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14. ABSTRACT

Triple--negative breast cancer (TNBC) is a heterogeneous mix of cancers defined only by their lack of estrogen receptor expression, lack of progesterone receptor expression, and lack of amplification of the oncogene HER2. This lack of molecular etiology has limited our ability to rationally design therapies for TNBC, resulting in shorter relapse-free survival and a worse overall prognosis than other breast cancer patients. Here, we systematically screened various drug combinations, looking for those that were synergistic in killing triple--.negative breast cancer cells. Using targeted inhibition of oncogenic signaling pathways combined with chemotherapy, we report the surprising finding that time-staggered EGFR inhibition, but not simultaneous co-administration, can dramatically sensitize the apoptotic response of a subset of triple-negative cells to conventional DNA damaging agents. Only a subset of triple-negative cells (4 of 10) were sensitized by this combination, and importantly, these cells could not have been identified by their EGFR gene amplification.

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DNA damage response, systems biology, triple-negative breast cancer

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Table of Contents

INTRODUCTION

In many forms of breast cancer aberrant hormonal and/or growth factor signaling play key roles in both tumor induction and resistance to treatment (Hanahan and Weinberg, 2000). Moreover, the identification of specific molecular drivers in various breast cancer subtypes has led to the development of more efficacious forms of targeted therapy (Schechter et al., 1984; Slamon et al., 1987). In spite of these advances, however, there are currently no targeted therapies, and no established molecular etiologies, for triple-negative breast cancers (TNBC)—a heterogeneous mix of breast cancers defined only by the absence of estrogen receptor (ER) or progesterone receptor (PR) expression, and lack of amplification of the HER2 oncogene (human epidermal growth factor receptor homologue 2; ErbB2) (Perou et al., 2000). Patients with triple-negative breast cancers have shorter relapse-free survival and a worse overall prognosis than other breast cancer patients, however, they tend to respond, at least initially, to genotoxic chemotherapy (Dent et al., 2007). Triple-negative patients generally do well if pathologic complete response is achieved following chemotherapy. When residual disease exists, however, the prognosis is typically worse than for other breast cancer subtypes (Abeloff et al., 2008). Thus, identifying new strategies to enhance the initial chemosensitivity of TNBC cells may have substantial therapeutic benefit. We wondered whether a systems biology approach, specifically focused on examining and manipulating the interface between growth factor signaling pathways and DNA damage signaling pathways in tumor cells, could modulate the therapeutic response of this recalcitrant tumor type.

BODY

AIM1: Identification of optimal co-treatments of ErbB inhibitors with various DNA damaging agents

Our previous data suggested that a more comprehensive assessment of pathway inhibitor/genotoxin combination efficacy would be beneficial for identifying efficacious forms of therapy. For example, previous studies by others using cell culture models of TNBC reported that EGFR inhibitors in combination with genotoxic compounds such as cisplatin resulted in less than a 10% survival benefit (Corkery et al., 2009); while a randomized phase II trial in TNBC patients reported that addition of cetuximab (an anti-EGFR antibody marketed as Erbitux®) to carboplatin did not improve outcome (Carey et al., 2008). Our preliminary data, on the other hand, suggested that combination efficacy was extremely sensitive to the order of drug presentation (Figure 1). The first goal of this project was to test this notion across a large panel of drugs used in various combinations and also in a larger panel of cell lines, which better represent the heterogeneous triple-negative subclass.

TASK 1: Establish appropriate dose range for measuring death in breast cancer cells.

Before testing drugs in combinations, we first needed to identify doses of each drug that would result in a moderate level of cell death. For this portion of our screen we focused on classic chemotherapeutic compounds, as these are widely used to treat all forms of breast cancer clinically. Our drug panel consisted of 7 different genotoxic agents, each with a different mechanism of action: ionizing radiation (IR), camptothecin, cisplatin, etoposide, doxorubicin, temozolomide, and paclitaxel. The primary assays used for this experiment were a flow cytometry-based assay of apoptotic cell death and a commercial cell proliferation assay (Cell TiterGlo, Promega),

and the assays were completed in cell lines representing hormone-sensitive breast cancer (MCF7), HER2 over-expressing breast cancer (MDA-MB-453), and triple-negative breast cancer (BT-20). These cell lines were chosen because each has a wild-type p53 protein, allowing chemosensitivity between cell lines to be directly compared. After initially characterizing combination efficacy in these cell lines, we intended to expand to other cell lines with p53 mutations and/or deletions (see below), as these are commonly found in the patient population. In addition, as a control, the human osteosarcoma cell line, U2OS, was used because of its prototypical response to genotoxic agents, as well as our lab's long history characterizing the DDR in this cell line (Reinhardt et al., 2007). Based on these data, optimal doses were chosen for each of the genotoxins (Figure 2A-D). Based on proliferation data, BT-20 cells were generally the most sensitive to genotoxic therapy, while MCF7 cells were the least sensitive. A similar method was used to determine optimal doses of each pathway inhibitor. We tested 9 different inhibitors targeted to protein in ErbB receptor signaling pathways. The final list, as shown in Figure 3A, included erlotinib (Tarceva), gefitinib (Iressa), lapatinib, MM-121, PD-98059, BMS-345541, rapamycin, NVP-BEZ235, and wortmannin. In general, these 9 pathway inhibitors did not induce cell death in any of our representative cell lines. Those drugs targeted to EGFR/ErbB1 (erlotinib, gefitinib, lapatinib), however, did reduce cell proliferation rates in all cell lines tested.

TASK 2: Test Co-treatments of DNA damaging chemotherapy with ErbB inhibitors.

Our preliminary data suggested that the precise timing of drug presentation could be a critical factor in combination efficacy. Here, we wanted to systematically analyze the extent to which this is true across a variety of drug combinations. Initially, we tested combinations of erlotinib and doxorubicin in BT-20 cells, to determine the optimal

lengths of time staggering and how these responses different between cancer subtypes (Figure 1 and Figure 4). We found that pre-treatment of BT-20 cells with erlotinib enhanced sensitivity to doxorubicin, and this effect was optimal when erlotinib was given 24 hours before doxorubicin. Conversely, erlotinib given after doxorubicin slightly desensitized the cells to doxorubicin, and this effect was optimal when erlotinib was given 4 hours after the genotoxic agent. These established conditions were used for our combination drug screen.

