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PRKACA Mediates Resistance to HER2-Targeted Therapy in Breast Cancer Cells and Restores Anti-Apoptotic Signaling

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Abstract

Targeting HER2 with antibodies or small molecule inhibitors in HER2-positive breast cancer leads to improved survival, but resistance is a common clinical problem. To uncover novel mechanisms of resistance to anti-HER2 therapy in breast cancer, we performed a kinase open reading frame (ORF) screen to identify genes that rescue *HER2*-amplified breast cancer cells from HER2 inhibition or suppression. In addition to multiple members of the MAPK and PI3K signaling pathways, we discovered that expression of the survival kinases PRKACA and PIM1 rescued cells from anti-HER2 therapy. Furthermore, we observed elevated PRKACA expression in trastuzumab-resistant breast cancer samples, indicating that this pathway is activated in breast cancers that are clinically resistant to trastuzumab-containing therapy. We found that neither PRKACA nor PIM1 restored MAPK or PI3K activation after lapatinib or trastuzumab treatment, but rather inactivated the pro-apoptotic protein BAD, thereby permitting survival signaling through BCL-XL. Pharmacological blockade of BCL-XL/BCL-2 partially abrogated the rescue effects conferred by PRKACA and PIM1, and sensitized cells to lapatinib treatment. These observations suggest that combined targeting of HER2 and the BCL-XL/BCL-2 anti-apoptotic pathway may increase responses to anti-HER2 therapy in breast cancer and decrease the emergence of resistant disease.

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CONFLICT OF INTEREST

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Keywords

breast cancer; drug resistance; HER2; PRKACA; PIM1

INTRODUCTION

The receptor tyrosine kinase HER2 is amplified and/or overexpressed in 20–30% of all breast cancers (1, 2), leading to constitutive proliferative and survival signaling via the downstream Ras/Erk and PI3K/Akt pathways. Amplification or overexpression of HER2 is associated with poor prognosis, and perturbation of HER2 signaling with trastuzumab or lapatinib has led to clinical benefit in HER2-positive breast cancer patients (3–7). However, many patients with early-stage disease experience tumor recurrence despite adjuvant treatment with trastuzumab, and patients with metastatic disease inevitably develop resistance to anti-HER2 therapies.

For trastuzumab, a monoclonal antibody that binds to the extracellular domain of HER2, a number of potential mechanisms of resistance have been identified. These include cleavage of the extracellular domain of HER2, as well as heterodimerization with HER3 or IGF1R; both of these mechanisms result in continued downstream MAPK and PI3K signaling (8–10). Resistance also has been shown to occur in the setting of *PTEN* loss, activating *PIK3CA* mutations, *CCNE* amplification, or c-SRC activation (11–17). Resistance to lapatinib, which inhibits intracellular tyrosine kinase activity, can be induced in HER2-amplified breast cancer cells by activating *PIK3CA* mutations, loss of *PTEN* (18), or by activation of mTORC1 (19). Since clinically resistant breast cancer samples have not been extensively molecularly characterized, due in part to limited sample availability, the extent to which each of these molecular mechanisms contributes to resistance in HER2-positive human breast cancers is largely unknown.

Although strategies to target the MAPK and PI3K pathways in resistant cancers are being pursued, these mechanisms likely fail to account for the development of resistant disease in all patients. Hence we conducted an unbiased screen to determine whether pathways other than those directly downstream of canonical HER2 signaling might also confer resistance. Here we describe a systematic interrogation of resistance mechanisms to suppression of HER2 to identify the major mechanisms of resistance to HER2-directed therapy.

RESULTS

We conducted two kinome ORF screens in parallel to identify genes that confer resistance to the lapatinib-like dual EGFR/HER2 inhibitor AEE788 and to suppression of *HER2* with a short hairpin RNA (shRNA). We reasoned that the “off-target” effects of a small molecule inhibitor and an shRNA should be different, such that the intersection of hits from both screens would help to identify biological pathways that can confer resistance to anti-HER2 therapy. We tested six independent anti-HER2 shRNAs in BT474 cells and found that there was a strong correlation between the degree of HER2 protein suppression and loss of viability/proliferation. We chose the most effective shRNA, sh4355, for the screen (Fig. S1A). We titrated the AEE788 dose in BT474 cells, and selected 0.85 μM for the screen

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because it reduced cell viability to approximately 40% that of control, allowing a sufficient window for rescue to be detected (Fig. S1B).

We then used the Broad Institute/Center for Cancer Systems Biology (CCSB) V5 epitope-tagged kinase ORF collection to identify genes that mediate resistance to these manipulations (20) (Fig. S2). Of the 597 ORFs, 14 scored more than two standard deviations (SD) above the median of all ORFs in the AEE788 screen, and 20 did so in the shRNA screen (Table 1 and Fig. 1A). Seven genes scored in both screens, including the activated forms of HRAS, KRAS, and MEK, which were screened as positive controls because they are known to signal downstream of HER2. AKT1, which signals downstream of HER2 to promote survival, scored strongly in both screens. In addition, MAP2K6, CRKL, and AKT3, which are known to signal through the Ras-ERK pathway or the PI3K-AKT pathway, scored more than two SD above the median in the shHER2 screen and more than 1.5 SD above the median in the AEE788 screen. These observations confirm prior work implicating MAPK and PI3K signaling as a major mechanism of resistance to HER2 inhibition (9–12, 21).

Three genes that have not been previously described as downstream targets of HER2 signaling scored more than 2 SD above the median in both screens: PRKACA, PIM1, and PIM2. In validation studies we found that, of these three molecules, PRKACA expression rescued BT474 cells most strongly from lapatinib, although PIM1 and PIM2 were expressed at much lower levels in these experiments (Fig. S3).

PRKACA is the alpha catalytic subunit of cyclic AMP (cAMP)-activated Protein Kinase A (PKA), whose activity is inhibited by PKA regulatory subunits. The second messenger cAMP activates PKA by causing the release of PRKACA or PRKACB from the regulatory subunits. Myriad effects of PKA activation have been described, including promotion of survival signaling. In addition, Vegran and colleagues demonstrated that *PRKACA* was one of 16 upregulated genes within a transcriptional signature that distinguishes breast cancers that failed to achieve a pathological complete remission (pCR) after trastuzumab plus docetaxel neoadjuvant chemotherapy from those that did achieve a pCR (22).

We validated our findings by performing dose titration curves for lapatinib in the setting of ectopic PRKACA expression in three HER2-amplified breast cancer cell lines. PRKACA expression increased the viability of BT474, SKBr3, and ZR-75-30 cells propagated in the presence of lapatinib (Fig. 1B). PRKACA expression also increased the viability of trastuzumab-treated HER2-amplified cells (Fig. S4). By counting viable cells, we found that lapatinib treatment of control cells expressing LACZ resulted in cell death, whereas overexpression of PRKACA in BT474 cells prevented cell death but failed to restore proliferation (Fig. 1C). Based on these observations, we hypothesized that PRKACA expression interferes with lapatinib-induced apoptosis. We found that, indeed, lapatinib treatment induced caspase 3/7 cleavage, and this event was significantly reduced by PRKACA overexpression (Fig. 1D, LACZ vs. PRKACA, $p = 0.0003$ for ZR-75-30, $p = 0.0002$ for BT474). These findings indicate that PRKACA rescues HER2-amplified cells from lapatinib treatment at least in part through the restoration of anti-apoptotic survival signaling.

