

AD _____

Award Number: W81XWH-10-1-0575

TITLE: Overcoming Resistance to Trastuzumab in HER2-Amplified Breast Cancers

PRINCIPAL INVESTIGATOR: DR. SUSAN MOODY

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
Boston, MA 02115

REPORT DATE: August 2011

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-08-2011		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 15 JUL 2010 - 14 JUL 2011	
4. TITLE AND SUBTITLE Overcoming Resistance to Trastuzumab in HER2-Amplified Breast Cancers				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0575	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) DR. SUSAN MOODY E-Mail: semoody@partners.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Dana-Farber Cancer Institute Boston, MA 02115				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The receptor tyrosine kinase HER2 is amplified and/or overexpressed in 25-30% of all breast cancers. Blockade of HER2 with drugs such as trastuzumab or lapatinib has led to clinical benefit in patients with both metastatic and early-stage HER2-amplified breast cancer. However, resistance and disease progression always occurs in patients with metastatic disease, and many patients with early-stage breast cancer experience recurrences despite adjuvant treatment with one of these agents. To identify novel mechanisms of resistance to anti-HER2 therapy, I conducted a screen for molecules whose forced expression in HER2-amplified breast cancer cells confers resistance to HER2 inhibition. I screened an open reading frame library of approximately 600 kinases and kinase-related molecules. I found that both activated HRAS and the catalytic subunit of Protein Kinase A (PRKACA) conferred significant resistance to both compound-mediated and genetic inhibition of HER2. Upon further mechanistic investigation, I found that activated HRAS fully restored phospho-ERK levels in the presence of either of two HER2 tyrosine kinase inhibitors, but did not restore phospho-AKT1 levels. By contrast, PRKACA expression provided resistance to anti-HER2 treatment, but did not restore phospho-ERK or phospho-AKT1 levels. The mechanisms by which resistance is conferred by PRKACA are under continued investigation.					
15. SUBJECT TERMS Drug resistance, HER2-amplified breast cancer, open reading frame screening, RNA interference screening, synthetic lethality					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	16	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	6
Reportable Outcomes	7
Conclusion	7
References.....	8
Appendices.....	n/a
Supporting Data	9

INTRODUCTION

The receptor tyrosine kinase HER2 is amplified and/or overexpressed in 25-30% of all breast cancers. Blockade of HER2 with drugs such as trastuzumab or lapatinib has led to clinical benefit in patients with both metastatic and early-stage HER2-amplified breast cancer. However, resistance and disease progression always occurs in patients with metastatic disease, and many patients with early-stage breast cancer experience recurrences despite adjuvant treatment with one of these agents. Most of the mechanisms of resistance to HER2 blockade identified to date result in the restoration of downstream receptor tyrosine kinase signaling. Given this observation, one approach to battling resistance is to block signaling via the two major oncogenic pathways downstream of HER2 activation, PI3K and Ras. While several PI3K inhibitors have shown promising results in pre-clinical testing, pharmacological targeting of Ras family members has not been successful. Given these difficulties in directly targeting Ras, we sought to discover synthetic lethal partners to oncogenic KRAS, the Ras family member most commonly mutated in cancer. Using high-throughput RNA interference (RNAi), we identified Tank-Binding-Kinase 1 (TBK1) as a molecule whose targeted downregulation led to lethality specifically in human cancer cell lines harboring KRAS mutations and/or displaying dependency on KRAS, but not in those that contained only wild-type KRAS and that were not dependent upon KRAS.

I hypothesized that in addition to inhibiting HER2, it would be necessary to target other molecules to achieve lasting remissions in HER2-amplified breast cancers. Because HER2 signaling leads to Ras activation, I hypothesized that inhibition of Ras or of TBK1 would impair the proliferation or viability of HER2-amplified breast cancer cells, whether or not they are sensitive to anti-HER2 therapy. I also hypothesized that additional genetic aberrations exist in HER2-amplified cells that are required for the HER2 amplification to persist, and that these serve as molecular vulnerabilities that are specific to HER2-amplified cells.

To discover novel molecular vulnerabilities in HER2-amplified breast cancers, I propose to: 1) test the effects of TBK1 and Ras inhibition on proliferation, viability, and the development of resistance to anti-HER2 therapy in HER2-amplified breast cancer cells; 2) test whether overexpression of any of approximately 1000 kinases and oncogenes is able to confer resistance to anti-HER2 therapy in HER2-amplified breast cancer cells; and 3) utilize genome-scale RNAi screening to identify synthetic lethal partners of amplified HER2.

BODY

Specific Aim 1: Determine whether suppression of TBK1 or of Ras family members affects the proliferation or viability of trastuzumab-sensitive and trastuzumab-resistant HER2-amplified breast cancer cell lines, and whether TBK1 or Ras inhibition in trastuzumab-sensitive cell lines enhances the lethality of trastuzumab or inhibits the outgrowth of trastuzumab-resistant cells.

To address the components of the first task associated with this aim, I first tested multiple shRNAs against HRAS, KRAS, and NRAS, and confirmed both their efficacy of target gene suppression and their specificity for the targeted Ras isoform (Fig. 1a). The TBK1 shRNAs had already been tested and confirmed (Barbie et al., 2009). I tested the effect of suppressing each of these molecules, along with negative controls, in two HER2-amplified and three non-HER2-amplified breast cancer cell lines. The results were, surprisingly, the opposite of that which I had hypothesized. The HER2-amplified cell lines were far less sensitive to suppression of any Ras isoform or of TBK1 than the non-HER2-amplified cell lines (Fig. 1b). At least three explanations for this observation exist: 1) HER2-amplified breast cancer cell lines are less

dependent upon Ras signaling than are non-HER2-amplified breast cancer cell lines, and/or 2) because the extent of suppression of each of these molecules by the shRNAs was not complete, and the HER2-amplified cell lines have hyper-activation of the Ras pathway at baseline, these cell lines are better able to tolerate partial suppression of the Ras pathways than are non-HER2 amplified cell lines, and/or 3) suppression of only one of the Ras isoform pathways at a time is not sufficient to impair the viability of HER2-amplified cell lines.