The data from our screen are summarized in Figure 5. Data were collected for both proliferation and apoptosis (cleaved-caspase-3 and cleaved-PARP double positive cells as measured by flow cytometry). Percent inhibition of proliferation and percent apoptosis tended to be very similar, suggesting that the primary means of cell death in response to the combinations tested was apoptotic. In general, we found that the order of drug presentation was a strong factor for only a small subset of the combinations. Synergistically enhanced cell death was observed only for combinations in BT-20 cells involving inhibition with erlotinib, gefitinib, lapatinib, or BMS-345441. The first three of these drugs all inhibit EGFR/ErbB1, and BMS-345441 is an inhibitor of NF-KB, a well-known pro-survival signal. In our view, it is not surprising that inhibiting NF-KB would result in greater cell death, but it is certainly notable that EGFR inhibition consistently enhanced cell death in BT-20 cells. Furthermore, this was also true when EGFR was knocked down using siRNA (Figure 6). Another surprise was that EGFR inhibition synergistically enhanced cell death across nearly all classes of genotoxin tested, with the only exception being the mitotic poison, taxol (paclitaxel). We were somewhat surprised to find such phenotypic specificity from the perspective of the inhibitors (with essentially only EGFR inhibitors resulting in time-staggered synergy), but such phenotypic promiscuity from the perspective of the genotoxins. Speculatively, we

suspect that this is likely the result of the oncogenic status of BT-20, and potentially other triple negative breast cancer cells. Although the driving oncogenes for this subtype are not currently known, this certainly warrants further study, and will also be discussed later in this report.

One fact in support of this notion was the sensitivity of HER2 over-expressing cells to various drug combinations. Whereas the molecular etiology of TNBC cells are not known, HER2 over-expressing cells are typically driven by HER2 signaling, and are typically sensitive to HER2 inhibition (Neve et al., 2006; Sorlie et al., 2001; Sorlie et al., 2003). When HER2 over-expressing MDA-MB-453 cells were treated with EGFR inhibitors like erlotinib, we did not observe enhanced sensitivity to DNA damaging agents. In fact (for reasons that are currently unclear), these cells were typically desensitized to DNA damage when pre-treated with erlotinib (Figures 4 and 7). However, when we inhibited HER2 in HER2-driven cells, we consistently observed enhanced sensitivity to genotoxic agents, similar to what was observed for BT-20 cells treated with erlotinib (Figure 7). These data may suggest that BT-20 cells are driven by EGFR signaling, and we intend to explore this more completely in the future.

We also monitored cell cycle progression and autophagy, in addition to apoptosis and proliferation. Our initial rationale for this was to capture any non-apoptotic cell death that may be occurring. As mentioned above, the mechanism of cell death in these cells treated with genotoxic agents tended to be apoptotic. Furthermore, at the doses tested, death was observed before significant changes in cell cycle status had taken effect (FIG). Nonetheless, these data are included in Figure 8. Although autophagy does not appear to contribute to death in these cells, genotoxic therapy does in some cases cause activation of autophagy (Figure 8). Determining how this contributes to the death/survival of these cells will be the subject of future study.

Following our screen, we found that the most efficacious combinations in BT-20 cells were those that inhibited EGFR 24 hours prior to the addition of a genotoxic compound. Because triple-negative breast cancer is a notably heterogeneous disease, we next wanted to test whether the efficacy of time-staggered erlotinib→doxorubicin treatment was unique to BT-20 cells or potentially a more general phenomenon of triple-negative breast cells. To answer this question we tested a handful of other widely available triple-negative cell lines (Neve et al., 2006). Unlike our original panel of cells, these selected cell lines have markedly different growth rates, EGFR expression levels, and even p53 states (Figure 9). Despite these differences, sustained EGFR inhibition enhanced sensitivity to doxorubicin in 9 of 10 triple-negative cell lines tested but a synergistic effect was observed in only 4 of the 10 TNBC lines (Figures 9 and 10). Interestingly, although there was no correlation between EGFR expression and sensitivity to erlotinib-based combinations, we found a very strong correlation between EGFR activity (i.e. EGFR phosphorylation on Y1173) and the level of sensitivity to the erlotinib-doxorubicin combination (Figure 10). Although the role of EGFR in triple-negative breast cancer had previously received a fair amount of attention, this was likely missed because 1) prior attempts to identify driving oncogenes relied only on measuring expression levels, not activity levels, and 2) EGFR targeted therapies had failed both in the pre-clinical and clinical setting. Our data, generated using a more systematic combination design as well as protein activity measurements, suggest that a significant subset of triple-negative cells may be driven by EGFR signaling. Testing this hypothesis, and also identifying reasons for the heterogeneity will be the focus of some of our future efforts.

AIM 2: Interrogate the integrated EGFR-DNA damage network in breast cancer cells following Tarceva-Doxorubicin treatment using a variety of high-throughput techniques.

TASK 1: Screen antibodies for use in protein lysate microarrays

From the screen performed in Aim 1 of this proposal, we've identified a synergistic drug combination that is dependent on the order of drug presentation. Here, we want determine the mechanism of this sensitivity, and our goal is to measure a variety of signaling events in the EGFR and DNA damage pathways, and eventually (as detailed in Aim 3) utilize mathematical modeling to provide insight into what changes are mediating the observed phenotypes. A critical factor in our ability to achieve this goal will be the availability of high-throughput techniques for measuring protein signaling activities. After previously evaluating a number of techniques, we've decided to pursue reverse phase protein lysate microarray (RPMA) (MacBeath, 2002; Sevecka and MacBeath, 2006). As is the case with many antibody based measurements, the success of this method depends largely on the availability of high fidelity antibodies, so our first task was to test antibodies to potential targets of interest.

Antibody validation began with the creation of 90 control lysates, which collectively were meant to function as "positive" and "negative" controls for all targets of interest. These lysates were printed on glass-backed nitrocellulose slides, and probed using established methods (MacBeath, 2002; Sevecka and MacBeath, 2006). Antibodies that showed greater than a 1.5-fold change between positive control and negative control conditions were considered candidates. Because RPMA is essentially a "dot blot," the signal emitted is likely to result from some combination of on-target and off-target antibody binding. Thus, candidate antibodies were then cross-examined in traditional Western blot format. Antibodies were considered "validated" if the

quantitative fold change between conditions were similar for RPMA and Western blot formats. The process is then completed for each cell line of interest, as antibody fidelity tends to be very cell line specific (Figure 11).