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We then investigated mechanism(s) by which PRKACA conferred resistance to trastuzumab and lapatinib treatment. Since HER2 is thought to signal primarily through the Ras/Raf/MAPK and PI3K/AKT pathways, we first examined the activation of these pathways in the presence of AEE788 or lapatinib in BT474 cells transduced with a control ORF, LACZ. As expected, both p-ERK and p-AKT levels decreased in a dose-dependent manner in these cells in response to AEE788 and lapatinib (Fig. 2A). Transduction of HRASV12 into BT474s prior to treatment with either inhibitor completely restored p-ERK levels at all doses examined, but only partially restored p-AKT levels in the setting of AEE788 treatment and had no effect at the doses of lapatinib tested. These observations may be the result of the failure of Ras signaling to fully restore HER2-mediated, Ras-independent PI3K activation (Fig. 2A)(23). By contrast, expression of PRKACA failed to restore p-AKT or p-ERK levels in the context of either drug (Fig. 2B). Although we noted a slight decrease in baseline p-ERK levels in BT474 cells in the context of ectopic PRKACA expression, we were unable to verify this finding in the other cell lines tested (Fig. 2D); it is therefore possible that a feedback mechanism is operative in BT474 cells that results in a small decrease in MAPK signaling when PRKACA is overexpressed, but which does not lead to a significant decrease in proliferation (Fig. 1C). These observations suggested that PRKACA rescues cells from HER2 tyrosine kinase inhibition by a mechanism that is not mediated by AKT or ERK activation.

PRKACA has previously been reported to phosphorylate β-catenin, resulting in its activation, as well as BAD, resulting in its inactivation (24–27). BAD phosphorylation, which is also mediated by AKT, prevents the inhibitory influence of BAD on BCL-2 and BCL-XL, thereby promoting the anti-apoptotic activities of these molecules (28). PRKACA has also been reported to phosphorylate and inactivate GSK3-beta, which could lead to increased β-catenin activation (29). We therefore investigated the phosphorylation status of β-catenin, BAD, and GSK3 in PRKACA-overexpressing cells in the context of trastuzumab or lapatinib treatment. We found that phosphorylation of BAD at ser112 and ser136 was strikingly diminished by lapatinib treatment, and was also inhibited by trastuzumab treatment (Fig. 2C). In contrast, exposure to lapatinib or trastuzumab failed to affect the phosphorylation of β-catenin or GSK3-beta (Fig. 2C). Similarly, phosphorylation of a canonical downstream target of PKA, CREB, and its closely related family member, ATF1, were increased as expected by PRKACA overexpression, but were not decreased by exposure to lapatinib or AEE788 (Fig. 2C). Phosphorylation of BAD at ser112 and ser136 in the setting of lapatinib treatment was fully restored by PRKACA expression in BT474, SKBr3, and ZR-75-30 cells (Fig. 2C, 2D). In consonance with the observation in BT474 cells that PRKACA did not restore MAPK or PI3K signaling in the context of lapatinib treatment, PRKACA expression in SKBr3 or ZR-75-30 cells failed to restore phosphorylation of AKT, ERK, mTOR, and p70-S6K (Fig. 2D). These observations suggest that lapatinib and trastuzumab exert some of their inhibitory effects on HER2-positive cells through BAD de-phosphorylation, likely as a result of AKT inactivation, and that PRKACA overexpression rescues these cells in part through BAD re-phosphorylation and inactivation.

We next examined whether the kinase activity of PRKACA was required for the observed phenotypes of lapatinib resistance and BAD phosphorylation. Expression of a kinase-dead PRKACA mutant (K72H, PRKACA-KD) (30) failed to rescue ZR-75-30 or SKBr3 cells

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from lapatinib treatment, whereas both a V5-tagged and untagged (closed) version of PRKACA-WT conferred robust rescue (Fig. 3A). In addition, PRKACA-KD failed to restore BAD phosphorylation, indicating that the kinase activity of PRKACA is required for this effect (Fig 3B). Moreover, PRKACA-WT levels, which were higher in the V5-tagged construct than in the closed construct, positively correlated with the extent of rescue from lapatinib, as well as with the extent of BAD phosphorylation observed (Fig. 3A, 3B). These findings confirm that the kinase activity of PRKACA is required for the observed phenotypes of rescue from lapatinib and of BAD phosphorylation.

To confirm that the phenotypes observed with exogenous PRKACA expression also occur upon activation of endogenous PRKACA, we conducted an experiment using forskolin to activate cyclic AMP, which in turn activates PRKACA. Forskolin pre-treatment of ZR-75-30 cells resulted in resistance to lapatinib treatment (Fig. S5A) as well as restoration of inactivating BAD phosphorylation (Fig. S5B). These results demonstrate that the phenotypes observed can be achieved by activation of physiologic levels of endogenous PRKACA.

Since we found that high levels of PRKACA expression conferred resistance to lapatinib and trastuzumab treatment, we wished to determine whether PRKACA is overexpressed in the setting of acquired resistance to anti-HER2 therapy in human breast cancer samples. To examine this question, we performed PRKACA immunohistochemistry on breast cancer samples taken from patients prior to the administration of anti-HER2 therapy and after the development of clinical resistance to trastuzumab-containing therapy. In three of five cases we found dramatically increased PRKACA expression in the clinically resistant sample as compared to the pre-treatment sample (Fig. 3C). Specifically, patient #1 was diagnosed with *de novo* HER2-positive metastatic breast cancer, confirmed by biopsy of a sternal lesion (Fig. 3C, panel a). She initially responded to trastuzumab-containing therapy for 17 months, but then developed clinical resistance and progression in her breast lesion while other areas of disease remained stable. At that time she underwent a mastectomy for local disease control (Fig. 3C, panel b). Patient #2 was diagnosed with early stage HER2-positive breast cancer, treated with lumpectomy, axillary lymph node dissection, and radiation. Twelve years later she experienced a recurrence in the same breast and the contralateral axilla (Fig. 3C, panel c, breast biopsy is shown). She then received a total of 24 months of trastuzumab-based treatment, with good response initially but with subsequent progression in the axilla only, which was resected at that time (Fig. 3C, panel d). Patient #3 was diagnosed with *de novo* metastatic breast cancer, confirmed by fine needle aspirate of a liver lesion. A biopsy of her breast cancer at diagnosis is shown (Fig. 3C, panel e). She was treated with trastuzumab plus vinorelbine, with an excellent response in both the breast and liver lesions. However, after nine months of treatment her breast lesion was clearly growing while her distant metastatic disease continued to respond to therapy. She therefore underwent mastectomy for local disease control (Fig. 3C, panel f). In each of these cases, the level of PRKACA in the epithelial tumor cells was strikingly higher in the clinically resistant sample than in the pre-treatment sample, which had demonstrated response to trastuzumab-based therapy. Together with our functional data demonstrating that PRKACA confers resistance to trastuzumab and lapatinib in HER2-amplified breast cancer cells, these observations

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strongly suggest that PRKACA overexpression contributes to trastuzumab resistance in a subset of human breast cancers.