The additional components of Specific Aim 1, Task 1 required the use of trastuzumab. Unfortunately, I encountered unexpected difficulties in obtaining trastuzumab for research purposes. A materials transfer agreement that was mutually satisfactory to both my sponsoring institution, Dana-Farber Cancer Institute, and to the manufacturers of trastuzumab, Genentech, could not be secured. As a result, the planned experiments in the setting of trastuzumab treatment could not be performed. I have recently been able to obtain trastuzumab via direct purchase from our institution's pharmacy, and these experiments are now underway. During the time that trastuzumab was unavailable to me, I did proceed with the experiments proposed for Specific Aim 2, using alternative methods of HER2 inhibition. These are described below under Specific Aim 2.

Milestone 1 was met for Specific Aim 1, in which I determined that neither HRAS, KRAS, NRAS, nor TBK1 is selectively essential in HER2-amplified breast cancer lines. IRB and IACUC approvals were also obtained during this time period for the proposed mouse experiments, thereby fulfilling Milestone 2.

Specific Aim 2: Identify novel mediators of trastuzumab resistance through high-throughput overexpression of a panel of approximately 1000 kinases and oncogenes.

Task 2 involved conducting an ORF screen to identify novel mediators of resistance to trastuzumab in HER2-amplified breast cancer cells. As noted above, I encountered unexpected difficulties obtaining trastuzumab for research use. Therefore, I developed an alternative strategy for HER2 inhibition to use in this screen that would allow me to identify molecules able to bypass the effects of HER2 blockade. I did have access to the Novartis compound AEE788, a kinase inhibitor that targets HER2 and EGFR in a similar manner to lapatinib, the Glaxo-Smith-Kline drug that is in clinical use. I found that treatment with AEE788 resulted in dose-dependent lethality in HER2+ BT474 cells (Fig. 2A). However, because in addition to its effect on HER2, AEE788 has effects on EGFR, and to a lesser extent on VEGFR1 and VEGFR2, I selected to perform a parallel screen using an anti-HER2 shRNA to block HER2. I tested six anti-HER2 shRNAs and found that sh4355 (targeted to the 3' UTR of HER2) resulted in both the greatest suppression of HER2 levels and the greatest impairment in viability in BT474 cells, so I selected this shRNA for the screen (Fig. 2B). I reasoned that the off-target effects of a kinase inhibitor and an shRNA targeting the 3' UTR are unlikely to overlap. Therefore, molecules that rescue from both of these mechanisms of HER2 inhibition are likely to be specific to HER2.

We conducted two parallel screens in which BT474 cells were first transduced in arrayed format with each molecule in the ORF library. Activated forms of HRAS and MEK (HRASV12 and MEK-DD, respectively) were included as positive controls. Two days later the cells were either treated with AEE788 or transduced again with the anti-HER2 shRNA. Viability was read out 6

days later using an ATP-based luminescence assay (Fig. 3). I then scored each screen for molecules whose viability readout was greater than 2 standard deviations above that of the median for the screen. AKT1, which lies downstream of PI3K signaling, scored highly in both screens, thereby validating the approach. In all, 7 molecules scored greater than 2 standard deviations above the median in both screens, and an additional 3 scored greater than 2 standard deviations above the mean in one screen and greater than one standard deviation above the median in the other (Fig. 4A, Fig. 4B).

I subsequently performed low-throughput validation of the top candidates from the screen at two doses of AEE788, along with HRASV12 as a positive control and LACZ as a negative control. Several of the candidates scored significantly better than the LACZ, including AKT1, CRKL, MAP2K6, MAP3K15, and PIM1; the one with the greatest magnitude of rescue from AEE788 is PRKACA, the catalytic subunit of Protein Kinase A (Fig. 5A). I subsequently was able to obtain lapatinib, and confirmed that PRKACA is able to rescue BT474 cells from lapatinib treatment as well (Fig. 5B). As mentioned above, I was recently able to obtain trastuzumab as well, and have initiated experiments to test the ability of PRKACA to rescue HER2-amplified cells from trastuzumab as well. I have also obtained shRNAs targeting PRKACA and am testing them in HER2-amplified cells, to address task 3 of this aim.

I also have begun to investigate the mechanism by which PRKACA rescues BT474 cells from AEE788 and lapatinib treatment. Since HER2 is thought to signal primarily through the MAPK and PI3K/AKT pathways, I first examined the activation of these pathways in the presence of the inhibitors in BT474 cells transduced with a neutral ORF, LacZ. As expected, both P-MAPK and P-AKT levels decreased in a dose-dependent manner in these cells in response to AEE788 and lapatinib. Transduction of HRASV12 into BT474s prior to treatment with either inhibitor completely restored P-MAPK levels at all doses examined, but only partially restored P-AKT levels in the setting of AEE788 treatment, and not at all in the setting of lapatinib treatment (Fig. 6A). By contrast, transduction of PRKACA restored neither P-AKT nor P-MAPK levels in the setting of either drug (Fig. 6B). This suggests that PRKACA is conferring increased survival upon HER2 tyrosine kinase inhibition by a mechanism that is not mediated via AKT or MAPK. I am continuing to investigate the potential mechanisms involved by examining additional pathways through which Protein Kinase A has been described to signal.

Specific Aim 3: Utilize genome-scale RNAi screening to identify synthetic lethal partners of amplified HER2.

In collaboration with the Broad Institute RNAi Platform under the direction of Dr. David Root, we have initiated the pooled, genome-scale screening of HER2-amplified and non-HER2-amplified breast cancer cell lines, as described in Task 4. To date, seven breast cancer cell lines have completed shRNA transduction, passaging, DNA harvest and are awaiting deep sequencing readout. An additional eight breast cancer cell lines have started the optimization process for transduction or are currently being passaged. I anticipate that deep sequencing of this group of cell lines will be completed in 3-4 months, at which time I will begin analysis of the data to identify synthetic lethal partners of amplified HER2 in breast cancer.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that suppression of any of the Ras isoforms HRAS, KRAS, and NRAS individually was not selectively lethal in the HER2-amplified cell lines BT474 and MDA-MB-453.
- Determined that suppression of TBK1 was not selectively lethal in the HER2-amplified cell lines BT474 and MDA-MB-453.
- Identified AKT1, CRKL, MAP2K6, MAP3K15, PIM1, and PRKACA as molecules whose forced expression partially rescues BT474 cells from AEE788 and lapatinib.