In total, we screened over 1000 antibodies to over 200 targets of interest in each BT-20, MCF7, and MDA-MB-453 cell lines. The full list of targets that we investigated is shown in Figure 12. In total, we were able to validate 24 antibodies for use in our analysis, which included antibodies targeting: B-RAF, DNA-PKcs, mTOR, phospho-4E-BP1, phospho-p38, phospho-53BP1, phospho-AKT, phospho-BRCA1, phospho-CDC25C, phospho-Cyclin E, phospho-ERK, phospho-H2AX, phospho-Histone H3, phospho-Hsp27, phospho-JNK, phospho-MEK, phospho-MK2, phospho-p90RSK, phospho-S6, phospho-S6K, phospho-SEK1, phospho-Wee1, and phospho-CHK2. The list of antibodies is included in the supporting data as Table 1.

Our list of 24 RPMA validated targets is substantial, however, there are a significant number of important targets for which RPMA-compatible antibodies were not found. For example, p53, a central player in mediating DNA damage signaling, could not be probed using the RPMA technology. As a complementary technique to RPMA, we had previously suggested using some combination of *in vitro* kinase assays or high-throughput Western blots (available through Invitrogen, sold as the iBLOT/ePAGE system). Based on considerations such as cost, time, and data quality, we have decided to use the Invitrogen high-throughput Western blot system to cover targets of interest that could not be probed using RPMA. One important note is that our preliminary data show quantitative similarity between the data produced using these two techniques (Figure 13).

KEY ACCOMPLISHMENTS

- Determined optimal drug combinations for a subset triple-negative breast cancer cells.
- Characterized a subset of triple-negative breast cancer cells as being oncogenically driven by EGFR signaling.
- Optimized Reverse Phase Protein Lysate Microarray for the study of EGFR signaling and DNA damage signaling.

REPORTABLE OUTCOMES

- Manuscript in preparation
- Invited to present work at Merrimack Pharmaceuticals (Cambridge, MA)
- Invited to speak about this work at AACR Conference on Systems Biology of Cancer: Confronting the Complexities of Cancer (San Diego, CA)

CONCLUSION

Triple-negative breast cancers continue to be the most aggressive and most poorly treated of the major breast cancer subtypes. In this early stage of this study, we aimed to identify drug combinations that could effectively kill triple-negative cells, and to move towards an understanding of why some treatments worked while others failed. Our goal was to use this information to better our understanding of the molecular nature of TNBC, and to drive the production of better treatment options for this disease. In this report, we present data for our systematic screen of drugs in the ErbB signaling network combined with traditional genotoxic chemotherapy compounds. We report the surprising finding that time-staggered EGFR inhibition, but not simultaneous co-administration, can dramatically sensitize the apoptotic response of a subset of triple-negative cells to

conventional DNA damaging agents. Only a subset of triple-negative cells (4 of 10) were sensitized by this combination, and importantly, these cells could not have been identified by their EGFR gene amplification status, mutation status, or EGFR protein expression level, but only by the level of phosphorylated (i.e. activated) EGFR protein. Our data suggest that EGFR inhibitors should be re-evaluated in the triple-negative setting, particularly if patient EGFR phosphorylation status can be determined. Future portions of this work, as previously outlined in the approved statement of work, can be completed as proposed, with our future efforts focusing on collecting a large dataset of signaling responses, and using computational approaches to analyze the dataset.

From a patient perspective, this work has should impact patient care in two ways. 1) Our data suggest that some TNBCs are oncogenically driven by EGFR, and that these cancers may be sensitive to EGFR targeted therapies. As these drugs are already approved for use in other contexts, triple-negative patients could see benefit in the relatively near future. 2) Phosphorylated-EGFR, but not total EGFR, could be a reliable biomarker for identifying patients who could see benefit from these therapies. This data suggests that we need to collect information about protein activities, instead of just protein abundance, and should change how we think about “personalized medicine.”

REFERENCES

Abeloff, M., Wolff, A., Weber, B., Zaks, T., Sacchini, V., and McCormick, B. (2008). Cancer of the breast. In Abeloff's Clinical Oncology, M. Abeloff, J. Armitage, J. Niederhuber, M. Kastan, and W. McKenna, eds. (Maryland Heights, MO, Churchill Livingstone).

Carey, L., Rugo, H., Marcom, P., Irvin, W., Ferraro, M., Burrows, E., He, X., Perou, C., and Winer, E. (2008). TBCRC 001: EGFR inhibition with cetuximab added to carboplatin in metastatic triple-negative (basal-like) breast cancer. J Clin Oncol *26*.

Chou, T.C., and Talalay, P. (1984). Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul *22*, 27-55.

Corkery, B., Crown, J., Clynes, M., and O'Donovan, N. (2009). Epidermal growth factor receptor as a potential therapeutic target in triple-negative breast cancer. Ann Oncol *20*, 862-867.

Dent, R., Trudeau, M., Pritchard, K.I., Hanna, W.M., Kahn, H.K., Sawka, C.A., Lickley, L.A., Rawlinson, E., Sun, P., and Narod, S.A. (2007). Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res *13*, 4429-4434.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

MacBeath, G. (2002). Protein microarrays and proteomics. Nat Genet *32 Suppl*, 526-532.

Neve, R.M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F.L., Fevr, T., Clark, L., Bayani, N., Coppe, J.P., Tong, F.*, et al.* (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell *10*, 515-527.

Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A.*, et al.* (2000). Molecular portraits of human breast tumours. Nature *406*, 747-752.

Reinhardt, H.C., Aslanian, A.S., Lees, J.A., and Yaffe, M.B. (2007). p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage. Cancer Cell *11*, 175-189.

Schechter, A.L., Stern, D.F., Vaidyanathan, L., Decker, S.J., Drebin, J.A., Greene, M.I., and Weinberg, R.A. (1984). The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. Nature *312*, 513-516.

Sevecka, M., and MacBeath, G. (2006). State-based discovery: a multidimensional screen for small-molecule modulators of EGF signaling. Nat Methods *3*, 825-831.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., and McGuire, W.L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science *235*, 177-182.

Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S.*, et al.* (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A *98*, 10869-10874.

Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J.S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S.*, et al.* (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A *100*, 8418-8423.

Tallarida, R.J. (2002). The interaction index: a measure of drug synergism. Pain *98*, 163-168.