The two samples that did not clearly demonstrate an increase in PRKACA expression in the post-treatment sample differed from the other three in that the pre-treatment sample was obtained from early stage disease which was completely resected (Fig. S6, panels a and c). No sample from the time of metastatic diagnosis was available for testing, so the level of PRKACA initially present in the metastatic disease is unknown. These patients received trastuzumab and/or lapatinib-based treatment in the metastatic setting, and were biopsied at a later point of progression (Fig. S6, panels b and d). PRKACA expression was low to moderate in these samples, suggesting that a different mechanism of resistance may have been operative in these cases.

Given the high PRKACA expression observed in several of the samples analyzed, we wished to examine a larger panel of breast cancers for PRKACA expression. Although we were unable to obtain more matched samples before and after the development of resistance to anti-HER2 therapy, we performed PRKACA immunohistochemistry on a commercially available breast tissue microarray. We found that, compared to normal breast tissue or benign breast disease, a large proportion of invasive breast cancers expressed high levels of PRKACA (Fig. S7). These findings demonstrate that PRKACA overexpression is a common feature of invasive breast cancers, and suggest that it may play a role in intrinsic as well as acquired resistance to anti-HER2 therapy.

The observations that PRKACA appears to confer resistance to HER2-targeted therapy via BAD phosphorylation and that PIM1 and PIM2, which also scored in our screens, have been reported to phosphorylate BAD suggested that BAD may be involved in PIM-mediated resistance to anti-HER2 directed therapy. We first confirmed that PIM1 rescues HER2-amplified cells from lapatinib and trastuzumab treatment (Fig. 4A and 4C). We further found that PIM1 expression restores BAD phosphorylation at ser112 in the presence of lapatinib (Fig. 4D) and also inhibits lapatinib-induced apoptosis (Fig. 1D). These findings support the hypothesis that PIM1 expression promotes survival in lapatinib-treated cells by restoring BAD phosphorylation and thereby suppressing apoptosis. Although PIM2 also scored in the initial screens, in validation studies we were unable to express PIM2 at levels comparable to other candidates and therefore did not pursue this candidate further (Fig. S3).

Based on our hypothesis that phosphorylation and inactivation of BAD in lapatinib-treated HER2-amplified cells allows continued survival signaling through modulation of BCL-XL activity, we reasoned that BCL-XL overexpression should also confer resistance to HER2 inhibition. Indeed, we found that overexpression of BCL-XL rescues cells from lapatinib treatment (Fig. 4B, 4C). We therefore hypothesized that combined inhibition of BCL-XL and HER2 would result in increased cell death of HER2-positive cells and would at least partially abrogate the resistance effects conferred by PRKACA and PIM1. Indeed, treatment of ZR-75-30 cells with a dual BCL-2/BCL-XL inhibitor, ABT-263, at a dose that on its own had no effect on cell viability, synergized with lapatinib to cause increased cell death (Fig. 4E and 4D, LACZ controls). Furthermore, the addition of this drug also partially abrogated the lapatinib rescue effect conferred by PRKACA, PIM1, and BCL-XL (Fig. 4E and 4D).

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These findings support the hypothesis that activation of BCL-2/BCL-XL at least partially underlies the lapatinib resistance conferred by PIM1 and PRKACA overexpression, and suggest that combined blockade of BCL-2/BCL-XL survival signaling and HER2 could enhance tumor responses and decrease rates of recurrent and resistant disease.

DISCUSSION

Resistance to targeted therapy in breast cancer remains a major clinical problem. Although significant clinical benefit has been achieved in HER2-positive breast cancers with the introduction of the anti-HER2 drugs trastuzumab, lapatinib, pertuzumab, and trastuzumab emtansine, such cancers remain nearly universally fatal once metastasis has occurred (31–34). Even in cases in which complete clinical responses are observed, it is evident that residual viable cancer cells remain which ultimately give rise to resistant disease. Eradication of residual disease is critical for preventing the emergence of resistance, and we hypothesize that this may be achieved by combining additional targeted therapies with anti-HER2 therapy.

Efforts to target resistant or residual disease in HER2-positive breast cancer thus far have largely focused on canonical downstream targets of HER2 signaling, such as PI3K, mTOR, and MEK, whose activation may serve to bypass the upstream HER2 blockade. However, such approaches have met with limited success in early phase clinical trials (35, 36). In addition, eradication of residual disease early in treatment may require targeting pathways that are independent of HER2 signaling, while simultaneously targeting HER2.

To identify a broad spectrum of biological pathways that help evade the blockade of HER2 signaling, we used an unbiased, systematic approach to interrogate 597 kinases and kinase-related molecules for their ability to confer resistance to a small molecule EGFR/HER2 inhibitor or to an anti-HER2 shRNA in HER2-dependent BT474 cells. We chose this dual approach in order to identify robust biological mediators of resistance to anti-HER2 therapy, irrespective of the specific mechanism by which HER2 signaling was blocked. We note that the expression of oncogenic versions of HRAS or KRAS or of activated MEK, as well as MAP2K6, CRKL, AKT1, or AKT3 conferred resistance to both the HER2 inhibitor and the anti-HER2 shRNA. These results confirm prior work implicating the MAPK and PI3K pathways in resistance to anti-HER2 therapy, and serve to validate the screening approach (8–15, 18, 19).

In addition to identifying expected mediators of PI3K and MAPK signaling, the most robust mediators of resistance that scored in both screens and that are not implicated in canonical signaling downstream of HER2 were PRKACA and PIM1. Consistent with these findings, downregulation of a regulatory subunit of PKA, *PRKAR2A*, has been reported to confer resistance to trastuzumab in HER2-amplified cells in vitro (37). Both PRKACA and PIM1 converge on activation of survival signaling through inactivation of BAD. We found that both lapatinib and trastuzumab treatment of HER2-positive breast cancer cells results in BAD de-phosphorylation, suggesting that the anti-cancer effects of these drugs are in part mediated by BAD activation and its subsequent inhibition of survival signaling. Since AKT and RSK are also known to phosphorylate BAD, it is possible that BAD inactivation

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represents a common downstream node through which resistance induced by PI3K or MAPK pathway activation is partly mediated and through which HER2 activation itself promotes survival in the absence of anti-HER2 treatment.