REPORTABLE OUTCOMES

Abstract: *Open Reading Frame Screening to Identify Novel Mechanisms of Resistance to Anti-HER2 Therapy*. Moody SE, Schinzel AC, Izzo F, Luo L, Boehm JS, Kim SY, Thomas SR, and Hahn WC. Era of Hope Conference, Orlando, FL. Aug. 4, 2011.

Promotion: The PI on this Award was promoted to Instructor in Medicine at the Dana-Farber Cancer Institute and Harvard Medical School, effective July 1, 2011.

CONCLUSIONS

The research supported by this Award has resulted in several valuable findings to date, with possible clinical applications. Although expression of activated HRAS appears sufficient to confer resistance to genetic or compound-mediated suppression of HER2 activity, suppression of the individual Ras isoforms HRAS, KRAS, or NRAS does not appear to be selectively detrimental to the proliferation or survival of HER2-amplified breast cancer cells. Similarly, suppression of TBK1, a synthetic lethal partner with KRAS activation, also failed to impair the proliferation of HER2-amplified breast cancer cell lines. Further research in this direction may aim to determine if suppression of all Ras isoforms simultaneously would be selectively lethal in HER2-amplified cells. However, given the pharmacological challenges of targeting Ras, I would defer from pursuing this direction further in favor of pursuing the novel findings from our ORF screen.

ORF screening using both genetic and compound-based approaches to target HER2 has yielded several valuable findings. AKT1, CRKL, MAP2K6, MAP3K15, PIM1, and PRKACA all appear to confer partial resistance to HER2 kinase inhibition; the magnitude of rescue from AEE788 and lapatinib with PRKACA is most pronounced. This finding is particularly exciting in light of the finding that overexpression of PRKACA is a component of a 28-gene signature that predicts failure to achieve a complete pathological response to trastuzumab-based neoadjuvant chemotherapy in human breast cancers (Vegran et al., 2009). As a serine/threonine kinase, PRKACA could thus represent a novel, druggable target to enhance treatment response in conjunction with or serially following anti-HER2 therapy. I am actively pursuing the mechanisms that underlie this finding, as well as determining whether PRKACA confers resistance to anti-HER2 therapies in additional HER2-amplified breast cancer cell lines.

Within the next year I will also have access to pooled shRNA screening data from HER2-amplified and non-HER2 amplified cancer cell lines. I anticipate that these data will provide

additional insights into targetable pathways in HER2-amplified breast cancers, and possibly in other classes of breast cancer as well.

REFERENCES

Barbie, D.A., Tamayo, P., Boehm, J.S., Kim, S.Y., Moody, S.E., Dunn, I.F., Schinzel, A.C., Sandy, P., Meylan, E., Scholl, C., et al. (2009). Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature* 462, 108-112.

Vegran, F., Boidot, R., Coudert, B., Fumoleau, P., Arnould, L., Garnier, J., Causeret, S., Fraise, J., Dembele, D., and Lizard-Nacol, S. (2009). Gene expression profile and response to trastuzumab-docetaxel-based treatment in breast carcinoma. *Br J Cancer* 101, 1357-1364.

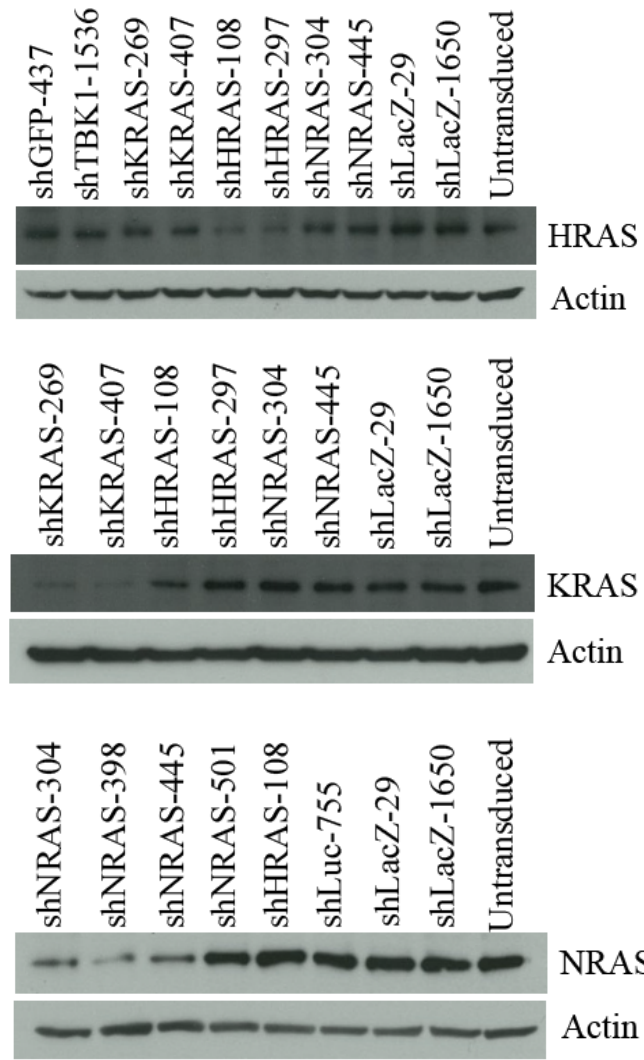


Figure 1A Immunoblot analyses testing the efficacy and specificity of pLKO-shRNAs targeted against HRAS (top panel), KRAS (middle panel), and NRAS (bottom panel). Cells were seeded on day 0, transduced with the indicated shRNAs on day 1, and puromycin-selected on day 2. Protein lysates were harvested on day 4, 72 hours after transduction. Blots were probed with the indicated antibodies against the Ras isoforms.

