 for up to 8 hours, followed by p110 $\alpha$  western blot.

943 (B) Taselisib and BYL719 treatment for 8 hours in HCC1954 PIK3CA H1047R mutant cells and  
944 HDQ-P1 *PIK3CA*-wild-type cells.

945 (C) Quantitative RT-PCR shows no reduction in *PIK3CA* RNA expression in HCC1954 cells  
946 treated with taselisib or BYL719 for up to 24 hours. Error bars are standard deviation of triplicates.

947 (D) Membrane-associated p110a expression following single oral dose of 15 mg/kg taselisib, 40  
948 mg/kg BYL719, or 50 mg/kg GDC-0077 treatment in HCC1954 xenograft tumors.

949

950 **Figure S4**

951 **Taselisib depletes mutant p110 $\alpha$  protein through ubiquitin and proteasome mechanism in a**  
952 **dose and time dependent manner**

953

954 **(A)** *PIK3CA*-mutant cells were treated with PI3K inhibitors at concentrations relevant to plasma  
955 concentrations achieved with clinically administered doses for pictilisib (GDC-0941) (330 mg),  
956 taselisib (6 mg), and GDC-0077 (9 mg).

957 **(B)** Quantitative mass spectrometry analysis of p110 $\alpha$  levels in HCC1954, HCC202 and HDQP1  
958 cells treated with either DMSO or 500 nM taselisib for 24 hours (n=4). For each cell line, the  
959 relative abundance of peptides from loci spanning the protein are compared between DMSO  
960 (above line) and taselisib-treated (below line) cells. For mutant loci (H1047R in HCC1954 cells,  
961 E545K in HCC202 cells), the protein sequence is split to depict the wild-type and mutant-specific  
962 peptides in parallel.

963 **(C)** Mass spectrometry analysis of peptides representing wild-type and E545K mutant p110 $\alpha$  from  
964 HCC202 breast cancer cells treated for 24 hours with either DMSO or 500 nM taselisib. The neo-  
965 tryptic peptide generated from p110 $\alpha$  E545K was used to assess mutant protein levels compared to  
966 wild-type protein in the same lysate (n=4).

967 **(D)** Lysosomotropic agents chloroquine and ammonium chloride (NH<sub>4</sub>Cl) do not rescue p110 $\alpha$   
968 degradation induced by 1.6  $\mu$ M taselisib in HCC1954 cells

969 **(E)** NGS and qRT-PCR for allele-specific mRNA expression in HCC1954 parental and isogenic  
970 HCC1954\_mutant and HCC1954\_wild-type cells. Error bars are standard deviation of triplicates.

971 **(F)** HCC1954\_mutant and HCC1954\_wild-type isogenic cells were treated with 1.6  $\mu$ M taselisib  
972 for up to 9 hours. Pull down of ubiquitinated protein was followed by western blotting with anti-  
973 p110 $\alpha$  antibody.

974 **(G)** Subcellular fractionation of HCC1954\_parental and isogenic HCC1954\_mutant and  
975 HCC1954\_WT cells. Pull down of ubiquitinated protein was followed by western blotting with  
976 anti-p110 $\alpha$  .

977

978 **Figure S5**

979 **Taselisib-mediated degradation of mutant p110 $\alpha$  occurs preferentially at the plasma**  
980 **membrane.**

981 (A) HCC1954\_mutant cells were treated with 1  $\mu$ M taselisib alone or combination with  
982 proteasome inhibitor MG132. Cell lysates were precipitated with p85 $\alpha$ , p85 $\beta$  or p55 $\gamma$  antibody,  
983 followed by immunoblot with antibodies indicated to the left. Real time qPCR assays of the p85  
984 isoforms in RNA collected from untreated cells.

985 (B) HCC1954 cells were treated with 1.6  $\mu$ M taselisib for 24 hours as indicated. Cell lysates were  
986 prepared and total protein were applied to pRTK arrays. Arrows indicate RTKs whose  
987 phosphorylation was up-regulated following the treatment.

988 (C) HCC1954\_mutant cell line was treated with 1  $\mu$ M taselisib alone or in combination with  
989 proteasome inhibitor MG132. Cell lysates were precipitated with p85 $\alpha$ , p85 $\beta$  or p55 $\gamma$  antibody,  
990 followed by immunoblot with antibodies indicated to the left.

991 (D) HCC1954\_mutant cell line was treated with 1  $\mu$ M taselisib alone or in combination with  
992 lapatinib. Cell lysates were precipitated with p85 $\alpha$  or p85 $\beta$  antibody, followed by immunoblot  
993 with antibodies indicated to the left.

994

995 **Figure S6**

996 **RTK activity plays a key role in regulating p110 $\alpha$  degradation**

997 (A) Western blot for membrane associated p110 $\alpha$  in a subset of treated (taselisib 20 mg/kg 4hr  
998 single dose) versus untreated PDX tumors HCI-003, HCI-013, WHIM20, HCI-011, WHIM18.  
999 Total lysates from untreated tumor were applied to pRTK arrays.

1000 **(B)** Cell lines were harvested after treatment with 0.5  $\mu\text{M}$  GDC-0077 or 1  $\mu\text{M}$  BYL719 at various  
1001 time points followed by western blot analysis with indicated antibodies

1002 **(C)** Mechanistic model of drug-induced p110 $\alpha$  degradation in heterozygous *PIK3CA*-mutant cells.  
1003 SKBR3 (HER2-positive *PIK3CA*-wild-type) cells were treated with 0.5  $\mu\text{M}$  GDC-0077 or 1  $\mu\text{M}$   
1004 BYL719 at various time points followed by western blot analysis with indicated antibodies.

1005 **(D)** HCC1954 parental and *PIK3CA*-wild-type and -mutant isogenic cell lines are harvested after  
1006 treatment with 0.5  $\mu\text{M}$  GDC-0077 or 1  $\mu\text{M}$  BYL719 at various time points followed by western  
1007 blot analysis with indicated antibodies. Cell viability  $\text{IC}_{50}$  values determined by quantifying ATP  
1008 from same cell lines at 5 days post-treatment.

