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Acquired HER2 mutations in ER+ metastatic breast cancer confer resistance to ER-directed therapies

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Despite the reduction in cancer recurrence and mortality provided by therapies that target the estrogen receptor (ER), resistant ER+ breast cancer remains the most common cause of breast cancer death. Beyond mutations in ER, which occur in 25-30% of patients treated with aromatase inhibitors, our understanding of clinical mechanisms of resistance to agents that target the ER remains incomplete. We identified activating HER2 mutations in metastatic biopsies from eight patients with ER+ metastatic breast cancer (MBC) who had developed resistance to ER-directed agents, including aromatase inhibitors, tamoxifen, and fulvestrant. Examination of the treatment-naïve primary tumors in five patients revealed no evidence of pre-existing mutations in four of the five patients, suggesting that these four mutations were acquired under the selective pressure of ER-directed therapy. These HER2 mutations were mutually exclusive with ER mutations, suggesting a distinct mechanism of acquired resistance to ER-directed therapies. *In vitro* analysis confirmed that these mutations conferred estrogen independence. In addition, and in contrast to ER mutations, these HER2 mutations resulted in resistance to tamoxifen, fulvestrant, and the CDK4/6 inhibitor palbociclib. One mutation observed in two patients, S653C, occurring in the transmembrane domain and not previously observed in breast cancer, was shown to likely function through constitutive dimerization. Resistance caused by all four mutations was overcome by combining ER-directed therapy with the irreversible HER2 kinase inhibitor neratinib, suggesting an effective treatment strategy in these patients.

Estrogen-receptor positive (ER+) breast cancer is the most common cause of breast cancer mortality, accounting for more than 20,000 deaths in the U.S. annually. Endocrine therapy is the

mainstay of therapy for patients with ER+ metastatic breast cancer (MBC); however, virtually all patients with ER+ MBC will develop therapeutic resistance. Beyond mutations in the ER itself, which occur in 25-30% of patients treated with aromatase inhibitors (AIs)[1-4], knowledge about clinical resistance mechanisms remains incomplete.

The epidermal growth factor receptor 2 (*ERBB2*/HER2) is frequently altered in cancer; in breast cancer this is manifested primarily via gene amplification[5] or HER2 overexpression. There is considerable *in vitro* evidence that HER2 signaling may play a complementary role to the estrogen pathway in ER+ breast cancer, through ER crosstalk or downstream signaling to provide survival signals in the context of estrogen deprivation[6-13] (See **Supplementary Note)**. Mutations in HER2 are comparatively rare in breast cancer, accounting for 1.6% of primary breast cancers in The Cancer Genome Atlas study[14]. Several activating hotspot mutations in the kinase and extracellular domains have been characterized[14-17]. However, the role of such mutations in MBC is less well understood.

As part of an ongoing sequencing study of ER+ MBC[18], we performed whole exome sequencing (WES) of metastatic tumor biopsies from 168 patients, nearly all of whom had received prior endocrine therapy[18]. In 12 patients, we identified mutations in *ERBB2*, including hotspot mutations in the kinase domain (KD), as well as in the extracellular (ECD), transmembrane (TM), and cytoplasmic (CD) domains (**Figure 1A**). The KD L755S, V777L, and L869R mutants have previously been identified and characterized as activating in breast cancer[14,15]. The TM mutation S653C (homologous to activating EGFR mutation S645C[19,20]) has not been described in

breast cancer, though was previously observed in one patient with urothelial bladder carcinoma, where it was characterized as activating and sensitive to lapatinib[21]. One metastatic biopsy had both G727A and V777L mutations; this combination has been reported in breast cancer[22], and homologous mutations to G727A are activating in EGFR (G719)[23] and found in combination with other EGFR mutations in NSCLC[24]. The ECD and CD mutants have not been previously reported. Overall, the increased prevalence of HER2 mutations in the metastatic setting seen here (compared to primary ER+ breast cancer[5,25,26]) is consistent with recent sequencing studies that included MBC[27-31] (**Supplementary Figure 1**; **Supplementary Table 1)**, though those studies did not specifically discuss HER2 mutations in MBC.

To determine if these 12 HER2 mutations were acquired in the metastatic setting, we obtained and performed WES on corresponding primary tumor biopsies collected prior to exposure to any endocrine therapy in eight patients[18]. In six of the eight primary tumors (75%), the HER2 mutations identified in the metastatic biopsies were not observed, suggesting that these mutations were acquired over the course of therapy (**Figure 1A, red triangles)**.

Figure 1B depicts clinical vignettes for the eight patients with activating HER2 mutations in their metastatic biopsies. Among these eight patients, the activating HER2 mutations were found to be acquired in four patients, shared in one, and indeterminate in three. All patients were treated with ER-directed therapy prior to their metastatic biopsies, including tamoxifen (five patients), AIs (six patients), and fulvestrant (two patients). Vignettes for the four patients with uncharacterized HER2 mutations are shown in **Supplementary Figure 2**. **Figure 2A** illustrates

all additional mutations observed in 593 cancer genes in metastatic biopsies and matched primaries when available. Detailed clinicopathological features, therapies, and genomic data for all patients and samples are found in **Supplementary Tables 2-6.**

To further explore the role of acquired HER2 mutations in these tumors we performed an evolutionary analysis to evaluate clonal structure and dynamics. **Figure 2B** illustrates the change in the estimated fraction of tumor cells harboring each genomic alteration (the cancer cell fraction, CCF) from the pretreatment primary biopsy to the resistant metastatic biopsy. In all four patients, HER2 mutations were not detected in the primary tumor, despite power to detect mutations at this locus (**Supplementary Table 5**). In three of the metastatic biopsies, the activating HER2 mutations were found to be clonally acquired. In the fourth biopsy, the HER2 mutation was subclonal, though evaluation of the clonality here was confounded by a concurrent HER2 amplification. Other alterations, including known driver mutations, were found to be clonal in both primary and metastasis in these patients. Evolutionary analysis for patients with uncharacterized HER2 mutations is shown in **Supplementary Figure 3.**

Although *ESR1* mutations are an established mechanism of resistance to AIs in ER+ MBC[1-4], none of the patients with acquired HER2 mutations had *ESR1* mutations in their metastatic biopsies, suggesting that these might be mutually exclusive events. Examination of publicly available sequencing data from the AACR Project GENIE database V1.0.1[30] demonstrated that *ERBB2* and *ESR1* mutations were indeed mutually exclusive, consistent with our results. Of 1,019 MBC samples (ER+ and ER-), 48 samples had an *ERBB2* mutation only, 109 had an *ESR1*

mutation only, and one had both mutations (odds ratio =0.17, p =0.0269, one-sided Fisher's exact test). Based on these findings, along with the higher overall incidence of acquired activating HER2 mutations in our cohort, we hypothesized that HER2 mutations may be a mechanism of acquired resistance to ER-directed therapy in ER+ MBC.

To investigate whether acquired HER2 mutations directly confer resistance to ER-directed therapy, we acutely expressed all HER2 mutants observed in the 12 patients in the ER+/HER2- breast cancer cell lines T47D and MCF7 through lentiviral transduction and examined the impact of acute infection on susceptibility to endocrine agents. These included estrogen-deprivation (charcoal dextran-stripped serum media), which recapitulates AI treatment *in vitro,* tamoxifen, and the selective estrogen receptor degraders (SERDs) fulvestrant and GDC-0810[32]. As controls, we transduced cells with wild-type HER2 and the kinase-dead HER2 mutant D845A[16].

T47D cells expressing the KD (L755S, V777L, and L869R) or TM (S653C) mutations were strongly resistant to estrogen deprivation *in vitro,* conferring a level of resistance equivalent to the previously described resistance-associated *ESR1* ligand binding domain (LBD) mutation Y537S (**Figure 3A**). Wild-type HER2 conferred only modest resistance, and the kinase-dead D845A mutation[16] did not alter sensitivity compared to GFP-expressing cells. We obtained similar results in MCF7 cells (**Supplementary Figure 4A**).