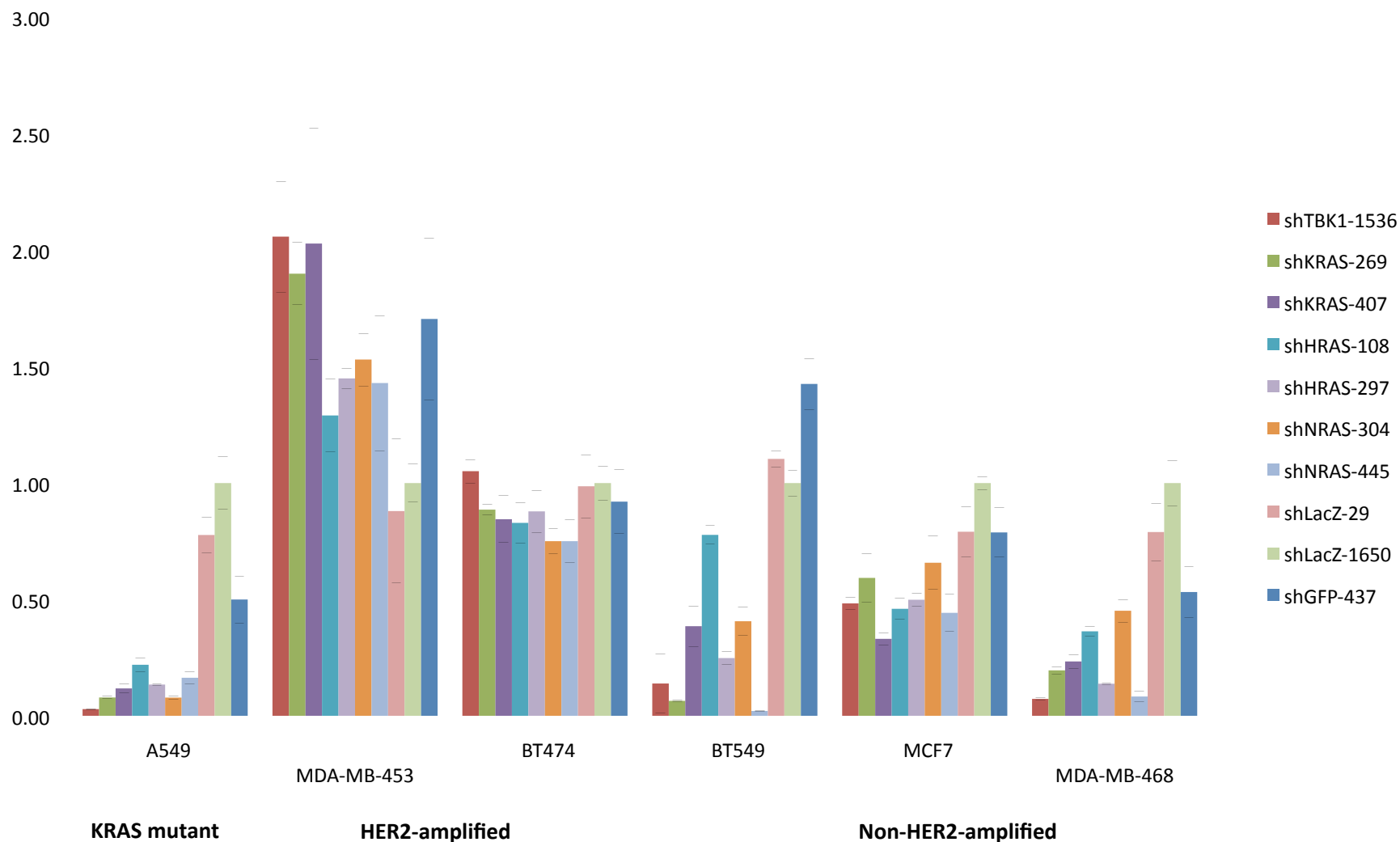


Figure 1B Relative viability of KRAS mutant lung cancer cells (A549), HER2-amplified breast cancer cells (MDA-MB-453 and BT474), and non-HER2-amplified breast cancer cells (BT549, MCF7, and MDA-MB-468). Cells were seeded on day 0, and transduced with the indicated pLKO-shRNAs on day 1. Replicate wells of cells were treated with puromycin on day 2 to confirm infection efficiency. Viability was assessed on day 7 by ATP-based luminescence assay. The results shown represent the mean and standard deviations of three replicate experiments in the absence of puromycin treatment.

Drug Titration and shRNA Selection for Screening

A.

AEE788

- Inhibitor of HER2, EGFR, VEGFR1 (FLT-1), VEGFR2 (KDR)
- Used at 0.85 μ M in screen

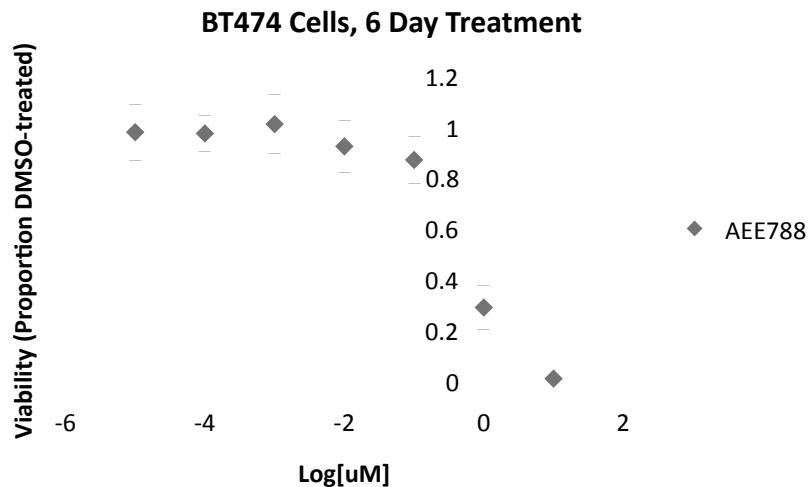
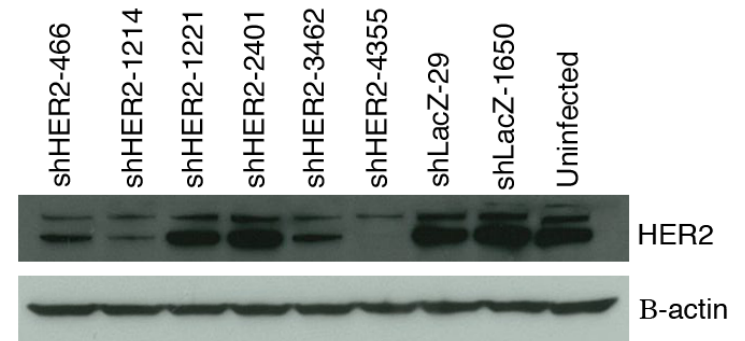


Fig. 2A Dose-response curve for AEE788 in BT474 cells.