The clinical relevance of our findings to human breast cancers is underscored by our finding of increased levels of PRKACA expression in three out of five breast cancer samples that developed clinical resistance to trastuzumab. Together with our results showing that PRKACA expression activates survival signaling in HER2-positive breast cancer cells treated with anti-HER2 therapy, these findings support the hypothesis that high levels of PRKACA expression mediate treatment resistance in HER2-positive breast cancer. Results from a tissue microarray of primary invasive breast cancers demonstrating strikingly high levels of PRKACA in a subset of samples, as compared to benign breast tissue, raises the possibility that PRKACA may play a role in intrinsic as well as acquired resistance. It is notable that only approximately 50% of trastuzumab-naïve, metastatic HER2-positive breast cancers undergo objective responses upon treatment with trastuzumab-based therapy, indicating that a subset of HER2-positive breast cancers do exhibit intrinsic resistance (33). The finding of high levels of PRKACA expression in a subset of primary invasive breast cancers also suggests that PRKACA may play a role in breast cancer progression. Indeed, a recent report showed that bi-allelic ablation of PRKAR1A, one of the regulatory subunits that inhibits PRKACA activity, results in spontaneous mammary tumorigenesis in mice (38). In addition, a chimeric transcript involving PRKACA that retains kinase activity has recently been identified in 15/15 human fibrolamellar hepatocellular carcinomas, and somatic activating mutations in PRKACA have been identified by two groups in 37–66% of adrenal adenomas from patients with Cushing's syndrome (39–41). These findings further support a role for PRKACA in tumorigenesis.

Our findings suggest that inhibition of PRKACA and/or its downstream anti-apoptotic effectors in combination with anti-HER2 therapy may prevent the emergence of resistant disease. Since activation of PIM1, AKT1, RSK, or p70S6K is also known to result in BAD phosphorylation and subsequent activation of BCL-XL/BCL-2 [reviewed in (28)], blockade of this signaling pathway may provide a robust means of simultaneously targeting multiple mechanisms of resistance to anti-HER2 therapy. Our finding that the BCL-XL/BCL-2 inhibitor ABT-263 synergizes with lapatinib in HER2-amplified cells supports this hypothesis. Indeed, recent work showed that blockade of this pathway also synergizes with tamoxifen in ER-positive breast cancers, suggesting that co-targeting of BCL-XL/BCL-2 with either ER- or HER2-directed therapies may represent a useful strategy across breast cancer subtypes (42). ABT-263 is currently under investigation in early phase clinical trials in a variety of cancers, although not in combination with anti-HER2 therapy in breast cancer (Clinicaltrials.gov).

Although our observations strongly suggest that the resistance effect conferred by both PRKACA and PIM1 overexpression is at least in part mediated through the restoration of anti-apoptotic signaling, it is possible that other pathways that contribute to this phenotype are also activated by PRKACA and/or PIM1. PRKACA in particular has been implicated in a variety of cellular processes, which vary by cell type and context. Moreover, the observation that the degree of rescue conferred by PRKACA is greater than that conferred

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by PIM1 suggests that PRKACA may induce additional antagonistic effects to anti-HER2 therapy. Further studies will be necessary to identify such mechanisms.

In summary, we have identified PRKACA as a molecule that is frequently overexpressed in invasive and trastuzumab-resistant breast cancers and have demonstrated that PRKACA overexpression leads to resistance to trastuzumab and lapatinib. We have also identified PIM1 as a novel mediator of resistance to anti-HER2 therapy and, by identifying the survival mechanism through which both PRKACA and PIM1 signal to promote resistance to anti-HER2 therapy, we have discovered a common resistance node whose simultaneous targeting along with HER2 may lead to improved tumor responses. Specifically, our study provides a rationale for further investigation of combined anti-HER2 and anti-BCL-XL/BCL-2 therapy in HER2-positive breast cancers. Such studies could lead to better responses and a decrease in residual disease, treatment resistance, and tumor recurrence.

MATERIALS AND METHODS

Cell Culture and Lentiviral Transduction

All cell lines were grown in media containing 10% FBS (Sigma) and 1% Penicillin/Streptomycin (GIBCO) (293T: DMEM; BT474 and ZR-75-30: RPMI; SkBr3: McCoy's 5A).

For lentivirus production, 293T cells were co-transfected with VSV-g and delta 8.9 packaging plasmids and the target plasmid using Mirus TransIT L1 transfection reagent according to manufacturer's instructions. The following morning, media was replaced with DMEM containing 30% FBS, and lentiviral supernatants were collected at 48 and 72 hours post-transfection, pooled, and stored at −80 degrees C.

Lentiviral transductions were performed at 1:20 to 1:10 dilution in media containing 8 ug/ml polybrene. Cells were centrifuged at 2000 rpm×15 min and allowed to infect for 24h at 37 degrees C, followed by a change to media with or without puromycin (pLKO) or blasticidin (pLX303 and pLX304). Puromycin doses were 2 ug/ml for all cell lines and blasticidin doses were 5 ug/ml for SKBr3 and 15 ug/ml for BT474 and ZR-75-30.

Kinase ORF Library Screening

Kinase library lentivirus production was performed as previously described (20). Optimal viral dilution was determined by titering in BT474 cells to ensure high infection efficiency with minimal viral toxicity. BT474 cells were seeded at a density of 5000 cells per well of 384-well plates. The following day, cells in each duplicate set of plates were transduced in quadruplicate with each lentivirally-delivered ORF (pLX304, Addgene) at 1:25 dilution in media containing 10 ug/ml polybrene; each ORF was tested in duplicate. Plates were centrifuged for 15 min at 930×g. After 24 h, virus was removed and cells were re-fed with media. On day 4, one set of kinase-library transduced ORFs was treated with 0.85 uM AEE788 or DMSO; blasticidin 15 ug/ml was also added to all wells at this time for selection of ORF-transduced cells. One parallel plate was run without blasticidin to confirm infection efficiency, which was >70% for >90% of ORFs, with an average of 91%. The second set of replicate plates was transduced with 4 ul (1:12.5 dilution) of pLKO-shHER2-4355, or mock

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transduction, in media containing 10 ug/ml polybrene. The latter set of plates underwent a media change on day 5 to remove virus. On day 10, 30 ul of Cell-Titer Glo reagent (Promega) was added to each well and luminescence was read 20 min later using Wallac Envision software.

Plasmids and Drugs

pLX304-ORF and pLKO-shRNA constructs were obtained from the Broad Institute Genetic Perturbation Platform (all available in Addgene). For the generation of PRKACA kinase-dead (K72H), the following PCR primers were used with pLX304-PRKACA-WT as template to generate overlapping 5' and 3' PRKACA fragments containing the appropriate mutations: PRKACA-KD-Fwd GAA CCA CTA TGC CAT GCA TAT CCT CGA CAA with pLX304-Rev 5'-CAA CAC CAC GGA ATT GTC AG-3'; PRKACA-KD-Rev TTG TCG AGG ATA TGC ATG GCA TAG TGG TTC with pLX304 Fwd 5'-CAC CAA AAT CAA CGG GAC TT-3'. The fragments generated were then gel purified (Qiagen) and combined as template for PCR with pLX304-Fwd and pLX304-Rev to generate the full-length PRKACA-KD coding sequence containing the K72H mutation. The mutant construct was confirmed by sequencing.