APPENDIX (updated CV)

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2003 - 2008 Ph.D. Pharmacology, University of North Carolina at Chapel Hill
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2008 - present Postdoctoral Fellow, Koch Institute for Integrative Cancer Research at MIT
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Research Experience

2008 - present Postdoctoral Associate, Koch Institute for Integrative Cancer Research, MIT
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2003 - 2008 Doctoral Student, University of North Carolina at Chapel Hill
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1998 - 2000 Undergraduate Research Intern, University of Washington
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Awards, Honors, and Fellowships

2010 - present Department of Defense Breast Cancer Research Program
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2009 - present Faculty of 1000 Associate Faculty Member

2007 - 2008 Ruth L. Kirschstein national Research Service Awards
Predoctoral Fellowship (F31), National Institutes of Mental Health

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2004 - 2006	Cell and Molecular Biology Predoctoral Fellowship CMB Program, University of North Carolina at Chapel Hill
2005	CMB Collaborative Research Award CMB Program, University of North Carolina at Chapel Hill
2005	National Science Foundation Predoctoral Fellowship Honorable Mention
1997-2002	DeEtte McAuslan Stuart Scholarship for Biological Sciences University of Washington

Teaching and Mentoring Experience

2004 - 2010	Rotation Mentor. Advised 13 rotation students and/or undergraduate students at MIT and UNC, including 4 student participants in the Initiative for Maximizing Student Diversity at UNC
2007	Course Instructor: Techniques in Biochemistry Initiative for Maximizing Student Diversity, University of North Carolina
2006	Student Instructor: Introduction to Neurobiology. Department of Neurobiology, University of North Carolina at Chapel Hill
2004	Discussion Leader: Cell 117 (Introduction to Cell biology) Department of Cell Biology, University of North Carolina at Chapel Hill
2000 - 2001	Teacher's Assistant: Introduction to Statistical Theory/Applied Statistics. Department of Psychology, University of Washington
1999 - 2001	Peer Instructor: Freshman Interest Group (Biological Sciences). New Student Programs, University of Washington

Invited Lectures

July 15, 2011	"New approaches for using network biology to rationally design combination therapies for cancer treatment." Merrimack Pharmaceuticals. Cambridge, MA
March 2, 2011	"Dynamic re-wiring of apoptotic signaling networks enhances tumor cell killing by DNA damage." AACR-NCI Conference on Systems Biology: Confronting the Complexity of Cancer. San Diego, CA.
July 22, 2010	"Systems Level Analysis of EGFR Inhibition-DNA Damage Combination Treatments in Breast Cancer." Integrative Cancer Biology Program Annual Meeting. Dedham, MA.

June 15, 2007 "Co-activation of G protein signaling by cell surface receptors and an intracellular exchange factor." Gordon Research Conference for Protein Phosphorylation and G protein signaling networks. Biddeford, ME.

Other Presentations

1. **Lee, M.J.,** A.S. Ye, G. MacBeath, and M.B. Yaffe. "Dynamic re-wiring of apoptotic signaling networks enhances tumor cell killing by DNA damage." Poster presented at Era of Hope: Annual Department of Defense Breast Cancer Research Program Meeting. Orlando, FL, March 2011.

2. **Lee, M.J.,** A. Ye, G. MacBeath, and M.B. Yaffe. "Systems Level Analysis of EGFR Inhibition-DNA Damage Combination Treatments in Breast Cancer." Poster presented at International Conference for Systems Biology for Human Disease, June 2010.

3. **Lee, M.J.** and Dohlman, H.G. "Activation of an endosomal G alpha subunit by a non-GPCR GEF." Poster presented at the Gordon Research Conference for Protein phosphorylation and G protein signaling networks, June 2006.

4. **Lee, M.J.** and Dohlman, H.G. "Localization and trafficking of the unmyristoylated yeast G alpha subunit, Gpa1." Poster presented at the American Society for Cell Biology Annual Conference, December 2004.

Research Publications

1. Torres, M.P.*, **M.J. Lee***, F. Ding, C. Purbeck, B. Kuhlman, N.V. Dokholyan, and H.G Dohlman. (2009) G protein mono-ubiquitination by the Rsp5 ubiquitin ligase. *J Biol Chem. 13:* 8940-50.
   *** Authors contributed equally to this work**

2. **Lee, M.J.** and H.G. Dohlman. (2008) Co-Activation of G protein Signaling by Cell-Surface Receptors and an Intracellular Exchange-Factor. *Current Biology*. *18,* 211-215.

3. Wang, Y., L.A. Marotti, **M.J. Lee**, and H.G. Dohlman. (2005) Differential regulation of G protein alpha subunit trafficking by mono- and poly-ubiquitination. *J Biol Chem. 280,* 284-291.

4. **Lee, M.J.,** A.S. Ye, A.K. Gardino, A.M. Heijink, P.K. Sorger, G. MacBeath, and M.B. Yaffe. Dynamic re-wiring of apoptotic signaling networks enhances tumor cell killing by DNA damage *(in review)*

5. Floyd, S.R., M.E. Pacold, S.M. Clarke, E.J. Blake, A. Fydrych, **M.J. Lee,** S.E. Silver, D.E. Root, W.C. Hahn, A.E. Carpenter, D.M. Sabatini, C.A. French, J.E. Bradner, and M.B. Yaffe. The Acetyl-Lysine Binding Protein Brd4 is an Endogenous Suppressor of the DNA Damage Response (*in review*)

6. Tentner, A.R., G.J. Ostheimer, **M.J. Lee**, L.D. Samson, D.A. Lauffenburger, and M.B. Yaffe. Combined Experimental and Computational Analysis of DNA Damage Signaling Reveals Context-Dependent Roles for Erk in Apoptosis and G1/S Arrest after Genotoxic Stress *(in review)*

Review Articles and Book Chapters

1. **Lee, M.J.** and M.B. Yaffe. Chapter 2: Protein Regulation. *Signal Transduction. (in preparation).*

2. **Lee, M.J.** and H.G. Dohlman. Development of a cell viability assay to monitor for palmitoylation of the yeast G alpha subunit Gpa1. (*in preparation*).

3. **Lee, M.J.** and H.G. Dohlman. $Gpa1^{N388D}$: A novel tool for identifying non-receptor GEFs for G alpha subunits. (*in preparation*).