While *ESR1* LBD mutations confer robust resistance to estrogen deprivation, they only confer partial resistance to tamoxifen and fulvestrant[1-4]. In contrast, HER2 KD and TM mutants were

completely resistant to tamoxifen (**Figure 3B**), fulvestrant (**Figure 3C),** and GDC-0810 **(Figure 3D)**. Similar results were observed in MCF7 cells (**Supplementary Figure 4**). Wild-type HER2 as well as ECD and CD mutants conferred intermediate resistance between that of *ESR1* Y537S and the KD/TM mutants (**Figures 3B-D; Supplementary Figures 4-6**). Expression levels of HER2 were similar for all HER2 constructs (**Figure 3E-F)**. Expressing the compound G727A and V777L kinase domain mutation (observed in patient *0300252*) conferred resistance to all ER-directed therapies (**Supplementary Figure 7**). All *in vitro* drug sensitivities were consistent with clinical resistance phenotypes in patients bearing the respective HER2 mutations (**Figure 1B**). Similar findings were seen at low levels of mutant HER2 expression, demonstrated using HER2 mutants expressed under a tetracycline responsive promoter in cells grown in low doses of doxycycline (**Supplementary Figure 8**).

HER2 activates pro-survival signaling pathways in cells, including RAS/RAF/MAPK and PI3K/AKT. HER2 mutants were associated with hyperphosphorylation of both ERK and AKT under conditions of estrogen-deprivation or inhibition (**Figures 3E-F**). *ESR1* Y537S did not hyperactivate MAPK or AKT signaling, while ECD and CD mutants had effects similar to wild-type HER2 (**Supplementary Figure 5E-F**). HER2 mutant cells also had lower levels of ER than controls (**Figure 3E**), consistent with prior studies in tamoxifen-resistant cells that also demonstrated lower levels of ER[33]. Treatment with fulvestrant resulted in an additional decrease of ER in HER2 mutant cells, but did not impact AKT or ERK phosphorylation (**Figure 3F**).

To investigate whether ER signaling is suppressed in HER2 mutant cells, we examined transcript levels of *ESR1* and the ER targets progesterone receptor (PR/*PGR*), *GREB1,* and *TFF1* by quantitative RT-PCR. *ESR1* transcript levels were significantly downregulated in HER2 mutant cells along with *PGR* and *GREB1* (**Figure 3G**), although *TFF1* was upregulated. The downregulation of *PGR* was consistent with the phenotype observed in the metastatic biopsies from patients, where acquisition of activating HER2 mutations coincided with loss of PR expression by immunohistochemistry (**Figure 1B, Figure 2**). The ECD and CD HER2 mutants suppressed ER, PR, and GREB1 only slightly (**Supplementary Figure 5G**).

To further examine transcriptional changes associated with HER2 mutants, we performed RNA sequencing on cells expressing the four activating HER2 mutants as well as GFP, wild-type HER2, kinase-dead HER2, and *ESR1* Y537S, all treated with either DMSO or fulvestrant (**Figure 4A**). Principal component analysis of the transcriptomes demonstrated that the four HER2 activating mutants clustered together and remained separate from a cluster containing cells expressing *ESR1* Y573S, as well as from a cluster containing cells expressing GFP, kinase-dead HER2, and one containing wild-type HER2 (**Figure 4B**). Under treatment with fulvestrant, 5,293 genes were significantly differentially expressed (q.value = 0.01) between all activating HER2 mutants compared to GFP (**Figure 4C; Supplementary Table 7** and **8)**. We next defined a shared HER2-MUT expression signature across the four activating HER2 mutants. Gene Set Enrichment Analysis[34] showed that the shared signature is enriched for *ERBB1/2* and RAS/MAPK signaling compared to GFP and wild-type HER2 cells treated with fulvestrant or DMSO (**Figure 4D, panels 1 and 2; Supplementary Table 9-12**).

Canonical ER targets were among both the upregulated and downregulated HER2-MUT genes (**Figure 4D, panel 3**), consistent with our quantitative RT-PCR of *PGR, GREB1,* and *TFF1* (**Figure 3G**). We hypothesized that HER2 mutants may result in reprogrammed ER signaling, similar to the previously described growth factor-induced ER cistrome[35]. Indeed, the HER2-MUT signature was significantly enriched for induction of growth factor-induced ER targets in HER2 mutant cells (**Figure 4D, panel 4**), along with a concomitant suppression of targets induced by estradiol, as previously observed in tamoxifen-resistant MCF7 cells[33]. Individually, all four HER2 mutants demonstrated the elevated RAS/MAPK transcriptional signature (**Figure 4E**) as well as the *ERBB1/2* and growth factor-driven ER signatures (**Supplementary Figure 9**).

HER2 KD mutations are known to activate HER2 signaling through conformational changes to the catalytic domain that destabilize the inactive conformation of the kinase[14] (**Figure 5A)**. The G727A/V777L co-mutations are in apposition as indicated, and likely modify KD conformation and sensitivity to reversible kinase inhibitors, as shown for EGFR previously[36]. In contrast, the mechanism of activation of the S653C TM mutation has not been reported (**Figure 5B**). We hypothesized that the cysteine residues form disulfide bridges, resulting in constitutive HER2 homodimerization. To test this, we performed immunoblotting under non-reducing conditions to look for intact HER2 dimers. HER2 S653C but none of the other mutants tested demonstrated higher molecular weight species, indicating reduction-sensitive dimers (**Figure 5C)**. Thus,

S653C likely functions by constitutive dimerization, as previously observed for HER2 ECD mutants G309E and E321G[16] as well as other TM domain mutants[37,38] (**Figure 5D**).

Neratinib, an irreversible kinase inhibitor with anti-pan-HER activity, inhibits HER2 L755S, V777L, and L869R *in vitro*[14,15] and has shown promise in monotherapy clinical trials in breast cancer patients with activating HER2 mutations[39,40]. Low doses of neratinib re-sensitized HER2 mutant cells to fulvestrant (**Figure 6A)**. These cells were also sensitive to neratinib monotherapy at a higher dose (**Supplementary Figure 10C)**. In all cases, combination with fulvestrant inhibited viability better than neratinib alone, suggesting that inhibition of HER2 mutants restored sensitivity to fulvestrant. In contrast, neratinib only partially resensitized *ESR1* Y537S cells to fulvestrant (**Figure 6A-B**), and high dose neratinib monotherapy was also ineffective in these cells (**Supplementary Figure 10A, C**) (see **Supplementary Note).**

Neratinib treatment of HER2 mutant cells decreased both ERK and AKT phosphorylation (**Figure 6B; Supplementary Figure 10B**). In contrast, pharmacologic inhibition of individual downstream effectors MEK, ERK, PI3K, AKT, and MTOR, alone or in combination with fulvestrant, failed to restore sensitivity to fulvestrant in HER2 mutants cells (**Supplementary Figure 10E-F**).

Combination treatment with endocrine therapy and CDK4/6 inhibitors is currently standard-of-care treatment for ER+ MBC. HER2 mutant cells were cross-resistant to the CDK4/6 inhibitor palbociclib, both alone and in combination with fulvestrant (**Figure 6C;**

Supplementary Figure 10D). Consistent with this, RNA-seq analysis revealed that HER2 mutant cells treated with palbociclib, either as single agent or with fulvestrant, grouped with DMSO and fulvestrant treated cells; cells treated with neratinib, with or without fulvestrant, grouped separately (**Figure 6D**). RAS-MAPK transcriptional activity remained robust in the presence of palbociclib monotherapy or combination therapy (**Figure 6E**). However, neratinib treatment, both as a single agent and with fulvestrant, resulted in repression of RAS/MAPK transcriptional activity (**Figure 6E)** and other transcriptional effects of HER2 mutants **(Supplementary Figure 11**). This was borne out clinically in one patient in the cohort with an acquired HER2 V777L mutation after progression on tamoxifen, who had intrinsic resistance to subsequent letrozole/palbociclib therapy (**Figure 6F**). The discovery of the acquired HER2 mutation in the metastatic biopsy prompted enrollment of the patient onto a Phase II trial of fulvestrant plus neratinib[41], resulting in a partial response lasting 6 months, consistent with our *in vitro* findings (**Figure 6A**).