1009 **(E)** HCC2911 parental and *PIK3CA*-wild-type and -mutant isogenic cell lines are harvested after  
1010 treatment with 0.5  $\mu\text{M}$  GDC-0077 or 1  $\mu\text{M}$  BYL719 at various time points followed by western  
1011 blot analysis with indicated antibodies. Cell viability  $\text{IC}_{50}$  values determined by quantifying ATP  
1012 from same cell lines at 5 days posttreatment.

1013

Figure 1

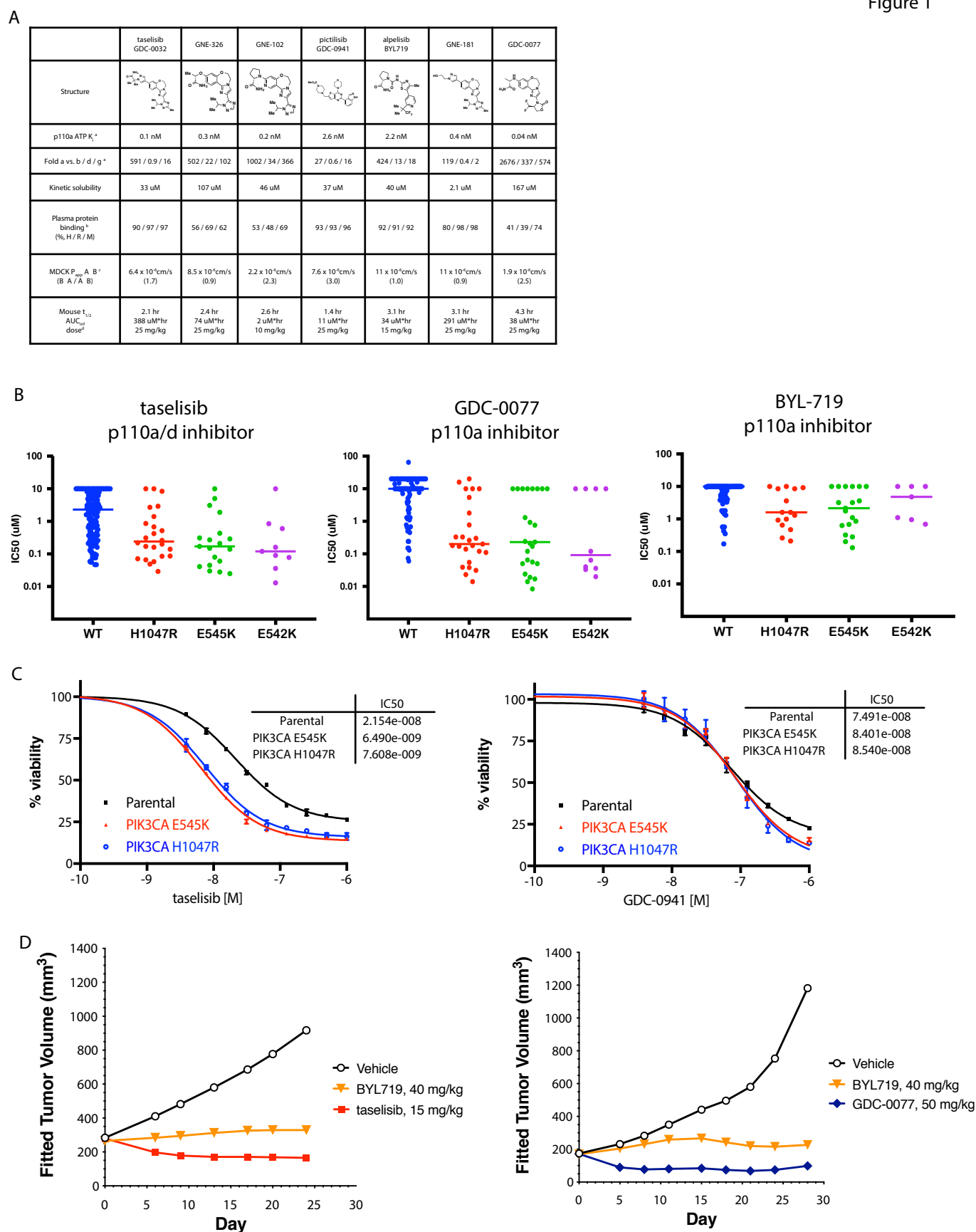


Figure 2

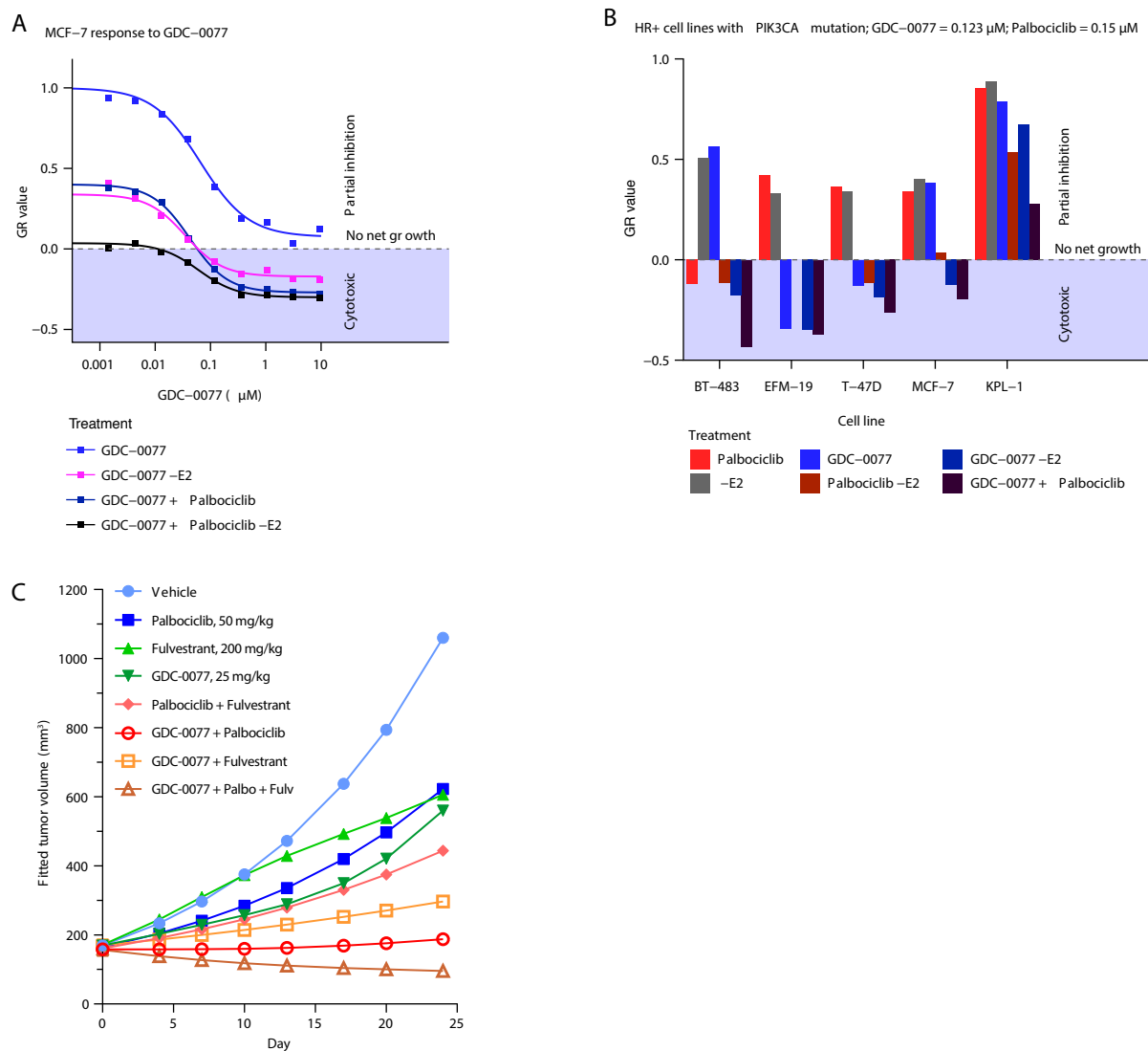


Figure 3

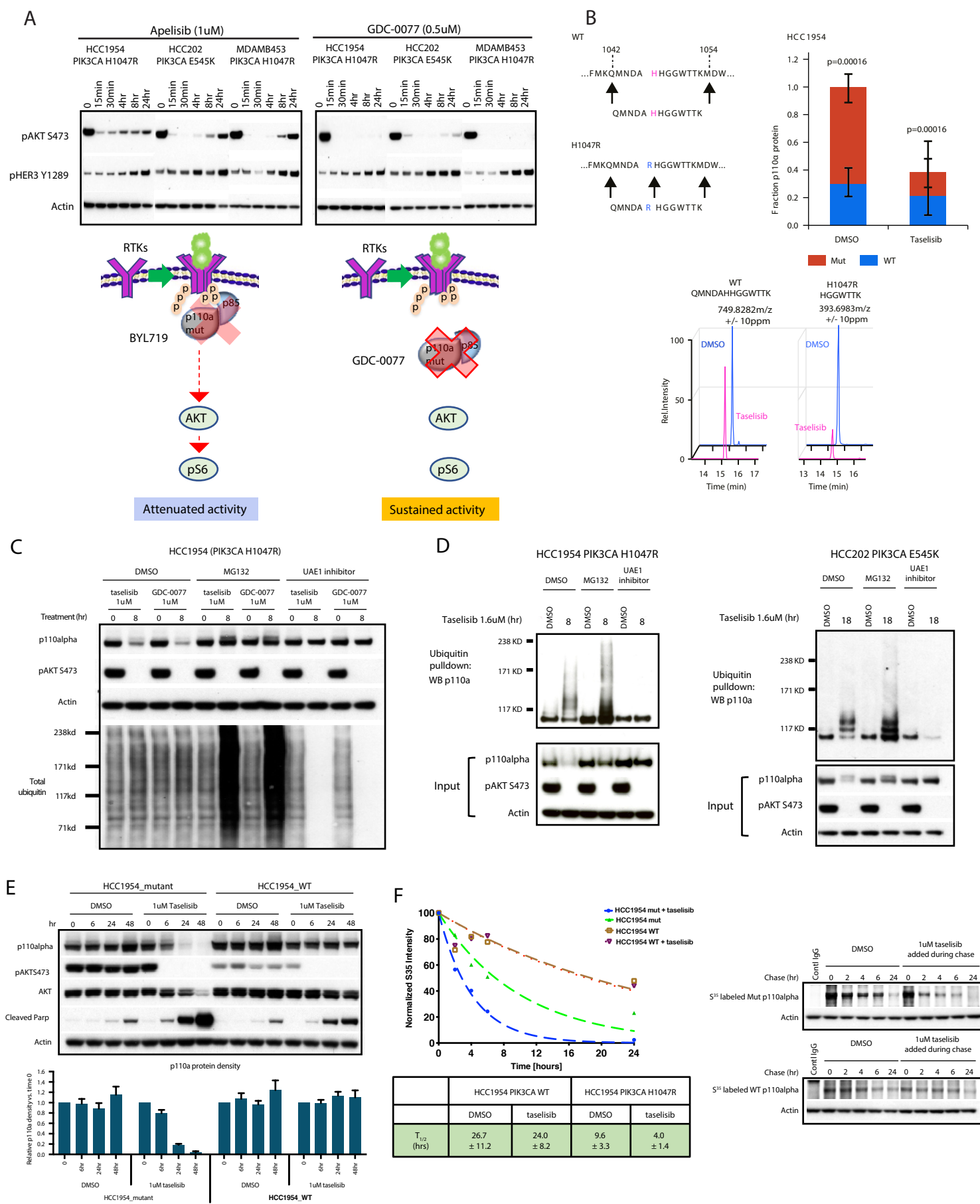


Figure 4