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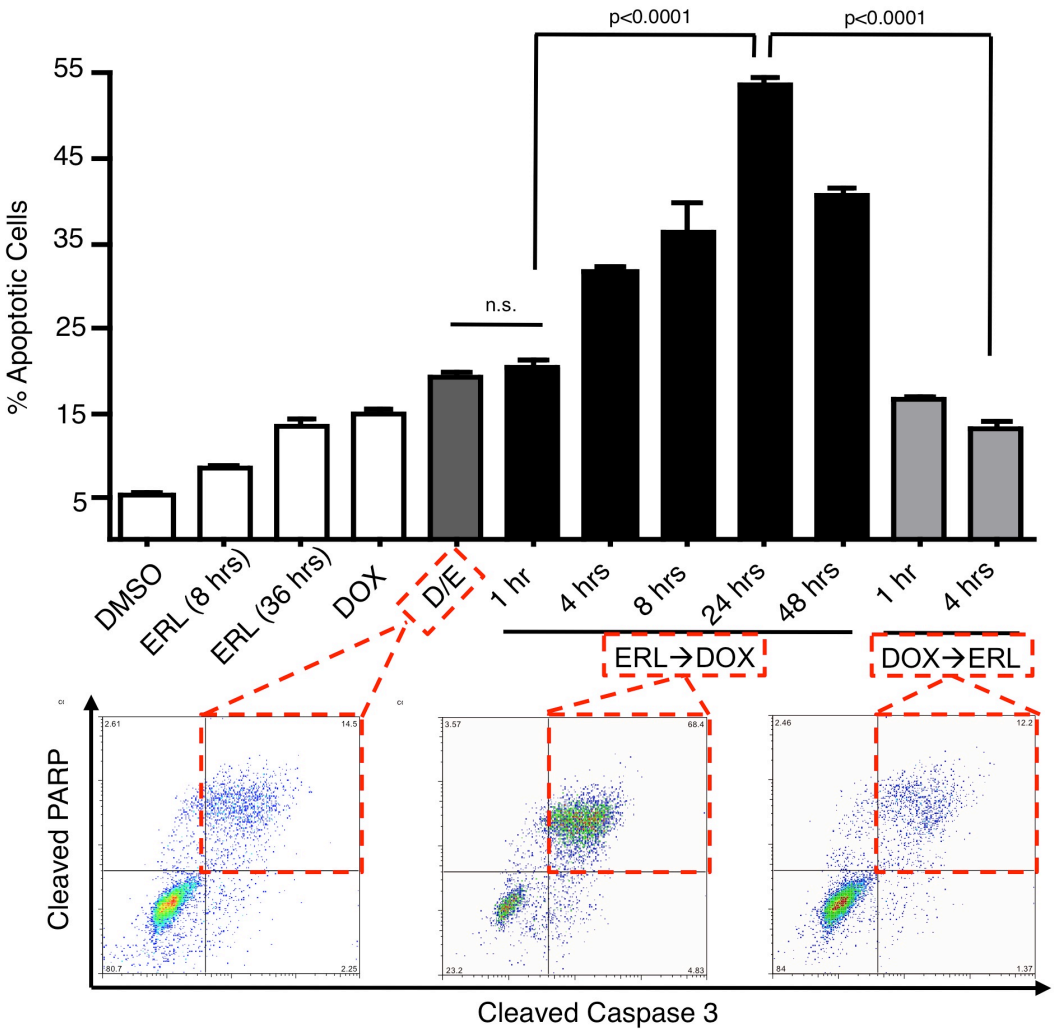
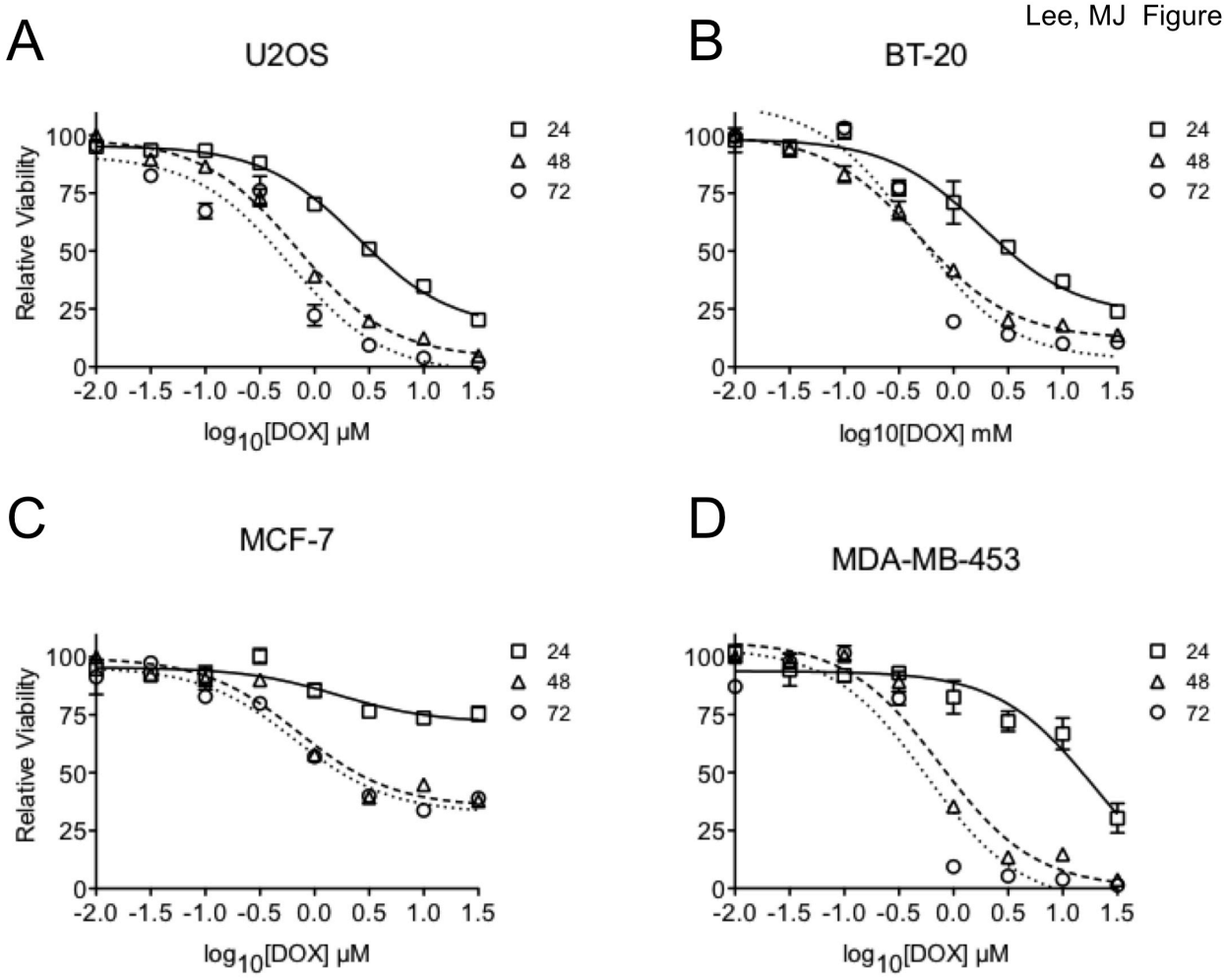
SUPPORTING DATA