Taken together, our results suggest that activating HER2 mutations are a distinct mechanism of acquired resistance to multiple forms of ER-directed therapy in MBC that can be overcome by an irreversible HER2 inhibitor. Our observations also suggest that, with the increasing clinical use of SERDs, which can overcome *ESR1* mutations, the prevalence of HER2 mutations might increase. The acquisition of targetable activating mutations in the metastatic setting highlights the importance of serial profiling of metastatic tumor biopsies or cell free DNA from blood at the time of resistance in patients with ER+ MBC. Identification of HER2 mutations in real time may help identify patients who will not benefit from ER-directed therapies or should be directed to

clinical trials testing strategies to overcome this mechanism of resistance. One such strategy, the combination of fulvestrant and neratinib is being tested in Phase II clinical trials[42] with promising preliminary results reported in individual patients[15] including within our cohort (**Figure 6F**). Ultimately, the use of up-front combinations to preempt the emergence of HER2-mutant resistant clones may lead to more durable responses in patients with ER+ MBC.

DATA AVAILABILITY

Tumor and germline whole exome sequencing data generated and analyzed for this study have been deposited in the access-controlled public repository dbGAP with the accession codes phs001285 (https://www.ncbi.nlm.nih.gov/gap). Additional data generated in this study including tumor exome analysis and RNA-seq data are available within the paper and in the supplementary information files.

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AUTHOR CONTRIBUTIONS

UN, OC, and NW conceived and designed the study; UN, CK, and MSC performed experiments; OC performed the computational analyses; OC and SF evaluated the evolutionary trajectories; AGW, SAW, and NW performed clinical data abstraction and annotation; CP and NSP performed kinase structural modeling; OR and AR supervised the RNA-seq experiment; LM, KH, and NO assisted with acquisition and annotation of clinical samples; CXM is the principle investigator on the clinical trial of fulvestrant/neratinib on which Patient 315 was treated; EPW, NUL, and NW oversaw patient enrollment and sample collection on the metastatic biopsy protocol; UN, OC, and NW wrote the manuscript with input from all authors; NW supervised the study.

COMPETING FINANCIAL INTEREST STATEMENT

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FIGURE LEGENDS

Figure 1: Acquired HER2 mutations in patients with endocrine resistance. (A) The location of HER2 mutations identified by sequencing metastatic biopsies is depicted along the length of the protein. Protein domains are indicated by color coding. Evolutionary classification for mutations are indicated by Red triangles = acquired mutations, Blue triangles = mutations shared with primary tumor, Grey triangles = indeterminate/unknown. (B) Clinical timelines for the 8 ER+ MBC patients bearing HER2 mutations in metastatic biopsies. Patient histories are shown from breast cancer diagnosis until metastatic biopsy sequenced in this study; arrows represent distinct therapies and durations, described in the legend. In each case, asterisks demarcate the time of diagnosis of metastatic disease.

Figure 2: Mutational landscape, clonal structure, and evolutionary dynamics in HER2 mutant metastatic tumors and matched primary tumors. (A) Single nucleotide variants (SNVs) and small insertions/deletions (indels) are depicted if observed in 593 cancer genes across the eight metastatic tumors with activating *ERBB2* mutations (top row) and, when available, matched primary tumors. All non-silent mutations in coding regions of the protein with high clonality (cancer cell fraction, CCF>50%) are shown. The primary tumor for patient *0300397* was sequenced using a targeted gene panel[43-45], so genes not included in this panel are coded in grey for this sample. For patient *0300322*, the *CDH1* mutation results in p.658_665LEVGDYKI>F (the annotation is truncated in the Figure). Clinical tracks annotate each sample by their ER status, PR status, and HER2 status. (B) The clonal dynamics are shown for four metastatic samples with activating HER2 (*ERBB2*) mutations by comparing the

metastatic cancer cell fraction, CCF (y-axis) to the matched primary CCF (x-axis). *ERBB2* mutations are mapped to metastatic acquired clones in all four patients (colored in red, upper left corner), not detected in the primary tumor, and found as clonal in the metastatic tumor in three patients (S653C, V777L, V777L/G727A) and sub-clonal in one patient (L755S). 'Truncal' mutations that are shared between the metastatic and the primary tumors are found in all patients, demonstrating that the primary and metastatic samples are clonally related (colored in grey, upper right corner, CCF ~1 in both the primary and the met). Primary-specific mutations are found in clones that were dominant in the primary but these clones are not observed in the metastatic tumor (colored in blue). The phylogenetic relationships among clones is reconstructed for each patient starting from the normal cell (white circle) connected to the ancestral cancer cells (grey trunk). The phylogenetic divergence to the primary clones (and sub-clones) is depicted with blue edges, and phylogenetic divergence to the metastatic clones (and sub-clones) is depicted in red. Selected mutations in cancer genes are marked on the corresponding branches of the cancer phylogeny.

Figure 3: HER2 mutations confer endocrine resistance. (A-D) T47D HER2 mutant and control cells were compared on the basis of sensitivity to estrogen deprivation and anti-ER agents tamoxifen, fulvestrant, and GDC-0810. (A) T47D cells expressing the indicated HER2 mutants or controls were serum-starved for two days, followed by treatment with vehicle or 10nM estradiol (E2), as indicated. After a week, relative viability compared to cells grown in complete media was analyzed by CellTiter-Glo. Results shown are mean±SEM of three technical replicates, and representative of 14 independent experiments. (B-D) Cells were plated as in (A),

and switched to full media containing a range of concentrations of tamoxifen (B), fulvestrant (C), or GDC-0810 (D) after two days. Cells were re-treated after 3 days. Viability was determined by CellTiter-Glo assay after a week, and normalized to untreated wells. Results shown are mean±SEM and representative of 13, 15, or nine independent experiments, respectively. (E-F) Levels of HER2 activation markers phospho-ERK and phospho-AKT were examined by western blotting in T47D HER2 mutant and control cells. T47D HER2 mutant and control cells were plated in estrogen-deprived media for 48 hours, then switched to fresh media supplemented with CSS (E), or complete media containing DMSO or 1μM fulvestrant (F), for 24 hours. Whole-cell extracts were analyzed by western blotting using the indicated antibodies. Results shown are representative of six and two independent experiments, respectively. (G) Levels of ER downstream target transcripts were examined by qPCR in T47D HER2 mutant and control cells. T47D HER2 mutant and control cells were plated in estrogen-deprived media for 48 hours, followed by RNA extraction and qRT-PCR using primers for *ESR1*, *PGR*, *GREB1*, or *TFF1*, as indicated. Results shown are the mean±SEM of three independent experiments.

Figure 4: Transcriptional cell-state analysis of HER2 mutant cells. Gene expression analysis by RNA sequencing of T47D cells expressing the indicated mutants or controls was performed after two days of serum starvation followed by treatment with DMSO or 1μM fulvestrant for 24 hours. Six replicates were performed for each specific construct and drug condition (with at least 5 passing QC). (A) Unsupervised hierarchical clustering of HER2 activating mutants, wild-type HER2, kinase-dead HER2 D845A, and *ESR1* Y537S transcriptomes treated with either DMSO or fulvestrant. (B) Principal component analysis of all biological replicates of HER2 mutants and

controls under treatment with fulvestrant. (C) Differentially expressed genes (DEG) contrasting all HER2 activating mutants (S653C, L755S, V777L, and L869R) with GFP, under treatment with fulvestrant. DEGs with the highest magnitude (by log-fold change) and significance are labelled. (D) Using DEG between all HER2 activating mutants and GFP under treatment with fulvestrant, a common transcriptional footprint for the HER2 mutants was inferred, termed "HER2-MUT". Gene set enrichment analysis was performed using "HER2-MUT" and showed significant enrichment in signatures representing growth factor-induced gene expression[46] (ERBB1/2 up) and mitogen activated protein kinase (MAPK) signaling[47] (RAS/MAPK up). A mixed profile was observed for the canonical (estradiol-driven) ER-signature[46] (Estradiol-driven ER up); however, a highly enriched profile was found for a growth-factor-driven ER-signature[35] (Growth factor-driven ER up). (E) The RAS/MAPK signature strength is shown for each of the HER2 mutants and the controls, across all replicates, in cells treated with fulvestrant.