AEE788 was a generous gift from Novartis, lapatinib (Tykerb) was purchased from Selleck Chemicals, ABT-263 (Navitoclax) was purchased from Toronto Research Chemicals, and trastuzumab (Herceptin) was purchased from the Dana-Farber Cancer Institute pharmacy.

Luminescence Assays

For ATP-based viability assays, 40 ul of Cell Titer Glo (Promega) was added to each well of a 96 well plate containing 100 ul media. For caspase 3/7 cleavage assays, 50 ul of Caspase 3/7 Glo reagent (Promega) was added to wells containing 50 ul media. Luminescence was read 20 minutes later using Wallac Envision software. For Caspase 3/7 Glo experiments, p-values were determined by 2-tailed paired t-test.

Immunoblot Analyses

Cells were washed with PBS and lysed on ice in RIPA buffer containing Complete protease inhibitor cocktail (Roche) and Phos-stop phosphatase inhibitor cocktail (Roche). Lysates were cleared by centrifugation at 13,000 rpm at 4 degrees C for 20 minutes and protein was quantified using BCA reagent (Thermo Scientific). 30–50 ug of protein lysate were run on Bis-Tris gels (Invitrogen) using MOPS or MES buffer (Invitrogen). Proteins were transferred to nitrocellulose membranes by wet transfer or by iBlot (Invitrogen) dry transfer. Membranes were blocked in 10% milk in 1X PBS containing 0.01% Tween 20 (PBST) and were incubated with antibodies in 5% milk or BSA in 1X PBST, according to manufacturers instructions. Membranes were washed with 1X PBST and incubated with secondary antibodies (Bio-Rad) in 5% milk/1X PBST at room temperature. Membranes were washed with 1X PBST and visualized with enhanced chemiluminescence (Amersham). Antibodies used were: ERBB2/Neu (Santa Cruz, #sc-7301), HRP-V5 (Invitrogen, #P/N 46-0708), HRP-b-actin (Santa Cruz, #sc-47778 HRP), PRKACA (BD Transduction Labs, #610980), HRAS (Santa Cruz, #sc-29), BAD (Cell Signaling, #9268), p-BAD ser112 (Cell Signaling, #5284), p-BAD ser136 (Cell Signaling, #4366), p-BAD ser155 (Cell Signaling, #9297), ERK p42/44

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(Cell Signaling, #9102), p-ERK p42/44 (Cell Signaling, #9101), AKT1 (Cell Signaling, #9272), p-AKT ser473 (Cell Signaling, #9271), p-b-catenin ser552 (Cell Signaling, #9566), p-b-catenin ser675 (Cell Signaling, #4176), b-catenin (Cell Signaling, #9562), p-GSK3 a/b ser21/9 (Cell Signaling, #9331), p-GSK3a ser21 (Cell Signaling, # 9316), GSK3 a b (Cell Signaling, #5676), CREB (Cell Signaling, #9197), p-CREB (Cell Signaling, #9198), p-mTOR (Cell Signaling, #5536), mTOR (Cell Signaling, #2972), p-p70S6K (Cell Signaling, #9234), p70S6K (Cell Signaling, #9202), PIM1 (Cell Signaling, #3247), PIM2 (Cell Signaling, #4730).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tumor samples with associated clinical information were obtained for analysis under Dana-Farber Cancer Institute Institutional Review Board Protocols 93-085 and 11-264. All subjects gave informed consent. Breast tissue microarray BRC961 was purchased from U.S. Biomax. PRKACA immunohistochemistry was performed by the Dana-Farber/Harvard Cancer Center Research Pathology core using standard protocols. The PRKACA primary antibody (BD Transduction Labs, #610980) was used at 1:1000 dilution with an overnight incubation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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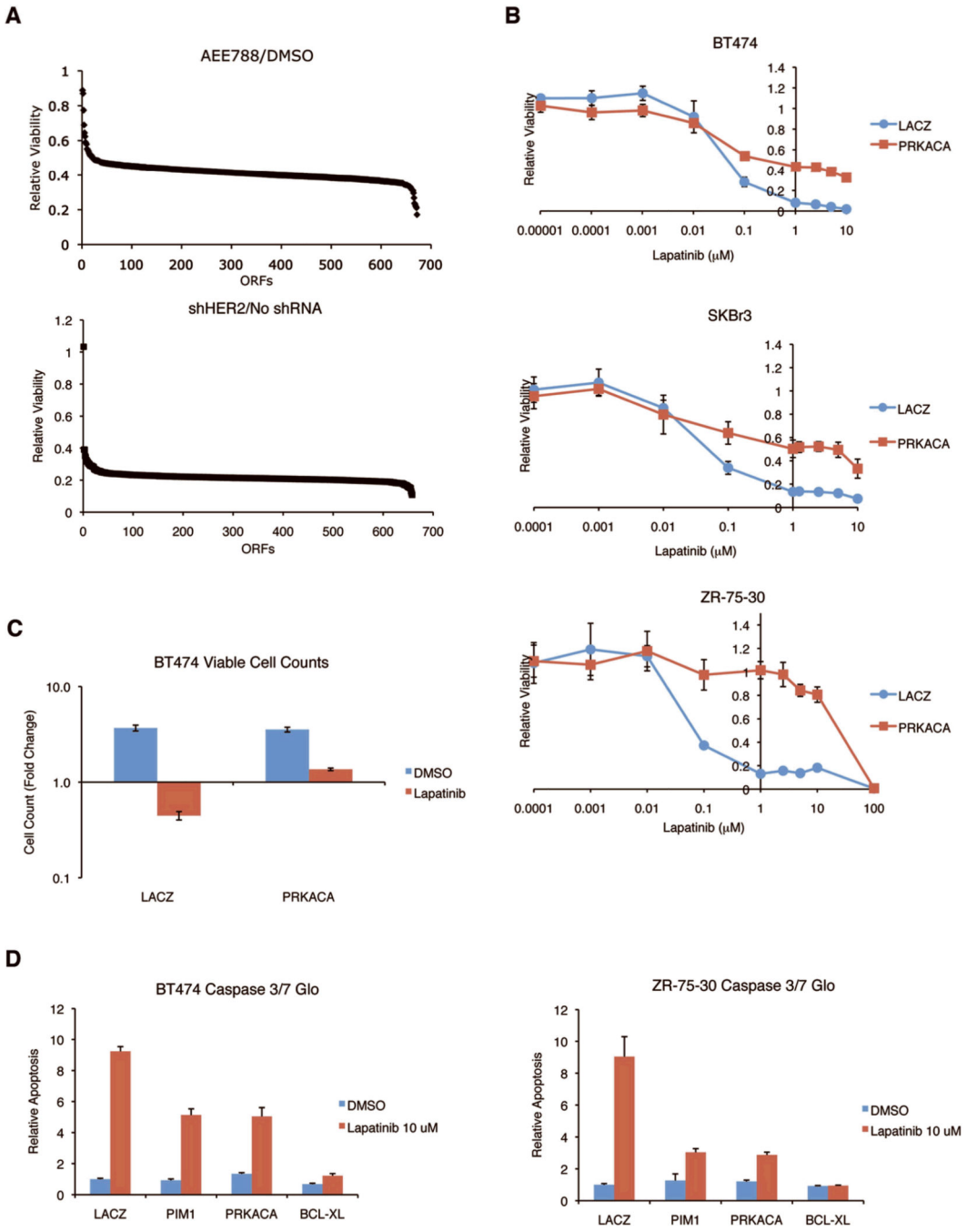

Figure 1.