Lee, MJ Figure 1

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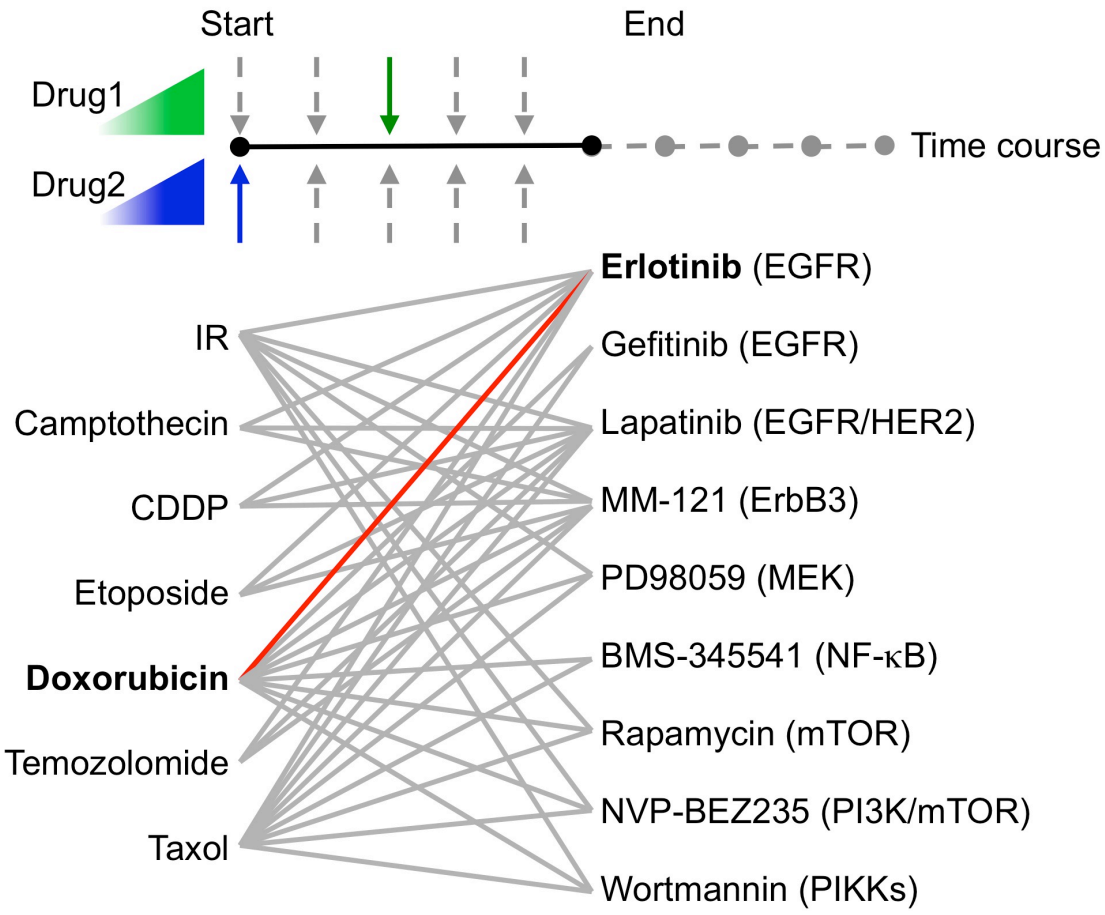
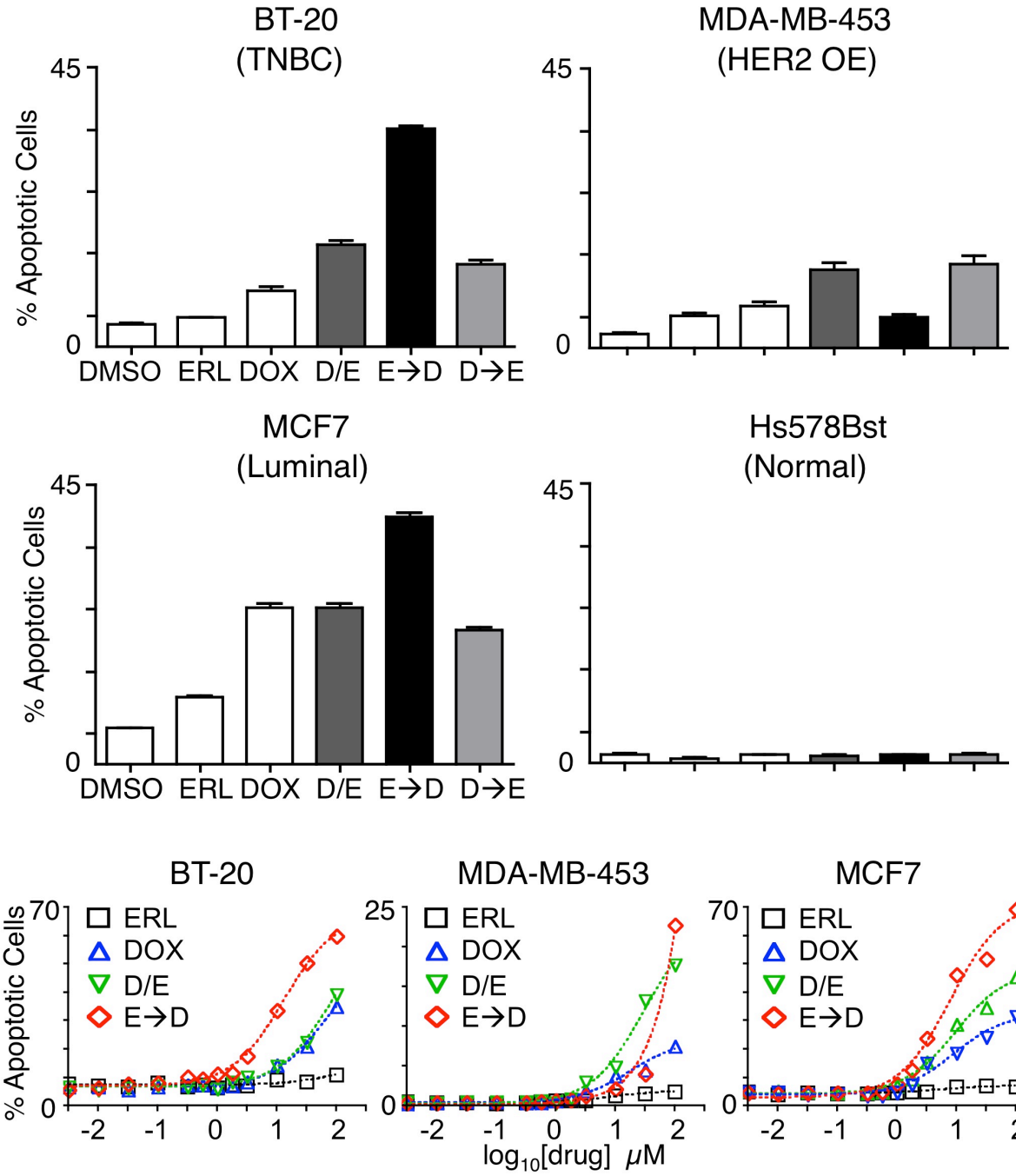