B.

BT474 (HER2+) Cells



BT474 Cells, 6 Day Cell Titer Glo

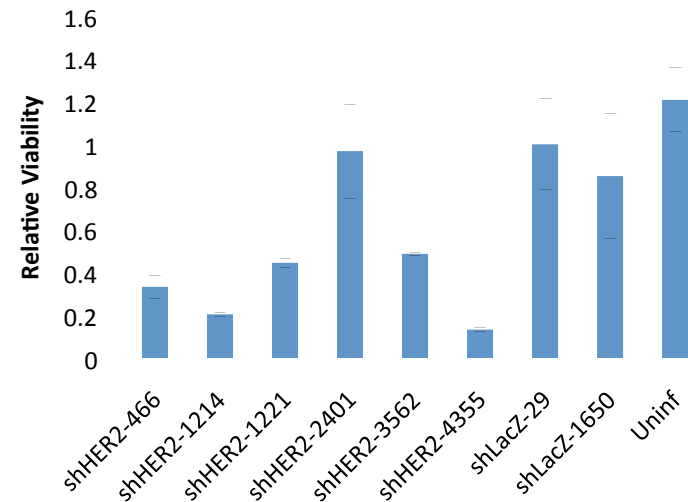


Fig. 2B Top panel: Western analysis of BT474 cells 72 hours after lentiviral infection with shRNAs targeting HER2 or control shRNAs. Bottom panel: Relative viability of BT474 cells 6 days after infection with shRNAs targeting HER2 or control shRNAs. Based on these analyses, anti-HER2 shRNA-4355 was chosen for the screen.

Open Reading Frame Screening Design and Timeline

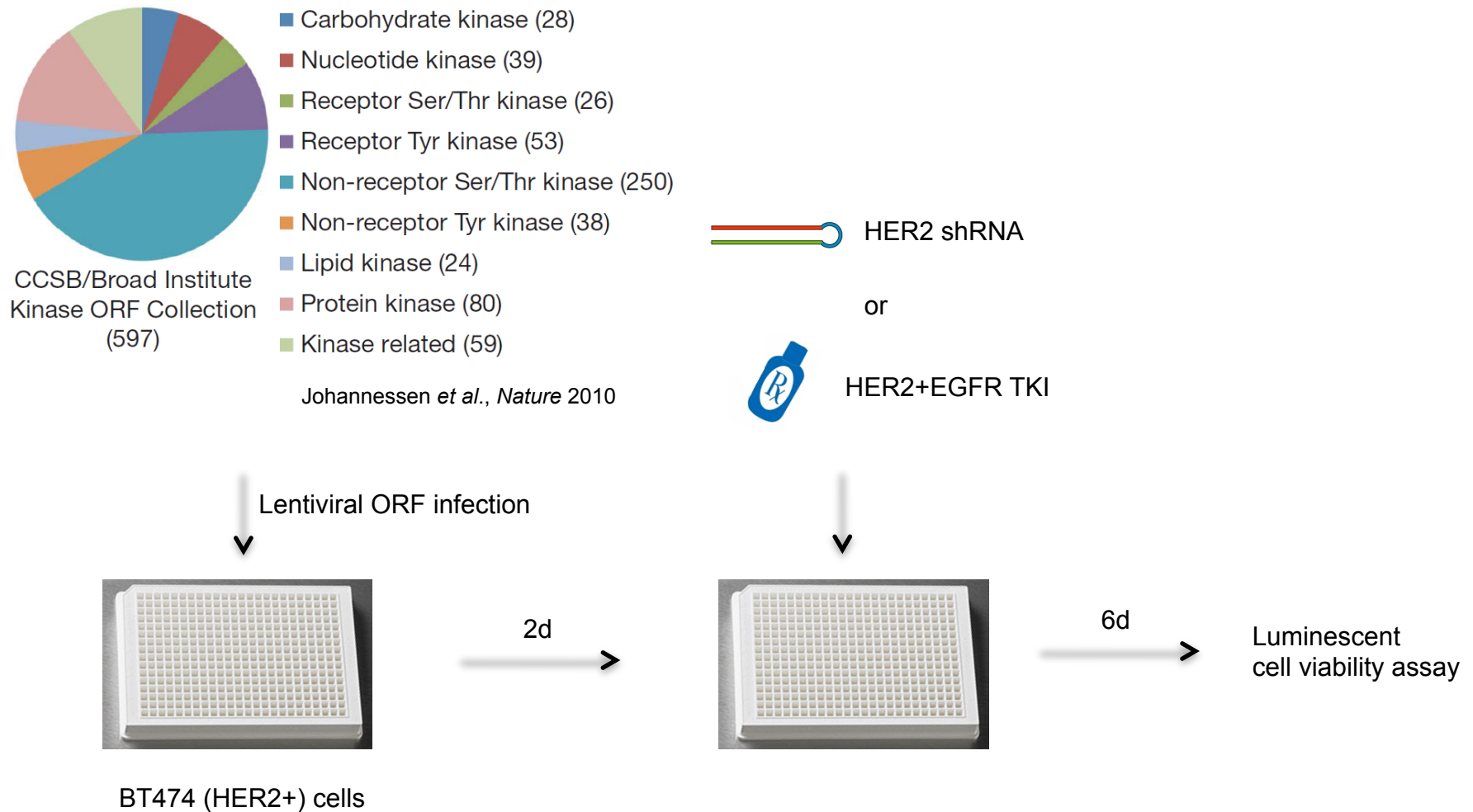


Figure 3: Schematic illustrating the ORF screen performed. BT474 cells were seeded 24 hours prior to spin infection in arrayed format with lentivirally-delivered ORFs. Two days later an anti-HER2 shRNA or the HER2/EGFR tyrosine kinase inhibitor (TKI) AEE788 was added. Viability was read out 6 days later by ATP-based luminescent cell viability assay. Each ORF was tested in duplicate and parallel plates were run under blasticidin selection to confirm infection efficiency.