Figure 5: Mechanism of activation of HER2 mutants. (A) Structure of the HER2 active kinase domain (PDB=3PP0) bound to a reversible kinase inhibitor (red molecule), with the locations of the KD mutations identified in the patients in this study indicated. L755S and L869R likely disrupt a cluster of hydrophobic residues that stabilize the inactive conformation of the molecule as in the case of EGFR[48]. In addition to activation by this mechanism, V777L and the G727A/V777L mutations are likely to impact the ability of HER2 to bind reversible kinase inhibitors as described for G719 in EGFR[36]. (B) Structure of the HER2 transmembrane domain (PDB=2N2A) with the location of the identified TM mutant S653C. The cysteine residue may form intermolecular disulfide bridges with other mutant HER2 monomers. (C) T47D HER2

mutant cells were examined for the presence of reduction-sensitive HER2 dimers, as a marker of constitutive disulfide bridge formation. T47D wild-type HER2 or mutant HER2 cells plated in complete media were extracted using non-reducing buffer containing iodoacetamide. Extracts were prepared and run under non-reducing conditions, then probed with anti-HER2 antibodies. The location of the HER2 monomer and dimers, as determined by expected molecular weight, is indicated. GAPDH was used as a loading control. Results shown are representative of three independent experiments. (D) A hypothetical model for hyperactive MAPK signaling arising from the HER2 mutants is depicted. (Left) In cells bearing wild-type HER2, HER2 monomers (blue) heterodimerize with other ERBB monomers (EGFR or HER3, purple) bound to their cognate ligand (pink), leading to C-terminal tail phosphorylation and signaling. (Middle) HER2 bearing KD mutations are in a constitutively active conformation and possibly hyper-autophosphorylated and capable of signaling as described previously for other KD mutations in HER2[49] and EGFR[50]. When these monomers heterodimerize with other members of the HER family such as EGFR or HER3 (blue) bound to their cognate ligand (pink), transphosphorylation leads to enhanced heterodimer signaling. KD mutants may also homodimerize as previously shown for wild-type HER2 overexpression (not depicted). (Right) HER2 TM domain mutants likely form intermolecular disulfide bridges with other HER2 monomers bearing the mutation, which would enable phosphorylation and activation in a ligand-independent manner.

Figure 6: ER+ cells with HER2 mutations are sensitive to fulvestrant plus neratinib. (A) T47D HER2 mutant and control cells were compared on the basis of sensitivity to pan-HER

kinase inhibitor neratinib alone or in combination with fulvestrant. T47D HER2 mutant and control cells serum-starved for two days were switched to complete media containing 100nM fulvestrant, 32nM neratinib, or the combination. Cells were re-treated after 3 days, and viability determined by CellTiter-Glo assay one week after the start of treatment. Results shown are mean ±SEM, and representative of 14 independent experiments. (B) Levels of HER2 activation markers phospho-ERK and phospho-AKT were examined by western blotting in T47D HER2 mutant and control cells treated with neratinib. T47D HER2mut and control cells serum starved for 48 hours were switched to complete media containing DMSO or 1μM neratinib for 24 hours. Whole cell extracts were analyzed for the indicated proteins by immunoblotting. Results shown are representative of three independent experiments. (C) The response to the CDK4/6 inhibitor palbociclib was examined in T47D HER2 mutant and control cells, both alone and in combination with fulvestrant. Cells plated as in (A) were treated with 100nM fulvestrant, 1μM palbociclib, or the combination. Cells were re-treated after 3 days, and viability determined by CellTiter-Glo assay 1 week after the start of treatment. Results shown are mean±SEM and representative of five independent experiments. (D-E) Gene expression analysis by RNA sequencing performed as described in Figure 4 included T47D cells expressing the four activating HER2 mutants, GFP, wild-type HER2, kinase-dead HER2, or *ESR1* Y537S, as indicated. Cells were serum-starved then treated with 1μM fulvestrant, 1μM neratinib, 10μM palbociclib, or combinations (1μM fulvestrant + 1μM neratinib, 1μM fulvestrant + 10μM palbociclib). 6 replicates were performed for each specific construct and drug condition, for a total of 288 transcriptomes (8 constructs x 6 drug conditions x 6 replicates). (D) Unsupervised hierarchical clustering of the cells bearing the four activating HER2 mutants treated with DMSO,

fulvestrant, neratinib, palbociclib, fulvestrant + neratinib, or fulvestrant + palbociclib. (E) The RAS/MAPK signature strength was compared in mutant cell lines under various treatment conditions. (F) Treatment history of an endocrine-resistant patient with an acquired V777L mutation showed intrinsic resistance to the combination of palbociclib and letrozole, and subsequent partial response to the combination of fulvestrant and neratinib.

REFERENCES

1. Merenbakh-Lamin, K. *et al.* D538G mutation in estrogen receptor-alpha: A novel mechanism for acquired endocrine resistance in breast cancer. *Cancer Res* **73**, 6856-64 (2013).
2. Robinson, D.R. *et al.* Activating ESR1 mutations in hormone-resistant metastatic breast cancer. *Nat Genet* **45**, 1446-51 (2013).
3. Toy, W. *et al.* ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. *Nat Genet* **45**, 1439-45 (2013).
4. Jeselsohn, R. *et al.* Emergence of constitutively active estrogen receptor-alpha mutations in pretreated advanced estrogen receptor-positive breast cancer. *Clin Cancer Res* **20**, 1757-67 (2014).
5. Comprehensive molecular portraits of human breast tumours. *Nature* **490**, 61-70 (2012).
6. Giuliano, M., Trivedi, M.V. & Schiff, R. Bidirectional Crosstalk between the Estrogen Receptor and Human Epidermal Growth Factor Receptor 2 Signaling Pathways in Breast Cancer: Molecular Basis and Clinical Implications. *Breast Care (Basel)* **8**, 256-62 (2013).
7. Osborne, C.K. & Schiff, R. Mechanisms of endocrine resistance in breast cancer. *Annu Rev Med* **62**, 233-47 (2011).
8. Hurtado, A. *et al.* Regulation of ERBB2 by oestrogen receptor-PAX2 determines response to tamoxifen. *Nature* **456**, 663-6 (2008).
9. Liu, S. *et al.* Targeting tyrosine-kinases and estrogen receptor abrogates resistance to endocrine therapy in breast cancer. *Oncotarget* **5**, 9049-64 (2014).
10. Knowlden, J.M. *et al.* Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. *Endocrinology* **144**, 1032-44 (2003).
11. Frogne, T. *et al.* Activation of ErbB3, EGFR and Erk is essential for growth of human breast cancer cell lines with acquired resistance to fulvestrant. *Breast Cancer Res Treat* **114**, 263-75 (2009).
12. Benz, C.C. *et al.* Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. *Breast Cancer Res Treat* **24**, 85-95 (1992).
13. Elledge, R.M. *et al.* HER-2 expression and response to tamoxifen in estrogen receptor-positive breast cancer: a Southwest Oncology Group Study. *Clin Cancer Res* **4**, 7-12 (1998).
14. Bose, R. *et al.* Activating HER2 mutations in HER2 gene amplification negative breast cancer. *Cancer Discov* **3**, 224-37 (2013).
15. Hanker, A.B. *et al.* An Acquired HER2 T798I Gatekeeper Mutation Induces Resistance to Neratinib in a Patient with HER2 Mutant-Driven Breast Cancer. *Cancer Discov* (2017).
16. Greulich, H. *et al.* Functional analysis of receptor tyrosine kinase mutations in lung cancer identifies oncogenic extracellular domain mutations of ERBB2. *Proc Natl Acad Sci U S A* **109**, 14476-81 (2012).
17. Zabransky, D.J. *et al.* HER2 missense mutations have distinct effects on oncogenic signaling and migration. *Proc Natl Acad Sci U S A* **112**, E6205-14 (2015).
18. Cohen, O. *et al.* Abstract S1-01: Whole exome and transcriptome sequencing of resistant ER+ metastatic breast cancer. *Cancer Research* **77**, S1-01-S1-01 (2017).