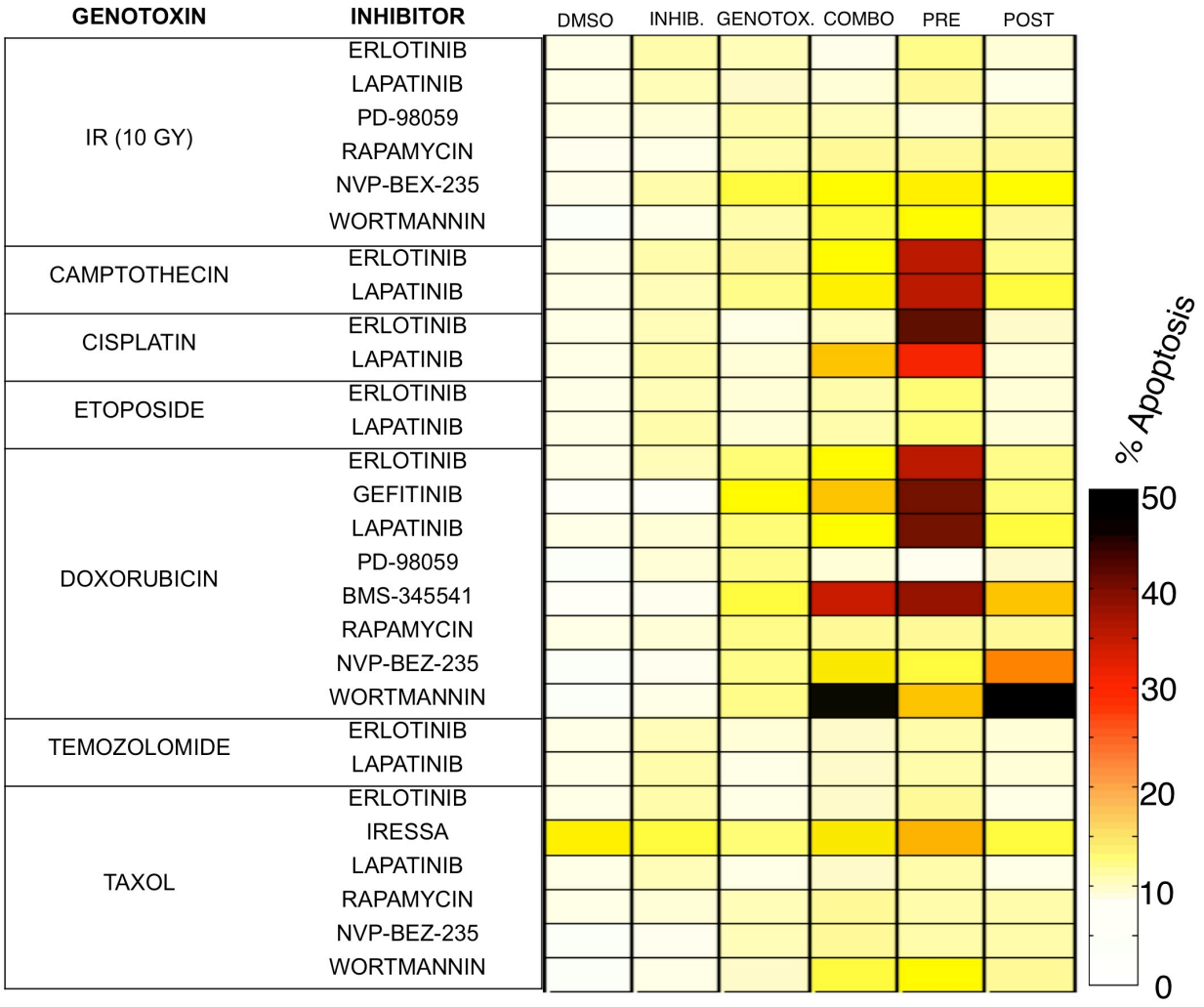
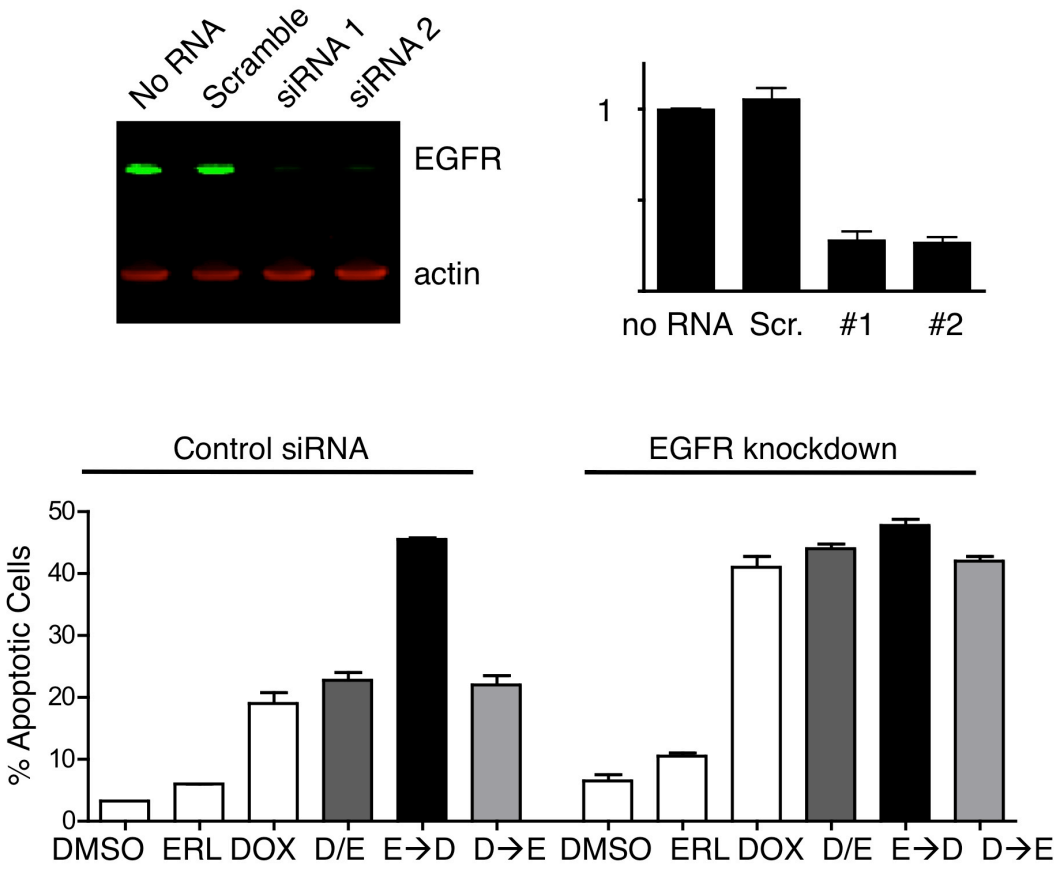
BT-20
log10[DOX] mM
C
MCF-7
D
MDA-MB-453