PRKACA confers resistance to anti-HER2 therapy and impairs apoptosis. A. Relative viability of screened BT474 cells containing each ORF and treated with AEE788 (top panel) or an shRNA targeting HER2 (bottom panel). B. PRKACA confers resistance to lapatinib. Cells were lentivirally-transduced with the indicated ORFs, treated with lapatinib at the indicated doses, and cell viability was assessed by ATP-based luminescence assay. Results are normalized to the DMSO control for each ORF and represent the mean and standard deviation (SD) of 6 replicates. C. PRKACA expression prevents lapatinib-induced cell

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death. BT474 cells were transduced with LACZ or PRKACA constructs and subsequently treated with lapatinib 5 uM or DMSO. Viable cells were counted by trypan blue exclusion 5 days after the addition of drug. Results show the mean and standard deviation of 3 replicates. D. Lapatinib treatment results in apoptosis of BT474 (top panel) and ZR-75-30 (bottom panel) cells, and this is partially rescued by PRKACA, PIM1, and BCL-XL. Cells were transduced with the indicated ORFs. Two days later lapatinib 10 uM or DMSO was added, and caspase 3/7 cleavage was measured 24h later by luminescent assay. Results represent the mean and SD of 5 replicates per cell line. For BT474, LACZ vs. PRKACA $p = 0.0002$, LACZ vs. PIM1 $p = 0.000007$. For ZR-75-30, LACZ vs. PRKACA $p = 0.0003$, LACZ vs. PIM1 $p = 0.0006$.

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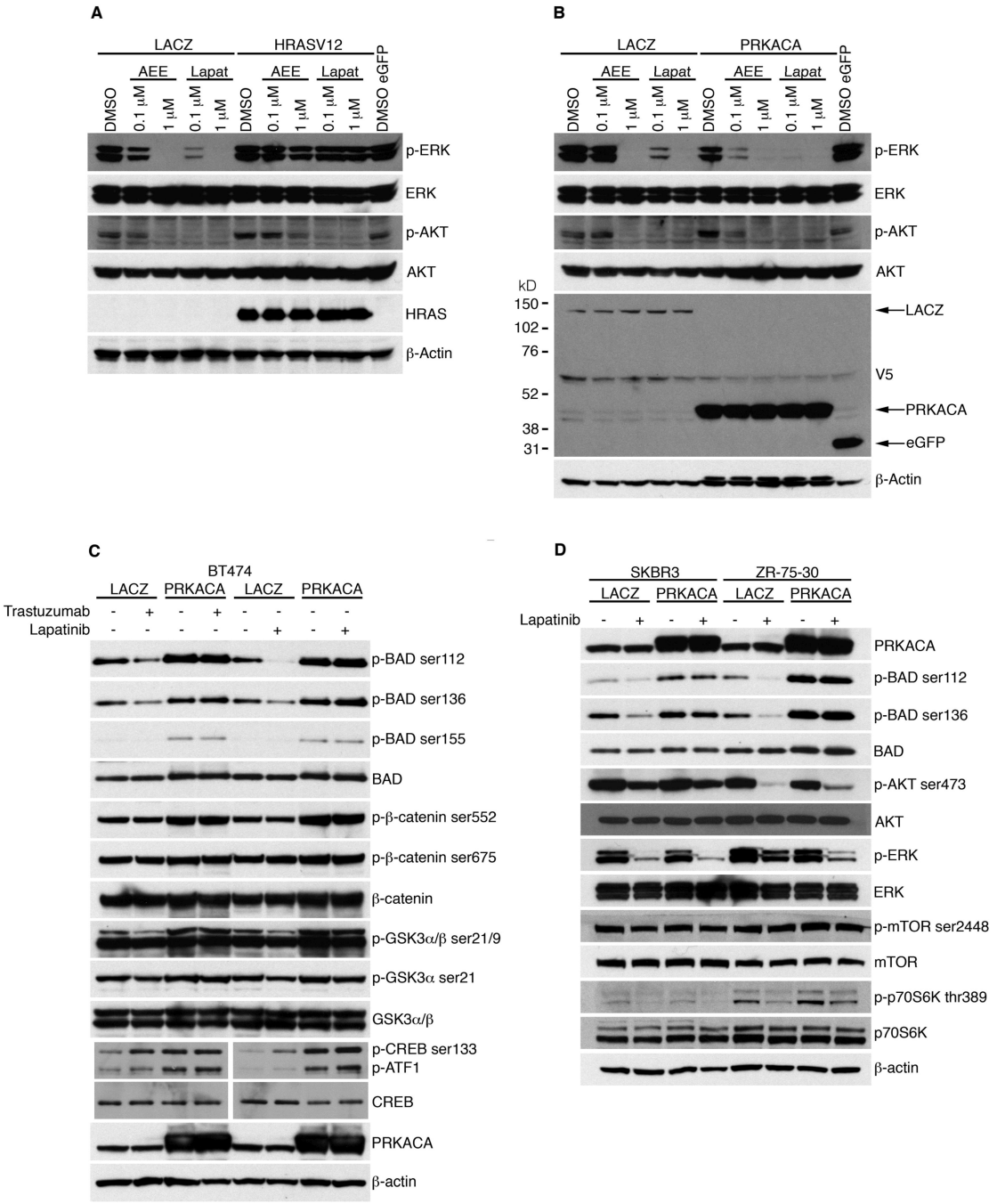

Figure 2.