19. Imielinski, M. *et al.* Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. *Cell* **150**, 1107-20 (2012).
20. Berger, A.H. *et al.* High-throughput Phenotyping of Lung Cancer Somatic Mutations. *Cancer Cell* **30**, 214-228 (2016).
21. de Martino, M. *et al.* Impact of ERBB2 mutations on in vitro sensitivity of bladder cancer to lapatinib. *Cancer Biol Ther* **15**, 1239-47 (2014).
22. Pereira, B. *et al.* The somatic mutation profiles of 2,433 breast cancers refines their genomic and transcriptomic landscapes. *Nat Commun* **7**, 11479 (2016).
23. Yun, C.H. *et al.* Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity. *Cancer Cell* **11**, 217-27 (2007).
24. Kobayashi, S. *et al.* Compound EGFR mutations and response to EGFR tyrosine kinase inhibitors. *J Thorac Oncol* **8**, 45-51 (2013).
25. Curtis, C. *et al.* The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* **486**, 346-52 (2012).
26. Ciriello, G. *et al.* Comprehensive Molecular Portraits of Invasive Lobular Breast Cancer. *Cell* **163**, 506-19 (2015).
27. Lefebvre, C. *et al.* Mutational Profile of Metastatic Breast Cancers: A Retrospective Analysis. *PLoS Med* **13**, e1002201 (2016).
28. Yates, L.R. *et al.* Genomic Evolution of Breast Cancer Metastasis and Relapse. *Cancer Cell* **32**, 169-184.e7 (2017).
29. Zehir, A. *et al.* Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. *Nat Med* **23**, 703-713 (2017).
30. AACR Project GENIE: Powering Precision Medicine Through An International Consortium. *Cancer Discov* (2017).
31. Robinson, D.R. *et al.* Integrative clinical genomics of metastatic cancer. *Nature* **548**, 297-303 (2017).
32. Lai, A. *et al.* Identification of GDC-0810 (ARN-810), an Orally Bioavailable Selective Estrogen Receptor Degrader (SERD) that Demonstrates Robust Activity in Tamoxifen-Resistant Breast Cancer Xenografts. *J Med Chem* (2015).
33. Massarweh, S. *et al.* Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. *Cancer Res* **68**, 826-33 (2008).
34. Sergushichev, A. An algorithm for fast preranked gene set enrichment analysis using cumulative statistic calculation. *bioRxiv* (2016).
35. Lupien, M. *et al.* Growth factor stimulation induces a distinct ER(alpha) cistrome underlying breast cancer endocrine resistance. *Genes Dev* **24**, 2219-27 (2010).
36. Jiang, J. *et al.* Epidermal growth factor-independent transformation of Ba/F3 cells with cancer-derived epidermal growth factor receptor mutants induces gefitinib-sensitive cell cycle progression. *Cancer Res* **65**, 8968-74 (2005).
37. Burke, C.L., Lemmon, M.A., Coren, B.A., Engelman, D.M. & Stern, D.F. Dimerization of the p185neu transmembrane domain is necessary but not sufficient for transformation. *Oncogene* **14**, 687-96 (1997).

38. Chen, L.I., Webster, M.K., Meyer, A.N. & Donoghue, D.J. Transmembrane domain sequence requirements for activation of the p185c-neu receptor tyrosine kinase. *J Cell Biol* **137**, 619-31 (1997).
39. Ma, C.X. *et al.* Neratinib Efficacy and Circulating Tumor DNA Detection of HER2 Mutations in HER2 Nonamplified Metastatic Breast Cancer. *Clinical Cancer Research* (2017).
40. Hyman, D.M. *et al.* HER kinase inhibition in patients with HER2- and HER3-mutant cancers. *Nature* **554**, 189-194 (2018).
41. Ma, C.X.B., R.; Gao, F.; Freedman, R.A.; Pegram, M.D.; Blackwell, K.; Bedard, P.L.; Hayes, D.F.; Goetz, M.P.; Niravath, P.A.; Russell, C.A.; Tripathy, D.; Cobleigh, M.A.; Forero, A.; Pluard, T.J.; Anders, C.K.; Bumb, C.; Naughton, M.; Al-Kateb, H.; Ellis, M.J. Phase II trial of neratinib for HER2 mutated, non-amplified metastatic breast cancer (HER2mut MBC). *Journal of Clinical Oncology* **34**, abstr 516 (2016).
42. Hyman, D. *et al.* Abstract PD2-08: Neratinib + fulvestrant in ERBB2-mutant, HER2–non-amplified, estrogen receptor (ER)-positive, metastatic breast cancer (MBC): Preliminary analysis from the phase II SUMMIT trial. *Cancer Research* **77**, PD2-08-PD2-08 (2017).
43. Wagle, N. *et al.* High-throughput detection of actionable genomic alterations in clinical tumor samples by targeted, massively parallel sequencing. *Cancer Discov* **2**, 82-93 (2012).
44. MacConaill, L.E. *et al.* Prospective enterprise-level molecular genotyping of a cohort of cancer patients. *J Mol Diagn* **16**, 660-72 (2014).
45. Sholl, L.M. *et al.* Institutional implementation of clinical tumor profiling on an unselected cancer population. *JCI Insight* **1**, e87062 (2016).
46. Liberzon, A. *et al.* The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst* **1**, 417-425 (2015).
47. Bild, A.H. *et al.* Oncogenic pathway signatures in human cancers as a guide to targeted therapies. *Nature* **439**, 353-7 (2006).
48. He, M. *et al.* EGFR exon 19 insertions: a new family of sensitizing EGFR mutations in lung adenocarcinoma. *Clin Cancer Res* **18**, 1790-7 (2012).
49. Wang, S.E. *et al.* HER2 kinase domain mutation results in constitutive phosphorylation and activation of HER2 and EGFR and resistance to EGFR tyrosine kinase inhibitors. *Cancer Cell* **10**, 25-38 (2006).
50. Kancha, R.K., von Bubnoff, N., Peschel, C. & Duyster, J. Functional analysis of epidermal growth factor receptor (EGFR) mutations and potential implications for EGFR targeted therapy. *Clin Cancer Res* **15**, 460-7 (2009).

ONLINE METHODS

Patient and Tumor Samples

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

Whole Exome Sequencing

DNA was extracted from primary tumors, metastatic tumors, and normal samples for all patients and whole exome sequencing was performed, as detailed below.

DNA extraction: DNA extraction was performed as previously described[51]. For whole blood, DNA is extracted using magnetic bead-based chemistry in conjunction with the Chemagic MSM I instrument manufactured by Perkin Elmer. Following red blood cell lysis, magnetic beads bind to the DNA and are removed from solution using electromagnetized rods. Several wash steps follow to eliminate cell debris and protein residue from DNA bound to the magnetic beads. DNA is then eluted in TE buffer. For frozen tumor tissue, DNA and RNA are extracted simultaneously from a single frozen tissue or cell pellet sample using the AllPrep DNA/RNA kit

(Qiagen). For FFPE tumor tissues, DNA and RNA are extracted simultaneously using Qiagen's AllPrep DNA/RNA FFPE kit. All DNA is quantified using Picogreen

Library Construction: DNA libraries for massively parallel sequencing were generated as previously described[51] with the following modifications: the initial genomic DNA input into the shearing step was reduced from 3µg to 10-100ng in 50µL of solution. For adapter ligation, Illumina paired-end adapters were replaced with palindromic forked adapters (purchased from Integrated DNA Technologies) with unique dual indexed 8 base index molecular barcode sequences included in the adapter sequence to facilitate downstream pooling. With the exception of the palindromic forked adapters, all reagents used for end repair, A-base addition, adapter ligation, and library enrichment PCR were purchased from KAPA Biosciences in 96-reaction kits. In addition, during the post-enrichment solid phase reversible immobilization (SPRI) bead cleanup, elution volume was reduced to 30µL to maximize library concentration, and a vortexing step was added to maximize the amount of template eluted.

Solution-phase hybrid selection: After library construction, hybridization and capture were performed using the relevant components of Illumina's Rapid Capture Exome Kit and following the manufacturer's suggested protocol, with the following exceptions: first, all libraries within a library construction plate were pooled prior to hybridization. Second, the Midi plate from Illumina's Rapid Capture Exome kit was replaced with a skirted PCR plate to facilitate automation. All hybridization and capture steps were automated on the Agilent Bravo liquid handling system.

Preparation of libraries for cluster amplification and sequencing: After post-capture enrichment, library pools were then quantified using quantitative PCR (KAPA Biosystems) with probes specific to the ends of the adapters; this assay was automated using Agilent's Bravo liquid handling platform. Based on qPCR quantification, libraries were normalized and denatured using 0.1 N NaOH on the Hamilton Starlet.