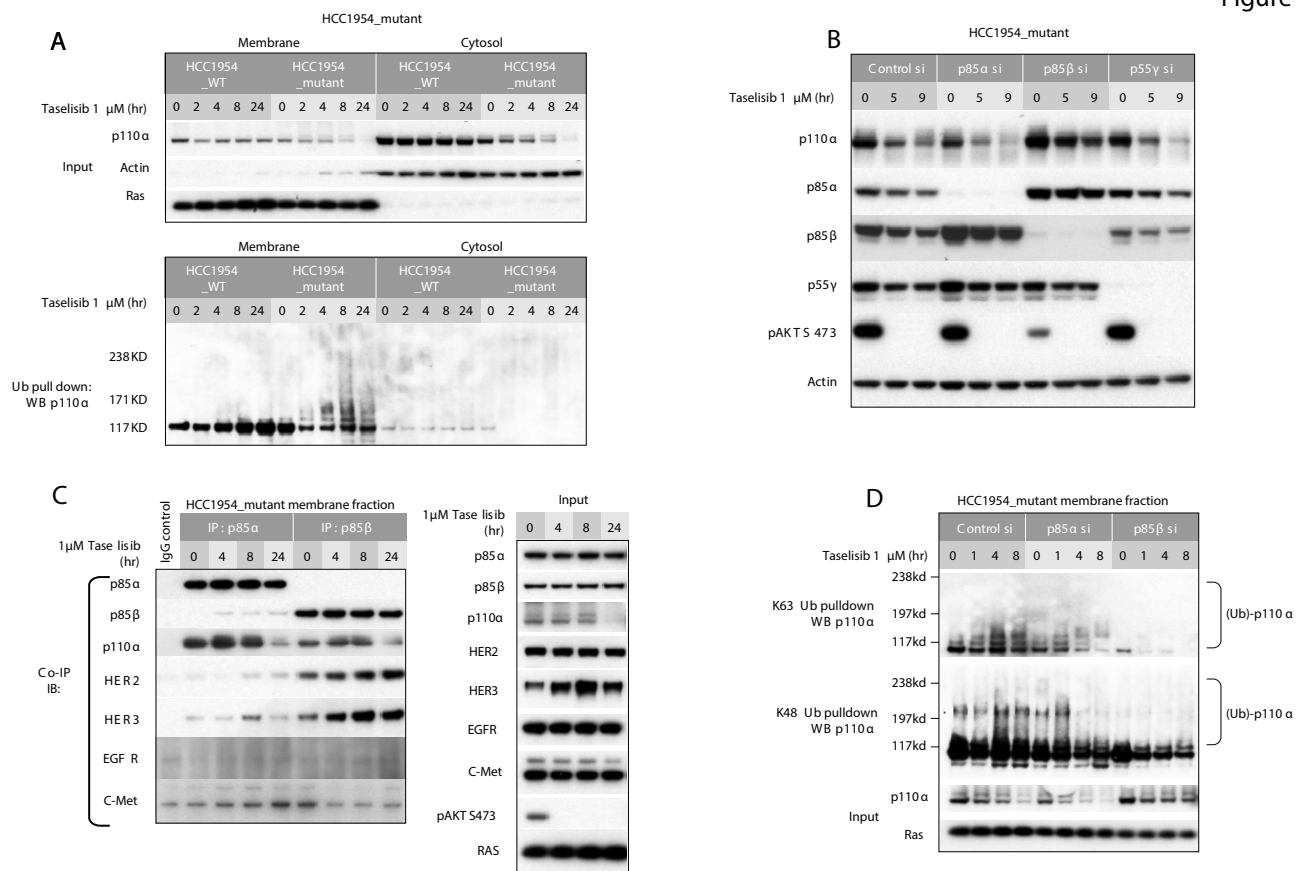




Figure 5

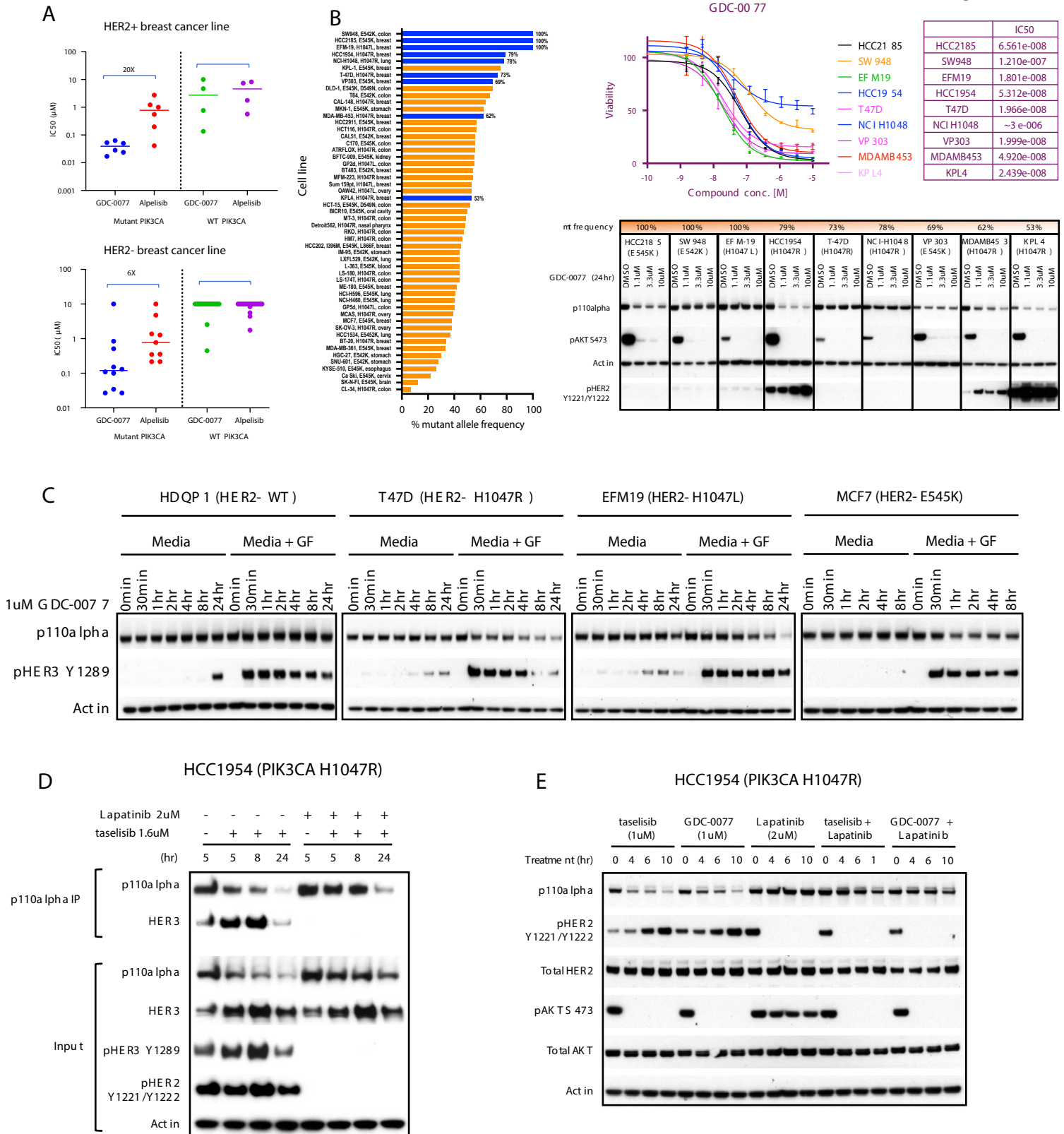


Figure 6

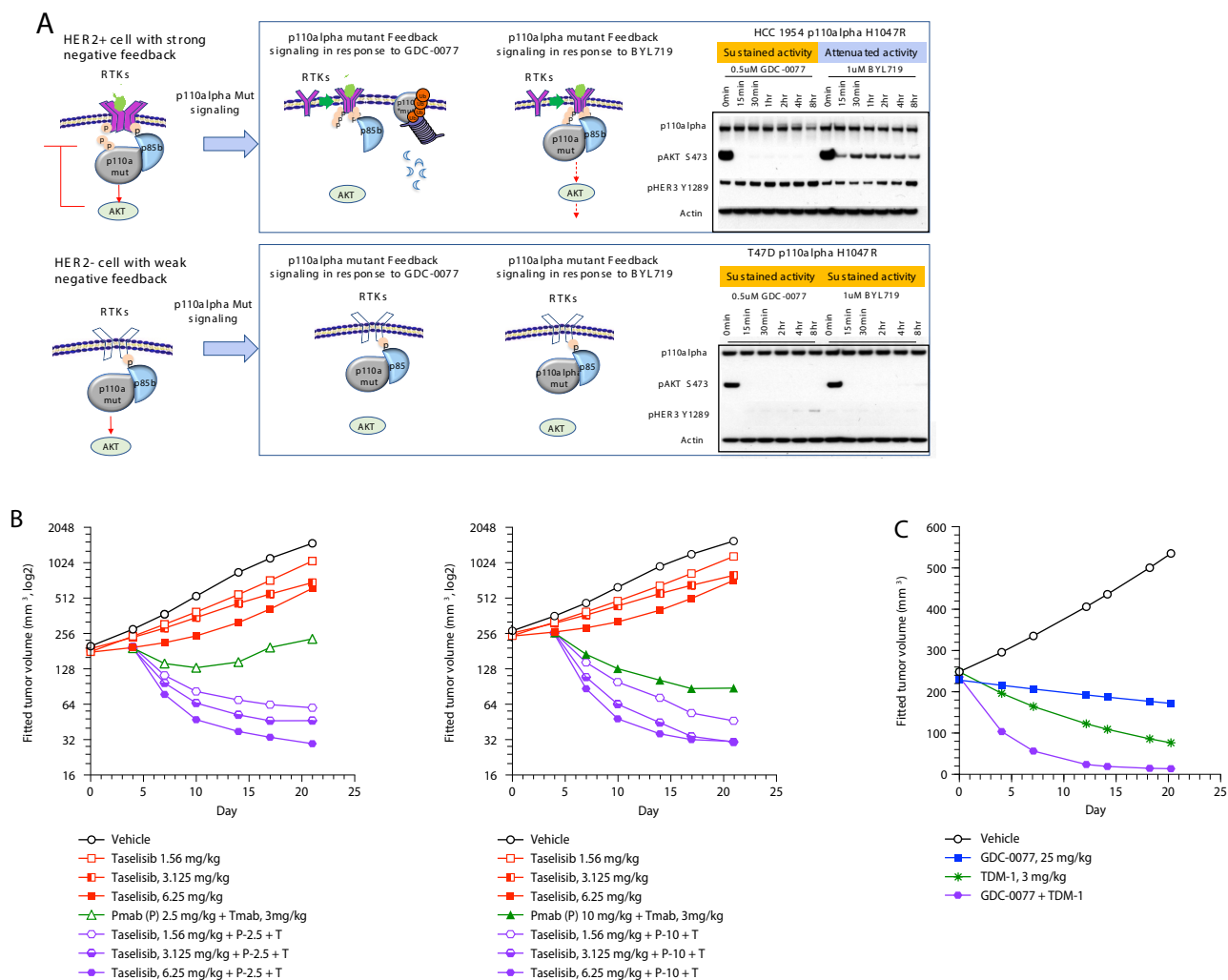