PRKACA does not restore MAPK or AKT signaling, but does restore BAD phosphorylation. A. AEE788 or lapatinib treatment decreases p-ERK and p-AKT levels in HER2-amplified cells, and p-ERK levels are fully restored by activated HRAS (HRASV12) overexpression. B. PRKACA overexpression does not restore p-ERK or p-AKT levels in AEE788- or lapatinib-treated cells. For both panels, BT474 cells were transduced with the indicated pLX304 constructs and blasticidin selected. Lapatinib, AEE788, or vehicle control (DMSO) was added at the indicated concentrations, and cell lysates were harvested 24 h

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later for immunoblot analysis with the indicated antibodies. The V5 antibody identifies each of the V5-tagged ORFs, as indicated. C. Lapatinib or trastuzumab treatment results in loss of BAD phosphorylation, which is restored by PRKACA overexpression. BT474 cells were lentivirally-transduced with the indicated pLX304 constructs, blasticidin selected, and treated with lapatinib 0.1 uM or trastuzumab 10 ug/ml for 24 h. Protein lysates were harvested and subjected to immunoblot analysis with the indicated antibodies. The samples for the p-CREB and CREB immunoblots were run on the same gel, but the lapatinib and trastuzumab lanes were run in inverse orientation from the other panels, so the image was cut and re-aligned for labeling consistency with the other panels. D. BAD phosphorylation was restored by PRKACA overexpression in multiple HER2-positive cell lines after lapatinib treatment. SKBr3 and ZR-75-30 cells were lentivirally-transduced with pLX304 constructs expressing LACZ or PRKACA, blasticidin selected, and then treated with lapatinib 0.1 uM or DMSO for 24 h prior to immunoblot analysis with the indicated antibodies.

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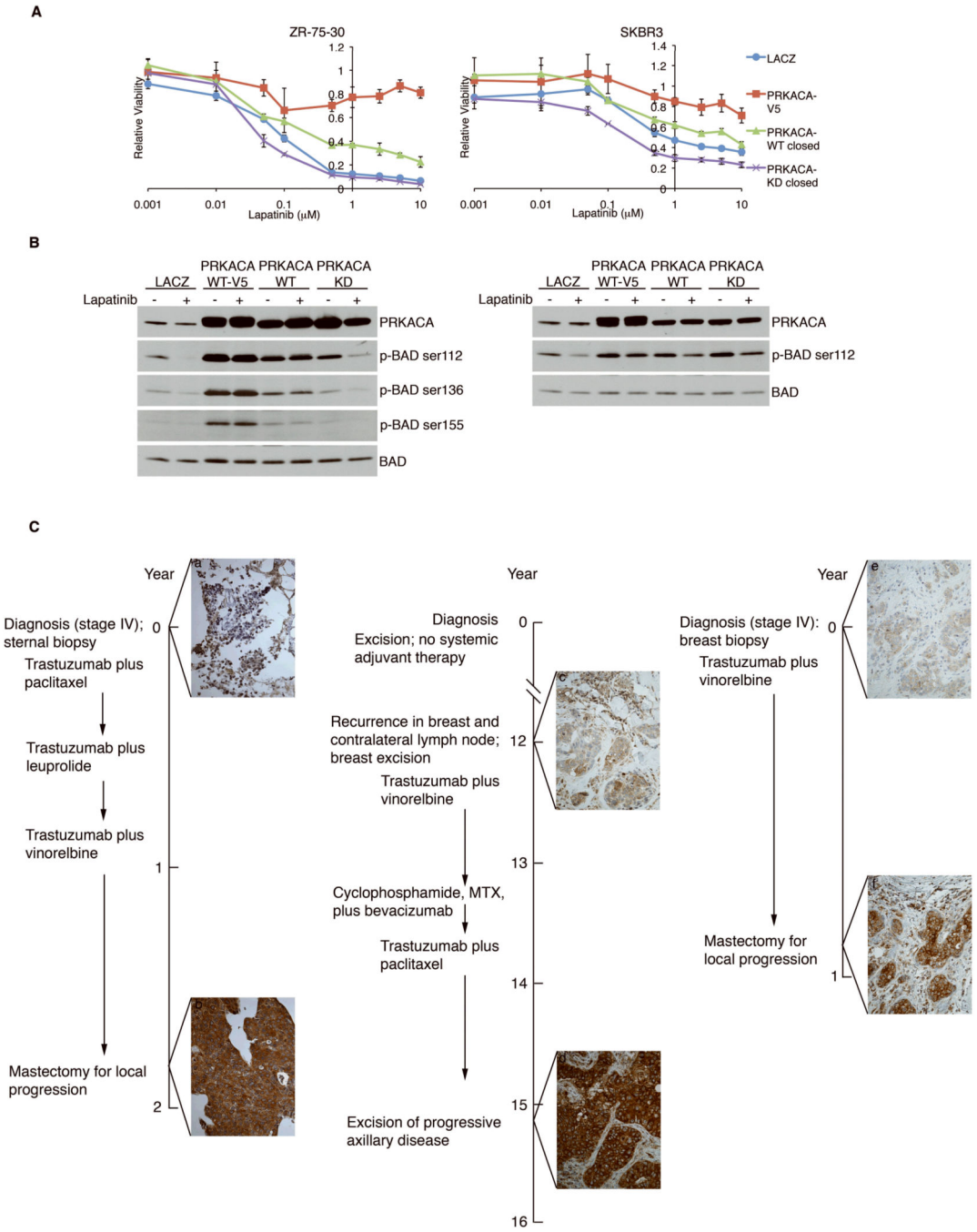

Figure 3.

PRKACA is overexpresed in trastuzumab-resistant breast cancers. A. The kinase activity of PRKACA is required to confer resistance against lapatinib and for BAD phosphorylation. A closed (untagged) kinase-dead mutant of PRKACA (PRKACA-KD) was transduced into ZR-75-30 and SKBr3 cells alongside wild-type PRKACA constructs that were either V5-tagged or closed, as well as a LACZ control. Cells were treated with lapatinib at the indicated doses or DMSO and viability was assessed 6 days later by ATP-based luminescence assay. Results are normalized to the DMSO control for each ORF and

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represent the mean and SD of 3 replicates. B. Cells were lentivirally-transduced with pLX304-ORFs expressing V5-tagged PRKACA, closed PRKACA, closed PRKACA-KD, or LAZ. After blasticidin selection cells were treated with DMSO or lapatinib 0.1 uM for 24 h. Protein lysates were harvested and subjected to immunoblot analysis with the indicated antibodies. C. PRKACA is overexpressed in trastuzumab resistant human breast cancers. Immunohistochemical analysis for PRKACA expression in matched breast cancer samples from patients prior to any treatment (panels a, c, e) and after the development of resistance to trastuzumab-containing therapy (panels d, e, f). Magnification 40X.