Cluster amplification and sequencing: Cluster amplification of denatured templates was performed according to the manufacturer's protocol (Illumina) using HiSeq 2500 Rapid Run v1/v2, HiSeq 2500 High Output v4 or HiSeq 4000 v1 cluster chemistry and HiSeq 2500 (Rapid or High Output) or HiSeq 4000 flowcells. Flowcells were sequenced on HiSeq 2500 using v1 (Rapid Run flowcells) or v4 (High Output flowcells) Sequencing-by-Synthesis chemistry or v1 Sequencing-by-Synthesis chemistry for HiSeq 4000 flowcells. The flowcells were then analyzed using RTA v.1.18.64 or later. Each pool of whole exome libraries was run on paired 76np runs, with a two 8 base index sequencing reads to identify molecular indices, across the number of lanes needed to meet coverage for all libraries in the pool.

Sequence data processing: Exome sequence data processing was performed using established analytical pipelines at the Broad Institute. A BAM file was produced with the Picard pipeline (see URLs) which aligns the tumor and normal sequences to the hg19 human genome build using Illumina sequencing reads. The BAM was uploaded into the Firehose pipeline (see URLs), which manages input and output files to be executed by GenePattern[52].

Sequencing quality control: Quality control modules within Firehose were applied to all sequencing data for comparison of the origin for tumor and normal genotypes and to assess fingerprinting concordance. Cross-contamination of samples was estimated using ContEst[53].

Somatic Alteration Assessment

MuTect[54] was applied to identify somatic single-nucleotide variants. Indelocator (see URLs), Strelka[55], and MuTect2 (see URLs) were applied to identify small insertions or deletions. A voting scheme with inferred indels requiring at least 2 out of 3 algorithms.

Artifacts introduced by DNA oxidation (so called OxoG) during sequencing were computationally removed using a filter-based method[56]. In the analysis of primary tumors that are formalin-fixed, paraffin-embedded samples [FFPE] we further applied a filter to remove FFPE-related artifacts[57].

Reads around mutated sites were realigned with Novoalign (see URLs) to filter out false positive that are due to regions of low reliability in the reads alignment. At the last step, we filtered mutations that are present in a comprehensive WES panel of 8,334 normal samples (using the Agilent technology for WES capture) aiming to filter either germline sites or recurrent artifactual sites. We further used a smaller WES panel of normal 355 normal samples that are based on Illumina technology for WES capture, and another panel of 140 normals sequenced without our cohort[18] to further capture possible batch-specific artifacts. Annotation of identified variants was done using Oncotator[58] (see URLs).

Copy Number and Copy Ratio Analysis

To infer somatic copy number from WES, we used ReCapSeg (see URLs), calculating proportional coverage for each target region (i.e., reads in the target/total reads) followed by segment normalization using the median coverage in a panel of normal samples. The resulting copy ratios were segmented using the circular binary segmentation algorithm[59].

To infer allele-specific copy ratios, we mapped all germline heterozygous sites in the germline normal sample using GATK Haplotype Caller[60] and then evaluated the read counts at the germline heterozygous sites in order to assess the copy profile of each homologous chromosome. The allele-specific copy profiles were segmented to produce allele specific copy ratios.

Cancer Cell Fraction and Evolutionary Analysis

Analysis using ABSOLUTE: To properly compare SNVs and indels in paired metastatic and primary samples, we considered the union of all mutations called in either of the two samples. We evaluated the reference and alternate reads in each patient's primary and metastatic tumors, including mutations that were not initially called in one of the samples. These mutations in matched samples were used as input for ABSOLUTE[61]. The ABSOLUTE algorithm uses mutation-specific variant allele fractions (VAF) together with the computed purity, ploidy, and segment-specific allelic copy-ratio to compute cancer cell fractions (CCFs).

Analysis of clonal dynamics using PHYLOGIC: To evaluate the mutation clonality in the patient-matched primary and metastatic samples, we used PHYLOGIC clustering of the

mutation-specific cancer cell fractions (CCFs), as previously described[62,63]. The CCF of indels may be systematically underestimated due to counting only a subset of the reads supporting the indel event in cases of longer indels. For example, the CDH1, c.437_447delCTCCTGGCCTC indel in Patient ID *300504* had underestimated CCF in both the Met and the Primary, but upon re-evaluation of the BAM files and supporting reads we assigned this mutation to the shared truncal mutations (clonal in both the primary in the met). Further details regarding CCF and Evolutionary analysis can be found online (see URLs).

Selected cancer genes depicted in Figure 2 (mutational landscape of potential drivers):
The list of mutated genes include: (A) ERBB2, PIK3CA, CDH1, PTEN, ATM, CDC42BPA, CTCF, ESR1, FOXP1, NF1, and SETDB1- significantly mutated genes in breast cancer[64] or significantly mutated genes in our metastatic cohort[18]; (B) ETV5, CDK12, MITF, PDGFRA, RARA, and TSC1- genes with plausible clinical impact[57]; and (C) MYH9, CHD4, FAT4, POT1, RMI2, ARID2, CASC5, CHCHD7, EBF1, ERCC5, HIP1, IL6ST, KAT6A, KMT2A, LZTR1, REL, RHOA, SALL4, WRN - genes from Cancer Gene Census (CGC), COSMIC database v82[65].

Cell culture

ER+/HER2- T47D (*American type culture collection #HTB-133*) and MCF7 cells (*ATCC #HTB-22*) were cultured in Phenol Red-free RPMI-1640 (*Gibco #11835-030*) or Phenol Red-free MEMα (*Gibco #41061-029*) supplemented with 10% fetal bovine serum (*Gemini #100-106*), respectively. HEK 293T/17 (*American Type Culture Collection #CRL-11268*) were cultured in

DMEM (*Gibco #11995-065*) supplemented with 10% fetal bovine serum (FBS). For serum starvation conditions, the appropriate media was supplemented with 10% charcoal dextran-stripped serum (*Gemini #100-119*).

Generation of HER2 mutant plasmids and cells

HER2 mutants were generated by site-directed mutagenesis of the HER2 open reading frame in a pDONR223 vector backbone, using the QuikChange II site-directed mutagenesis kit (*Agilent Technologies #200523*). After sequencing, the mutant constructs were cloned into the pLX307 vector backbone or the pDEST40 vector using the gateway LR clonase kit (*Life Technologies #11791019*), to facilitate generation of mutant-encoding lentivirus, as described below.

To generate HER2 mutant or control lentivirus, 293T cells were transfected to produce viral particles using FuGENE HD (*Promega #E2311*) with VSV-G and Δ8.91 envelope and packaging plasmids. After 72 hours of incubation the supernatant was filtered through a .45μm filter (*Corning #431225*), tested for the presence of lentiviral particles using Lenti-x GoStix (*Takara #631244*), and stored at -80°C until transduction. Lentiviral transduction of T47D cells plated in 6-well dishes was accomplished by viral spinoculation of cells in media containing 4μg/mL polybrene (*Santa Cruz #sc-134220*). After overnight incubation, media was replaced. The cells were selected in 1.5μg/mL Puromycin (*Gibco #A11138-03*) 48 hours after infection for two days, after which media was replaced with fresh media containing Puro, and selected for a further two days. After confirmation of complete selection in uninfected control wells, cells were

trypsinized, counted, and plated for viability, western blotting, qPCR, or HER2 dimerization assays as described below.

In cases where estrogen deprivation was required, cells were cultured in Phenol red-free RPMI containing 10% charcoal dextran-stripped fetal bovine serum (*Gemini #100-119*) after selection.

HER2 inducible cell lines

To generate HER2 inducible cell lines, pDONR223 constructs encoding various HER2 mutants or controls, as well as pDONR223-GFP and pDONR223-HER2 wild-type, were cloned into the Gateway-compatible pLX403 vector, which drives transgene expression using a Tet-On system. The pLX317-GFP and pLX317-HER2 controls and pDONR223-HER2 plasmid were obtained from the Broad Institute TRC portal. The VSV-G envelope and Δ8.91 packaging plasmids, pLX307 Gateway cloning destination vector, and pLX403 vector were kind gifts from Levi Garraway. Lentivirus encoding all mutants and controls was generated as described above, followed by transfection into T47D cells and bulk selection with Puromycin to generate a stable cell line in each case. The cells were treated with various concentrations of doxycycline (*Clontech #631311*) ranging from 25-500 ng/mL, for various time periods ranging from 3 hours to 1 week, to determine the minimal dose and induction time of doxycycline required to stimulate HER2 mutant expression over endogenous levels. Transgene expression was seen as early as 3 hours post-induction at 25ng/mL doxycycline.