Figure S1

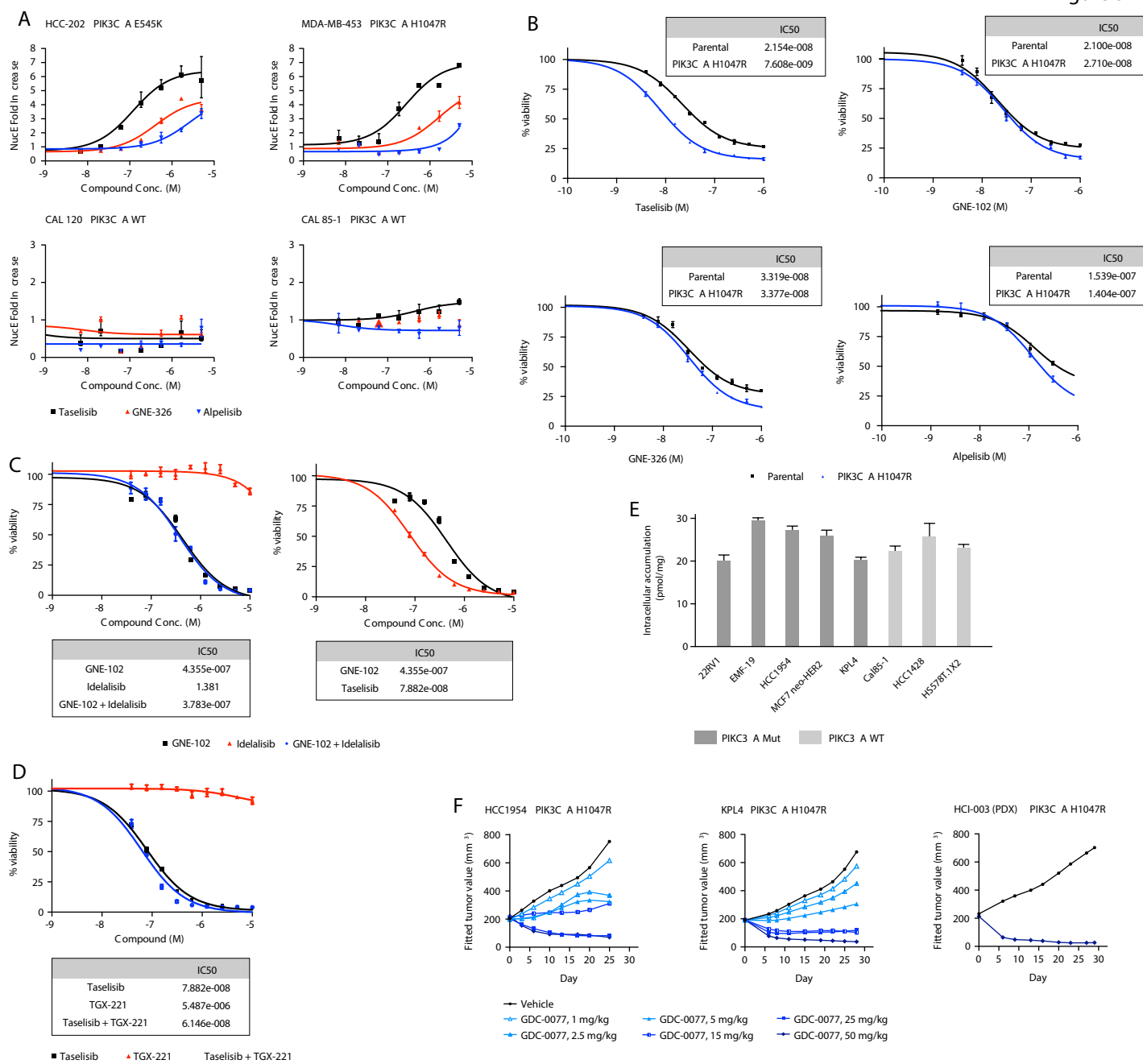


Figure S2

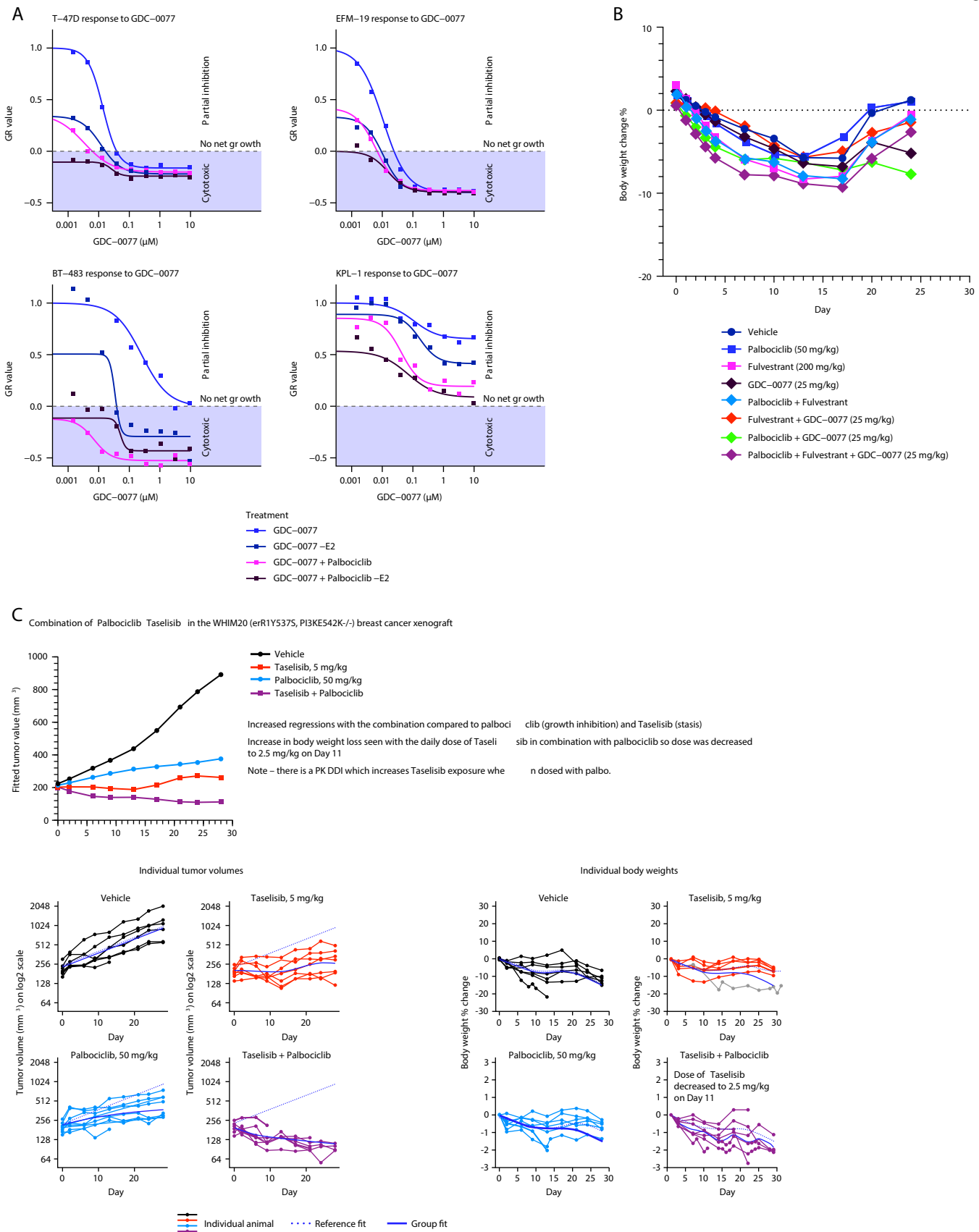


Figure S3