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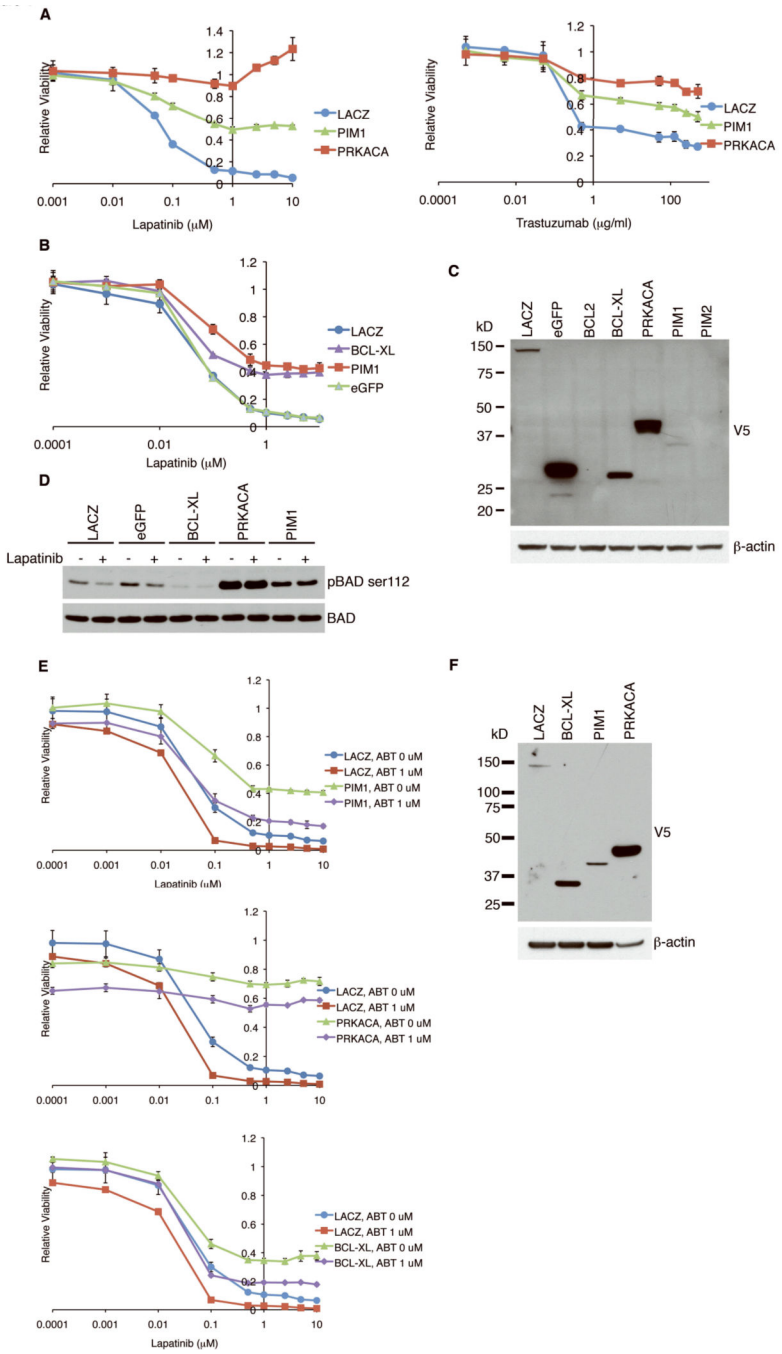

Figure 4.

PIM1 and BCL-XL confer resistance to anti-HER2 treatment in HER2-positive cells, and blockade of BCL-2/BCL-XL partially abrogates the resistance effect of PRKACA and PIM1. A. ZR-75-30 cells were transduced with the indicated pLX304 constructs. Two days later they were treated with increasing doses of lapatinib (left panel) or trastuzumab (right panel), and cell viability was assessed by ATP-based luminescence assay 6 days later. Results are normalized to the vehicle control for each ORF and represent the mean and SD of 3 replicates. B. BCL-XL overexpression confers resistance to lapatinib. ZR-75-30 cells

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were transduced with the indicated constructs. Two days later cells were treated with increasing doses of lapatinib, and cell viability was assessed by ATP-based luminescence assay 6 days later. Results are normalized to the vehicle control for each ORF and represent the mean and SD of 3 replicates. C. Immunoblot analysis of lysates from ZR-75-30 cells three days after ORF transduction in parallel with the cells used in (A) and (B) demonstrates exogenous expression of the indicated proteins. The anti-V5 antibody identifies each of the V5-tagged ORFs. D. PIM1 restores BAD ser112 phosphorylation after lapatinib treatment. ZR-75-30 cells were lentivirally-transduced with the indicated pLX304 constructs, blasticidin selected, and treated with DMSO or lapatinib 0.1 uM for 24h. Protein lysates were harvested and subjected for immunoblot analysis with the indicated antibodies. E. Pharmacological blockade of BCL-2 and BCL-XL synergizes with lapatinib treatment and partially abrogates the rescue effect conferred by PIM1 (top panel), PRKACA (middle panel), and BCL-XL (bottom panel). Two days after transduction with the indicated pLX304 constructs, ZR-75-30 cells were treated with the indicated doses of lapatinib and ABT-263 or DMSO. Cell viability was read out 6 days later by ATP-based luminescent assay. All results are normalized to the LACZ, DMSO-treated control and represent the mean and SD of 3 replicates. All three panels are from the same experiment using the same LACZ controls, but results are shown in separate graphs for ease of visualization. F. Immunoblot analysis of lysates from ZR-75-30 cells three days after ORF transduction in parallel with the cells used in (E) demonstrates exogenous expression of the indicated proteins. The anti-V5 antibody identifies each of the V5-tagged ORFs.

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Table 1

ORF screen identifies mediators of resistance to anti-HER2 treatment. Listed are ORFs that scored ≥ 1.5 standard deviations above the median of all ORFs for their ability to confer resistance to the anti-HER2 tyrosine kinase inhibitor AEE788 or an shRNA targeting HER2. ORFs that are listed in bold scored greater than two standard deviations above the median in one screen and at least greater than 1.5 standard deviations above the median in the other screen.

AEE788, > Median + 2 STDEV		**shHER2, > Median + 2 STDEV**	
Gene	**Relative Viability**	**Gene**	**Relative Viability**
FGR	0.889	**HRASV12**	**1.033**
HRASV12	**0.870**	STYK1	0.391
LIMK2	0.774	**MEKDD**	**0.390**
RP6-213H19.1	0.690	**MAP2K6**	**0.351**
PRKACA	**0.624**	**PIM2**	**0.343**
BLK	0.591	**PIM1**	**0.324**
PIM1	**0.585**	DDR1	0.315
ERBB3	0.579	**KRASV12**	**0.314**
PIM2	**0.553**	**PRKACA**	**0.306**
KRASV12	**0.550**	**CRKL**	**0.304**
AKT1	**0.543**	LYN	0.291
MEKDD	**0.538**	JAK2	0.290
HIPK1	0.529	ABL2	0.290
TNK1	0.523	**AKT3**	**0.286**
		AKT1	**0.285**
		ABL1	0.283
		MAP3K15	0.282
		PTK2B	0.272
		FER	0.267
		AK1	0.267
AEE788, > Median + 1.5 STDEV		**shHER2, > Median + 1.5 STDEV**	
Gene	**Relative Viability**	**Gene**	**Relative Viability**
DAPK3	0.516	HIPK4	0.265
MAP2K6	**0.515**	YES1	0.264
LOC646505	0.514	CDKL4	0.263
DYRK1B	0.514	SNX16	0.260
CRKL	**0.509**	KHK	0.258
NLK	0.500	LYK5	0.258
NEK5	0.498	ROR2	0.257
PRPS2	0.497	TSSK6	0.256
AKT3	**0.491**	NME3	0.255
		RPS6KA6	0.253
		NRBP	0.252