Kill curves

T47D HER2mut cells were plated in RPMI with 10% CSS at 1000 cells/well of 96-well Viewplates (*Perkin-Elmer #6005181*). After two days of serum starvation, cells were switched over to complete media or treated with estradiol (*Sigma-Aldrich #E2758*) as appropriate, and treated with a range of doses of the corresponding drug. Cells were re-treated after three days. One week after the start of treatment, viability was determined using CellTiter-Glo as described below. The normalized data was plotted in GraphPad Prism, and analyzed by nonlinear regression curve fit using the log (inhibitor) vs. response 3-parameter model.

CellTiter-Glo viability assays

One week after the start of treatment, the media in 96-well plates was replaced with 100µLs fresh media per well and brought to room temperature. CellTiter-Glo (*Promega #G7572*) was added to each well (20µL), lysed for 2 minutes at ~250rpm, then equilibrated at room temperature for 20 minutes. A plate reader was programmed to integrate luminescence for 500ms per well. Background luminescence from media-only wells were subtracted from all values, and raw values normalized against untreated wells for each cell line.

Chemicals and antibodies

Chemicals used: Iodoacetamide (*Sigma-Aldrich #A3221-10V*), fulvestrant (*Sigma-Aldrich #I4409-25mg*), neratinib (*Selleck Chem #S2150*), (Z)-4-HydroxyTamoxifen (*Sigma-Aldrich #H7904-25mg*), GDC-0810 (*Medkoo #206041*), AZD5363 (*Thermofisher Scientific #NC0488926*), VX-11e (*Selleck Chem #S7709*), trametinib/ GSK1120212 (*Selleck Chem #S2673*), BYL719 (*Selleck Chem #S2814*), RAD001/everolimus (*Selleck Chem #S1120*) and

palbociclib (*Selleck Chem #S1116*). Primary antibodies used: p-HER2 (*EMD Millipore – 06-229*), HER2 (*Cell Signaling Technology – Clone: 44E7 #2248S*), ERα (*Santa Cruz Biotechnology – Clone: HC-20 #SC-543*), p-ERK (1/2) (*CST – Clone: E10 #9106S*), ERK (1/2) (*CST – Clone: 137F5 #4695S*), p-AKT (*CST – Clone: D9W9U #12694S*), AKT (*CST #9272S*), GAPDH (*SCB – Clone: V-18 #SC-20357*), β-Actin (*Santa Cruz #47778*), Goat anti-rabbit (*Pierce #32260*), Rabbit anti-goat (*Invitrogen #81-1620*), Goat anti-mouse (*Novex #A16090*).

Western blotting

Approximately $5.0x10^5$ HER2 mutant or control cells were serum-starved for two days, then treated with fulvestrant 1μM or neratinib 1μM for 24 hours. Whole cell protein extracts were made by passive lysis with 50μl of lysis buffer, comprised of: RIPA buffer (*Sigma-Aldrich #R0278-50ml*), 1mM DL-Dithiothreitol *DTT* (*Sigma-Aldrich #D0632-5G*), 1mM PMSF (*Sigma-Aldrich #P7626-5G*), protease inhibitor (*Sigma-Aldrich #P8340-5ml*), and Halt™ phosphatase inhibitor (*Thermo scientific #78428*). Cell pellets were lysed on a rotator at 15rpm for 15 minutes at 4°C; lysates were then cleared by centrifugation at 14,000xg for 15 minutes at 4°C.

Protein was quantified by BCA assay (*Pierce™ BCA Protein Assay Kit #23225*) according to the manufacturer's microplate procedure. Samples were prepared using 30μg of protein, Bolt™ LDS Sample Buffer (*Life technologies #B0007*), and DTT, then heated to 70°C for 10 minutes according to novex's NuPAGE Bis-Tris Gel sample preparation recommendations. Samples were run on a Bolt™ 4-12% Bis-Tris Plus gel (*Thermo Fisher Scientific #NW04125BOX*) at 200V for 35 minutes in 1x Bolt™ MOPS SDS Running Buffer (*Life technologies #B0001*).

Protein was transferred to nitrocellulose membranes using the BioRAD transblot turbo according to the fast transfer protocol in the Trans-Blot® Turbo™ RTA Transfer Kit. Membranes were blocked at room temperature for 1 hour in 5% milk in tris-buffered saline/ TBS (*Biorad #170-6435*) with 0.1% Tween-20 (*Sigma-Aldrich #P9416-100ml*). The membranes were incubated overnight at 4°C with primary antibody at a 1:1000 dilution in 1% milk in TBS-Tween (TBS-T). The following day the membranes were washed 3x for 5 minutes with TBS-T, and incubated with secondary antibody at a 1:2000 dilution in 1% milk in TBS-T, for 1 hour at room temperature. After washing 4x 10 minutes with TBS-T, membranes were treated with Pierce™ ECL Plus Western Blotting Substrate (*Life technologies #32132*) for 5 minutes and exposed to autoradiography film (*Fisher Scientific #NC9648989*).

qRT-PCR

Approximately $8.0x10^5$ T47D HER2 mutant or control cells were serum starved in RPMI-1640 supplemented with 10% CSS for 48 hours. Cells were washed with PBS, trypsinized, and cell pellets collected and washed 1X with PBS. RNA was harvested using a RNeasy Mini Kit (*Qiagen #74104*) according to the supplied protocol. 1µg of RNA was reverse transcribed to cDNA on a ProFlex PCR System thermocycler, according to the RNase inhibitor-free protocol in the High-Capacity cDNA Reverse Transcription Kit (*Applied Biosystems #4368814*) user manual.

The cDNA was diluted 8-fold in ddH_2O and mixed with PowerUp™ SYBR® Green Master Mix (*Applied Biosystems #A25742*) and appropriate forward and reverse primer pairs specific for

ESR1 and selected targets in a 20µl reaction according to the manufacturers' specifications. The quantification was performed on an Applied Biosystems 7300 Real-Time PCR System. Each sample was run in triplicate and the raw data was analyzed by the delta delta CT method ($2^{-\Delta\Delta CT}$) and normalized to GFP.

Primers were ordered from Integrated DNA Technologies at 25nm scale and standard purification. Primer sequences can be found in **Supplementary Table 13**.

RNA sequencing on HER2 mutant cell lines and controls

RNA-seq experimental setup and treatment of cells

T47D cells were infected *de novo* with lentivirus encoding HER2 mutants and controls (GFP, wild-type HER2, kinase-dead HER2, and ESR1 Y537S) in the pLX307 plasmid as described above. Upon completion of selection with Puromycin, the cells were plated in 96-well plates in RPMI supplemented with 10% CSS for 48 hours. Cell were then treated with DMSO, 1µM fulvestrant, 1µM neratinib, 10µM palbociclib, 1µM fulvestrant + 1µM neratinib, or 1µM fulvestrant + 10µM palbociclib for 24 hours. The cells were washed 3X with ice-cold PBS and lysed with TCL buffer (*Qiagen 157013305*) containing 1% β-mercaptoethanol (*Sigma M3148*), transferred to PCR plates (*Qiagen 951020401*), sealed, spun down for one minute at 3,000 rpm, and immediately frozen at −80°C until RNA extraction. For each specific construct and drug condition we performed 6 biological replicates, for a total of 288 transcriptomes (8 constructs x 6 drugs x 6 replicates).

RNA-seq protocol

Plates with cell lysates were thawed and purified with 2.2x RNAClean SPRI beads (Beckman Coulter Genomics). The RNA captured beads were air-dried and processed immediately for RNA secondary structure denaturation (72˚C for three minutes) and cDNA synthesis. We performed SMART-Seq2 following the published protocol[66] with minor modifications in the reverse transcription step. We made a 15µl reaction mix for each PCR and performed 10 cycles for cDNA amplification. We used 0.2ng cDNA of each population and one-eighth of the standard Illumina NexteraXT (Illumina FC-131-1096) reaction volume in both the tagmentation and PCR amplification steps. Uniquely indexed libraries were pooled and sequenced with NextSeq 500 high output V2 75 cycle kits (Illumina FC-404-2005) and 38 × 38 paired-end reads on an Illumina NextSeq 500 instrument, aggregating three NextSeq runs

Reads alignments, expression quantification and QC

Reads were mapped to the human genome (hg19) with STAR aligner[67] [version 2.5.2b] with default parameters against hg19 of the human genome. Transcriptome quality and expression quantification was conducted using RNA-SeQC[68]. Samples with less than 12,000 unique genes were removed from subsequent analysis, excluding 26 samples and retaining 262 profiles, with a minimum of 3 replicates for each activating HER2 mutant (median of 6 and lower quantile of 5 replicates). Samples passing QC had a mean of 17,363 detected genes by at least three reads (s.d. = 755) and a mean of 7,060,935 uniquely mapped pairs of reads (s.d. = 1,191,466).