Kinase ORF Screening Identifies Candidates that Confer Resistance to Anti-HER2 Treatment

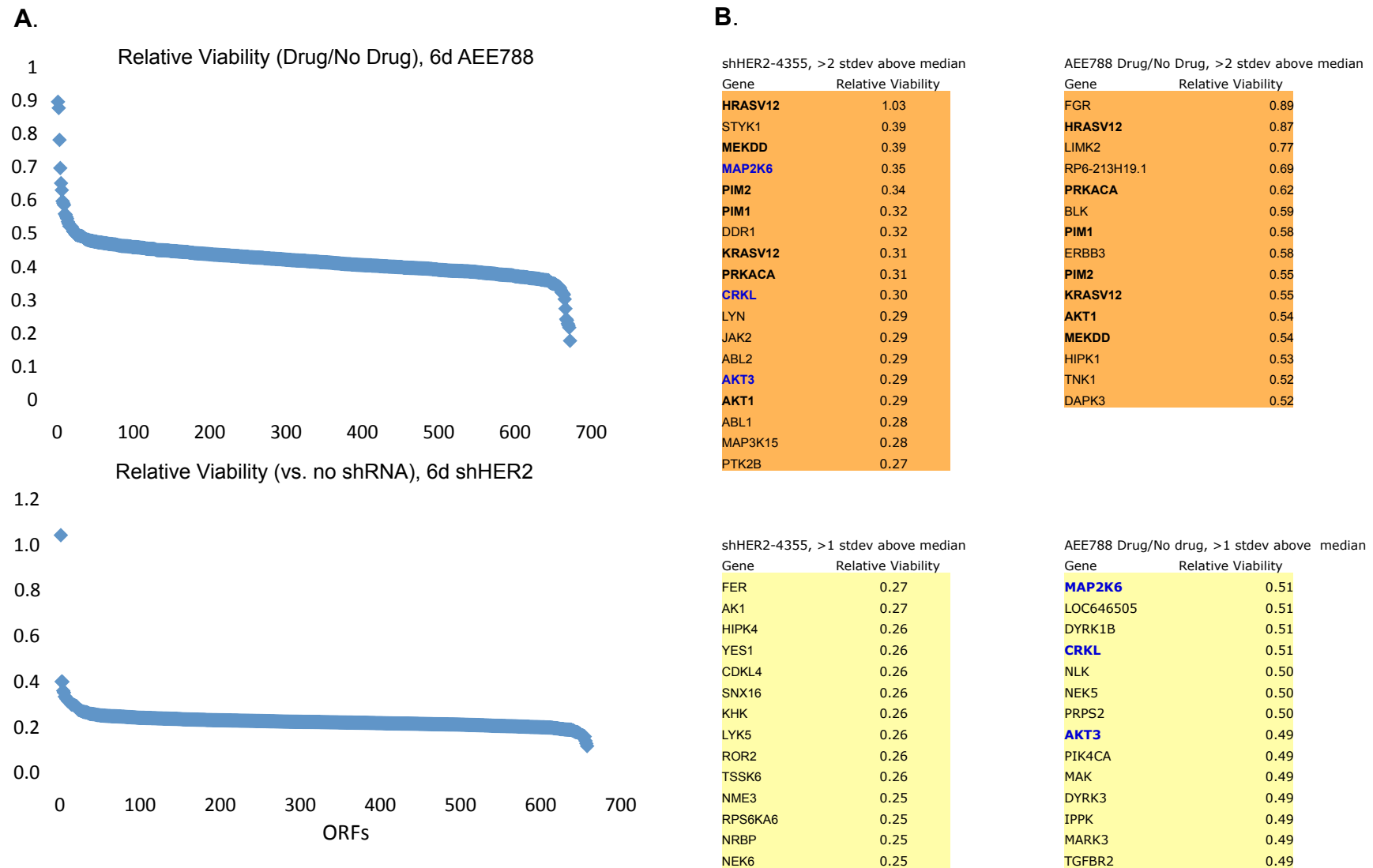


Figure 4 Kinase ORF Library Screening Identifies Candidates that Confer Resistance to Anti-HER2 Treatment. A. Relative viability of BT474 cells containing each ORF and treated with AEE788 (top panel) or an shRNA targeting HER2 (bottom panel). B. Summary of top candidates from both screens. ORFs in black bold scored greater than two standard deviations above the median in both screens. ORFs in blue bold scored greater than two standard deviations above the median in one screen and greater than one standard deviation above the median in the other.