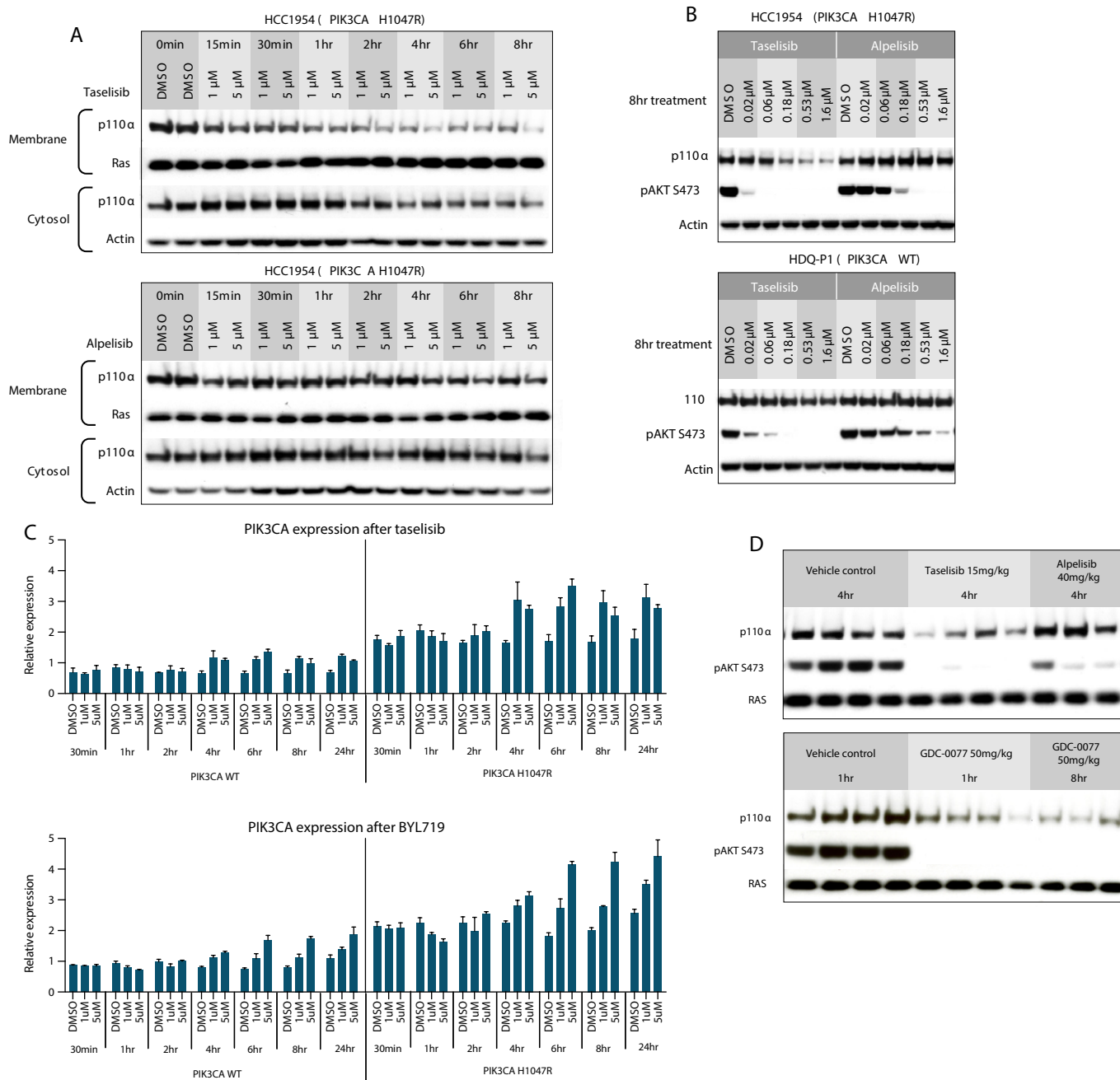


Figure S4

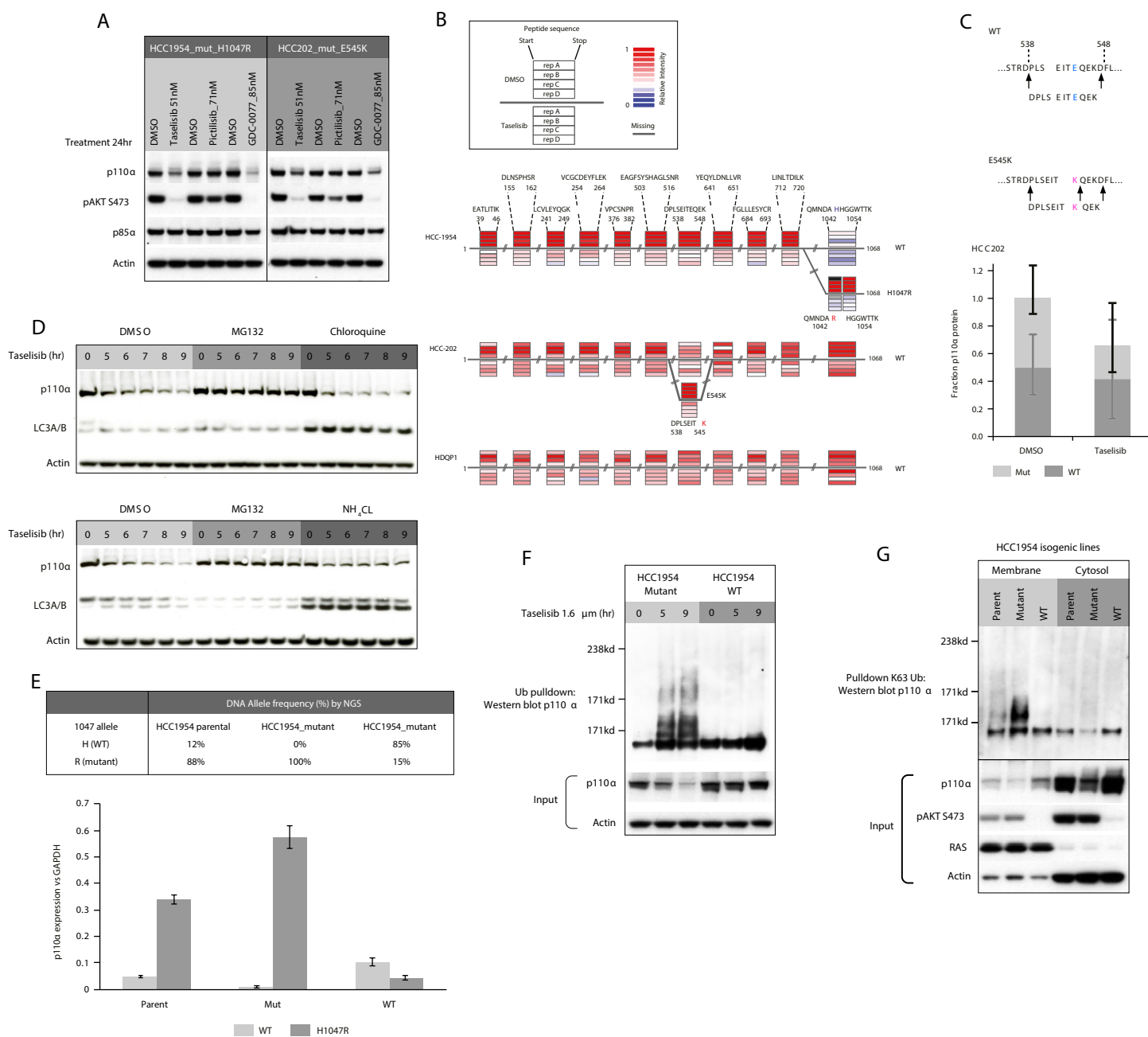


Figure S5

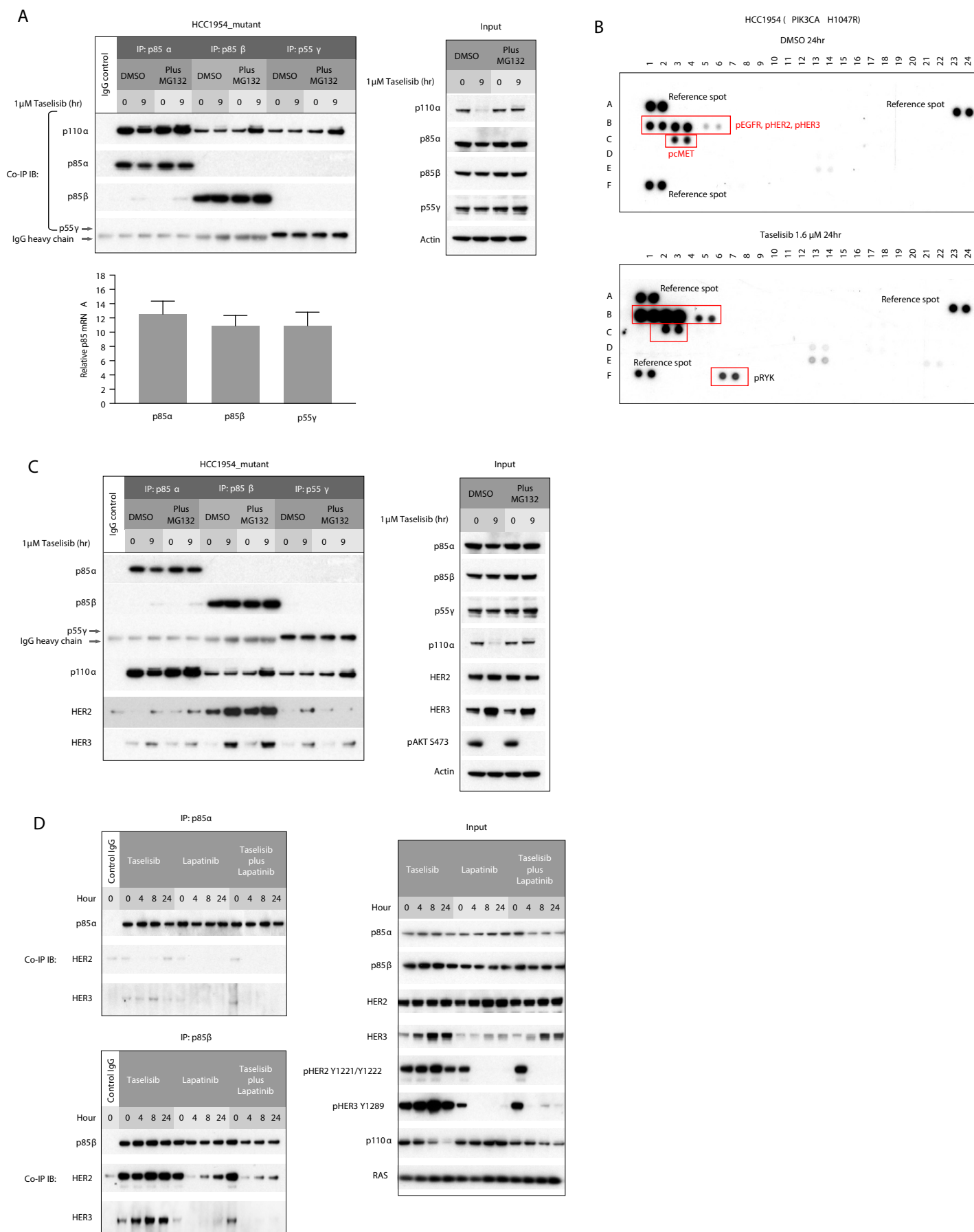


Figure S6