Principal Component Analysis (PCA)

The TPM (Transcripts Per Million) measured expression per gene was log normalized. To correct for batch effects between the three 96-well plates, we used the ComBat algorithm with default parameters[69]. Principal Component Analysis (PCA) was performed with the R implementations of the function *prcomp* as part of the *stats* package using the default parameters.

Heatmap Analysis

The clustering of transcriptional profiles across various drug conditions used genes with high dispersion among samples, as calculated LogVMR (variance-to-mean ratio (VMR)) as implemented in *FindVariableGenes* function, *Seurat* R package.

Differential expression analysis

Differential expression analysis was performed over the raw read counts using *limma* package[70] with *voom* assessment[70] of counts normalization and while accounting for the among-plates batch effect.

Definition of HER2-MUT signature

The HER2-MUT signature is inferred by the list of differentially expressed genes (DEGs), comparing the four activating HER2 mutations (V777L, S653C, L869R, and L755S) with control ORF and under a given drug condition. Supplementary tables 9 and 10 depict the HER2-MUT signature with DEG between the mutants to GFP under fulvestrant and DMSO, respectively. Supplementary tables 11 and 12 depict the HER2-MUT signature with DEG between the mutants to wild-type HER2 under fulvestrant and DMSO, respectively.

Transcriptional signatures analysis

We used Fast Gene Set Enrichment Analysis[34] with 50,000 permutations and gene set size limited to min of 3 and max of 1,000 genes, respectively. A total set of 5,095 gene sets was

analyzed including the c2, c6, and hallmark collections from MSigDB[46] augmented with a breast cancer gene set collection[35,71,72]. Significant gene sets, controlling for a False Discovery Rate (FDR) of 5% are found in Supplementary tables 9 and 10 for enrichments of HER2 mutants vs. GFP DEGs under treatment with fulvestrant and DMSO, respectively. Significant gene sets, controlling for a False Discovery Rate (FDR) of 5%, are found in supplementary tables 11 and 12 for enrichments of HER2 mutants vs. HER2 WT DEGs under treatment with fulvestrant and DMSO, respectively. The evaluation of signature strength for single samples was done using Gene Set Variation Analysis (GSVA)[73] with default parameters using the log2 normalized, batch-corrected expression for each sample.

HER2 dimerization assay

The HER2 dimerization assay was performed as previously described[16]. Briefly, T47D HER2 mutant or control cells were cultured in phenol red-free RPMI-1640 with 10% FBS in 6-well plates until nearly confluent (~3-4 days). Cells were washed twice with cold PBS containing 10mM idoacetamide (*Sigma-Aldrich #A3221*) while on ice. Cells were actively lysed with 200µl of TGP buffer, comprised of 50mM Tris base (*Sigma-Aldrich #T1503*), 10% glycerol (*Sigma-Aldrich #G5516*), 1% Triton x-100 (*Sigma-Aldrich #T8787*), 10mM iodoacetamide, 1mM phosphatase inhibitor, and 1mM protease inhibitor, for 20 minutes at 4°C. Lysates were cleared by centrifugation, and quantified by BCA. The samples were prepared in LDS sample buffer without DTT, and 25µg loaded on gels as described in the 'western blotting' section. After transfer, membranes were probed with anti-HER2 and anti-GAPDH antibodies for loading control as described above.

URLs

Picard (http://picard.sourceforge.net/); Firehose (http://www.broadinstitute.org/cancer/cga/Firehose); Indelocator (http://www.broadinstitute.org/cancer/cga/indelocator); MuTect2 (https://software.broadinstitute.org/gatk/documentation/tooldocs/current/org_broadinstitute_gatk_tools_walkers_cancer_m2_MuTect2); Novoalign (www.novocraft.com/products/novoalign/); ReCapSeg (http://gatkforums.broadinstitute.org/categories/recapseg-documentation); Oncotator (http://www.broadinstitute.org/cancer/cga/oncotator); CCF and Evolutionary analysis (http://www.broadinstitute.org/cancer/cga/acsbeta).

Life Sciences Reporting Summary

Further information on experimental design is available in the Nature Research Life Sciences Reporting Summary linked to this article.

METHODS-ONLY REFERENCES

51. Fisher, S. *et al.* A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries. *Genome Biol* **12**, R1 (2011).
52. Reich, M. *et al.* GenePattern 2.0. *Nat Genet* **38**, 500-1 (2006).
53. Cibulskis, K. *et al.* ContEst: estimating cross-contamination of human samples in next-generation sequencing data. *Bioinformatics* **27**, 2601-2 (2011).
54. Cibulskis, K. *et al.* Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat Biotechnol* **31**, 213-9 (2013).
55. Saunders, C.T. *et al.* Strelka: accurate somatic small-variant calling from sequenced tumor-normal sample pairs. *Bioinformatics* **28**, 1811-7 (2012).
56. Costello, M. *et al.* Discovery and characterization of artifactual mutations in deep coverage targeted capture sequencing data due to oxidative DNA damage during sample preparation. *Nucleic Acids Res* **41**, e67 (2013).

57. Van Allen, E.M. *et al.* Whole-exome sequencing and clinical interpretation of formalin-fixed, paraffin-embedded tumor samples to guide precision cancer medicine. *Nat Med* **20**, 682-8 (2014).
58. Ramos, A.H. *et al.* Oncotator: cancer variant annotation tool. *Hum Mutat* **36**, E2423-9 (2015).
59. Olshen, A.B., Venkatraman, E.S., Lucito, R. & Wigler, M. Circular binary segmentation for the analysis of array-based DNA copy number data. *Biostatistics* **5**, 557-72 (2004).
60. DePristo, M.A. *et al.* A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet* **43**, 491-8 (2011).
61. Carter, S.L. *et al.* Absolute quantification of somatic DNA alterations in human cancer. *Nat Biotechnol* **30**, 413-21 (2012).
62. Brastianos, P.K. *et al.* Genomic Characterization of Brain Metastases Reveals Branched Evolution and Potential Therapeutic Targets. *Cancer Discov* **5**, 1164-1177 (2015).
63. Stachler, M.D. *et al.* Paired exome analysis of Barrett's esophagus and adenocarcinoma. *Nat Genet* **47**, 1047-55 (2015).
64. Lawrence, M.S. *et al.* Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature* **505**, 495-501 (2014).
65. Futreal, P.A. *et al.* A census of human cancer genes. *Nat Rev Cancer* **4**, 177-83 (2004).
66. Picelli, S. *et al.* Smart-seq2 for sensitive full-length transcriptome profiling in single cells. *Nat Methods* **10**, 1096-8 (2013).
67. Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15-21 (2013).
68. DeLuca, D.S. *et al.* RNA-SeQC: RNA-seq metrics for quality control and process optimization. *Bioinformatics* **28**, 1530-2 (2012).
69. Leek, J.T., Johnson, W.E., Parker, H.S., Jaffe, A.E. & Storey, J.D. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics* **28**, 882-3 (2012).
70. Ritchie, M.E. *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* **43**, e47 (2015).
71. Stover, D.G. *et al.* The Role of Proliferation in Determining Response to Neoadjuvant Chemotherapy in Breast Cancer: A Gene Expression-Based Meta-Analysis. *Clin Cancer Res* **22**, 6039-6050 (2016).
72. Creighton, C.J. *et al.* Development of resistance to targeted therapies transforms the clinically associated molecular profile subtype of breast tumor xenografts. *Cancer Res* **68**, 7493-501 (2008).
73. Hanzelmann, S., Castelo, R. & Guinney, J. GSVA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinformatics* **14**, 7 (2013).