Validation of Top Candidates From Primary Screen

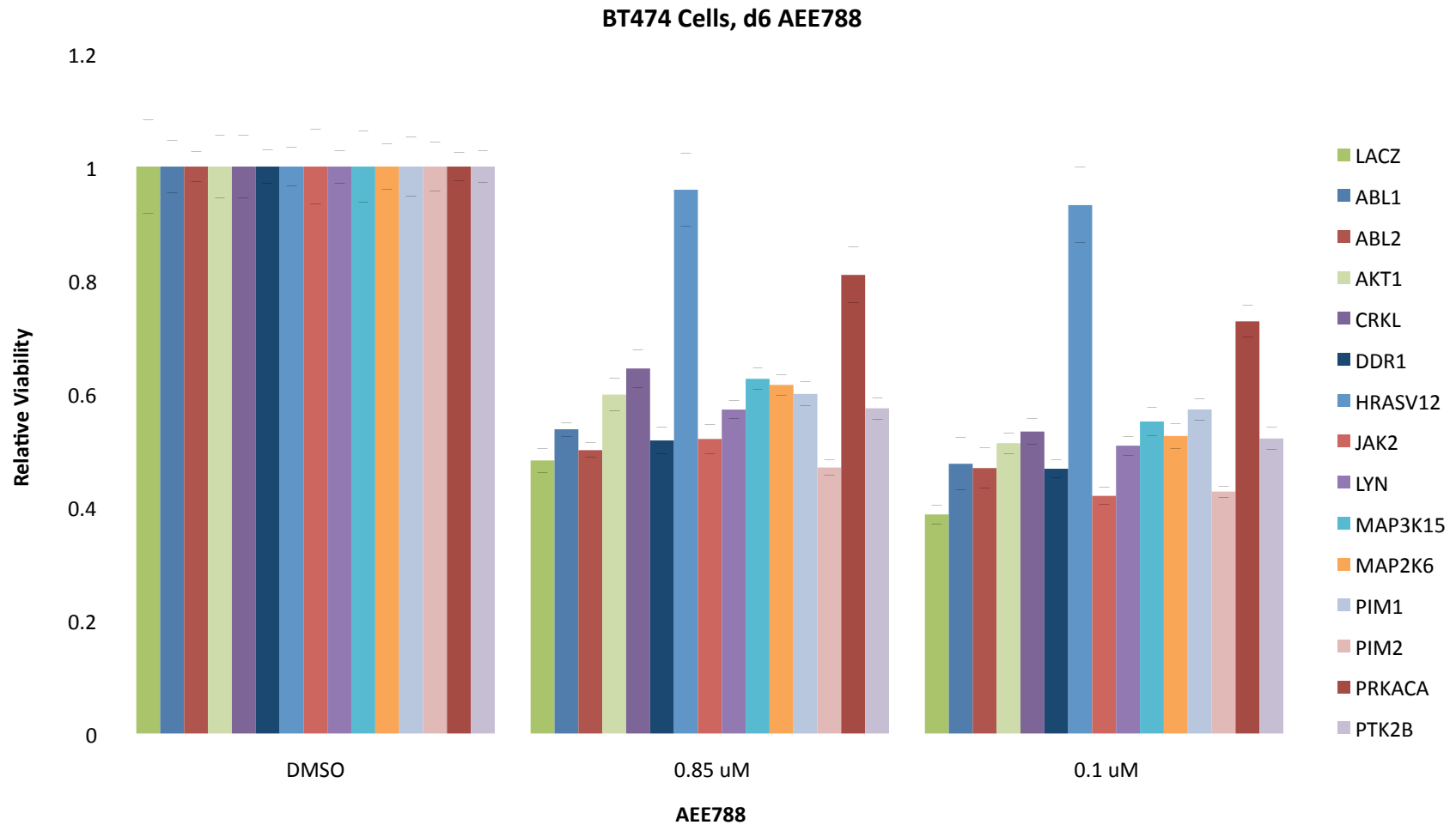


Figure 5A Low-throughput validation confirms strong rescue from AEE788 by HRASV12 and PRKACA. BT474 cells were transduced with the indicated ORFs and selected with blasticidin for 4 days. Cells were then seeded and treated with drug, and 6 days later relative viability was determined via ATP-based luminescence assay. The results shown represent the mean and standard deviation of 6 replicate wells.

PRKACA and Activated HRAS Expression Confer Resistance to Lapatinib

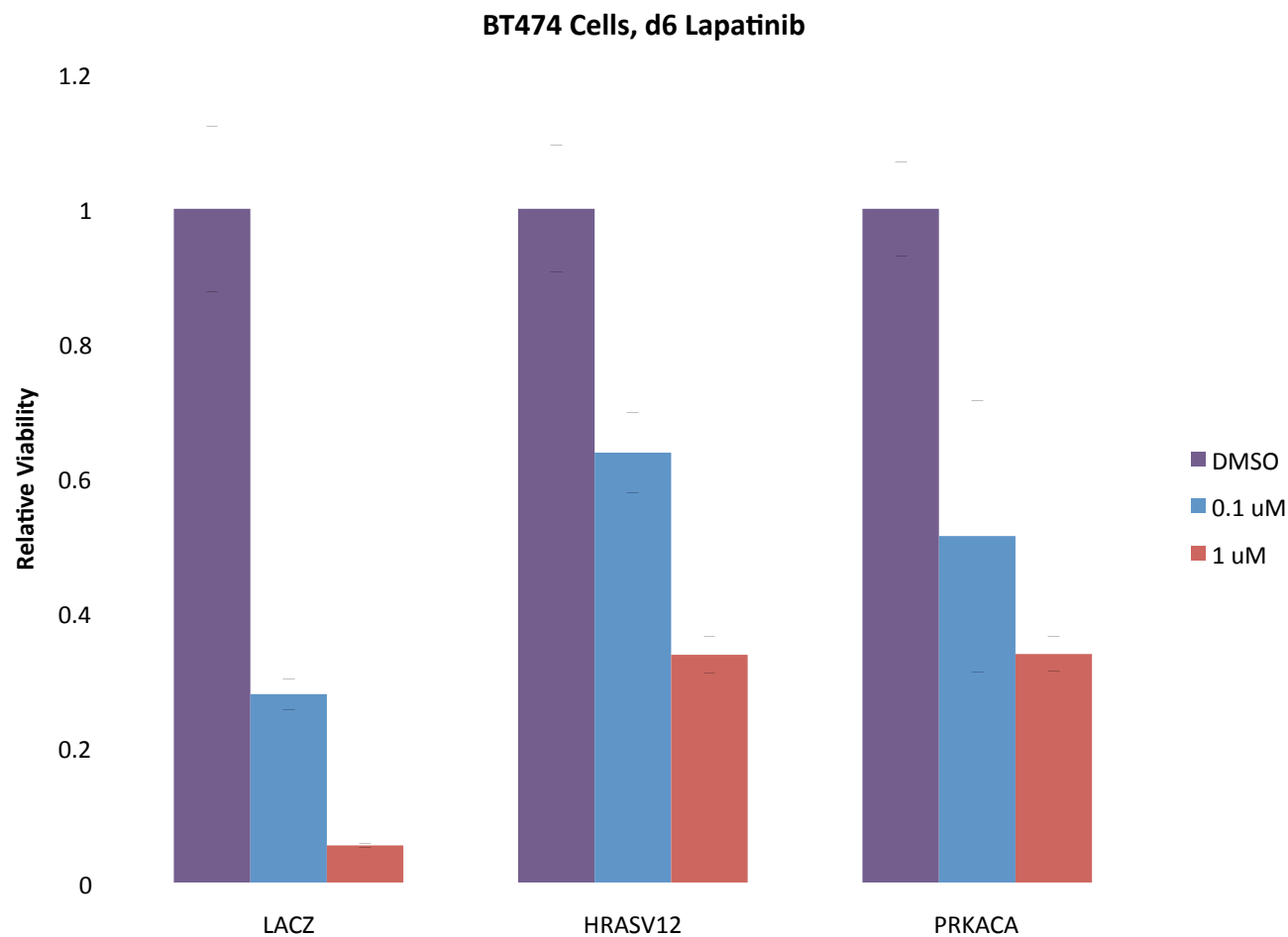


Figure 5B HRASV12 and PRKACA confer resistance to lapatinib in BT474 cells. BT474 cells were seeded and transduced with the indicated ORFs. Two days later lapatinib was added at the indicated doses. Cell viability was read out by ATP-based viability assay five days later. The results shown represent the mean and standard deviation of 6 replicate wells per condition.

Effect of HRASV12 or PRKACA Expression on MAPK and AKT Activation in BT474 Cells Treated with HER2/EGFR Inhibitors

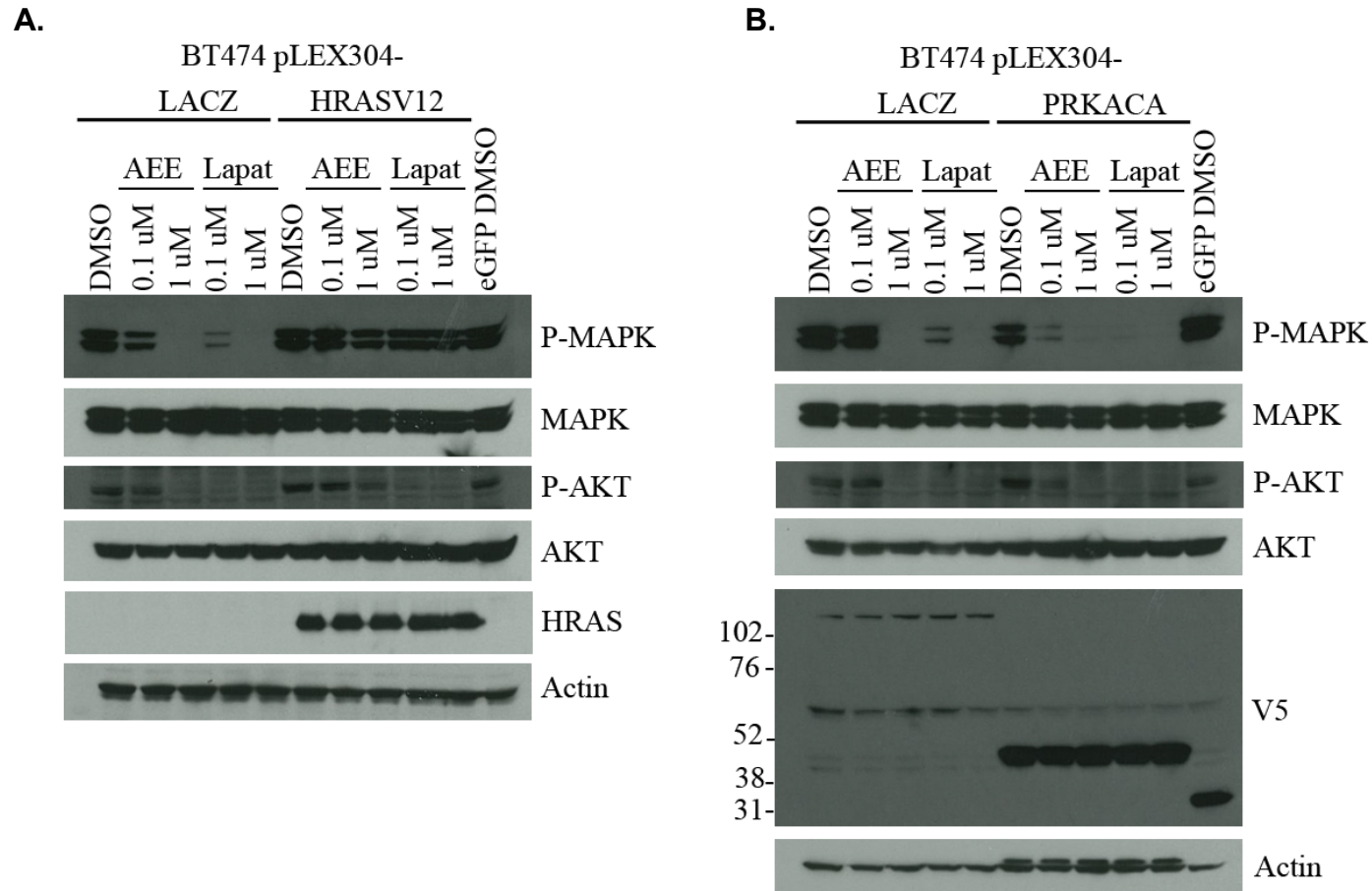


Figure 6 Effect of ORF expression on MAPK (ERK) and AKT activation in the context of AEE788 (AEE) or lapatinib (Lapat) in BT474 cells. **A.** HRASV12 expression restores MAPK activation in the setting of both AEE788 and lapatinib, and partially restores AKT activation in the presence of AEE788 (AEE). **B.** PRKACA expression does not detectably restore MAPK (ERK) or AKT phosphorylation in the context of AEE788 or lapatinib. LACZ, PRKACA, and eGFP are V5-tagged and are detected by the V5 antibody.