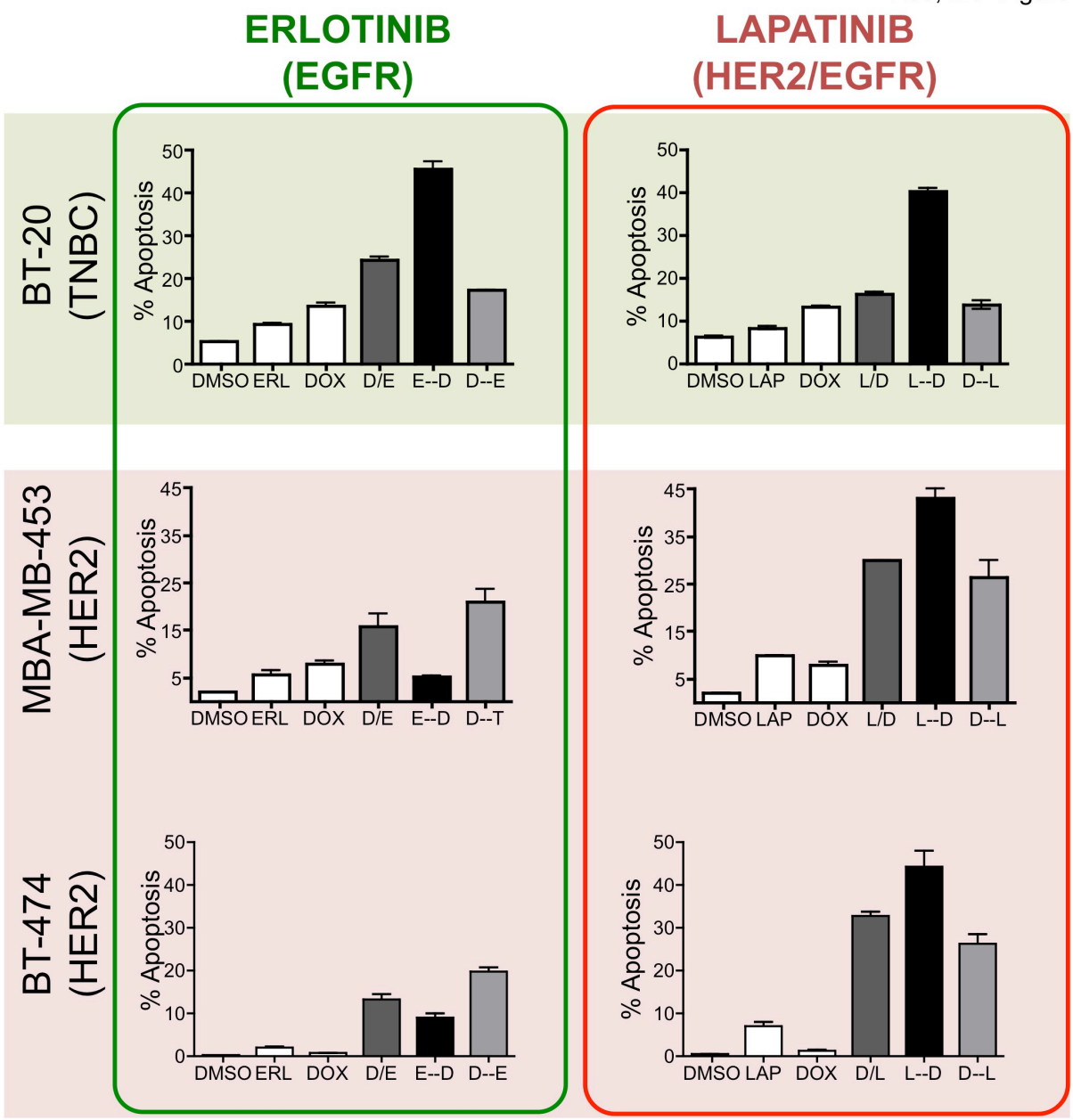

Start
End
Drug1
Drug2
Time course
IR
Camptothecin
CDDP
Etoposide
Doxorubicin
Temozolomide
Taxol
Erlotinib (EGFR)
Gefitinib (EGFR)
Lapatinib (EGFR/HER2)
MM-121 (ErbB3)
PD98059 (MEK)
BMS-345541 (NF-κB)
Rapamycin (mTOR)
NVP-BEZ235 (PI3K/mTOR)
Wortmannin (PIKKs)

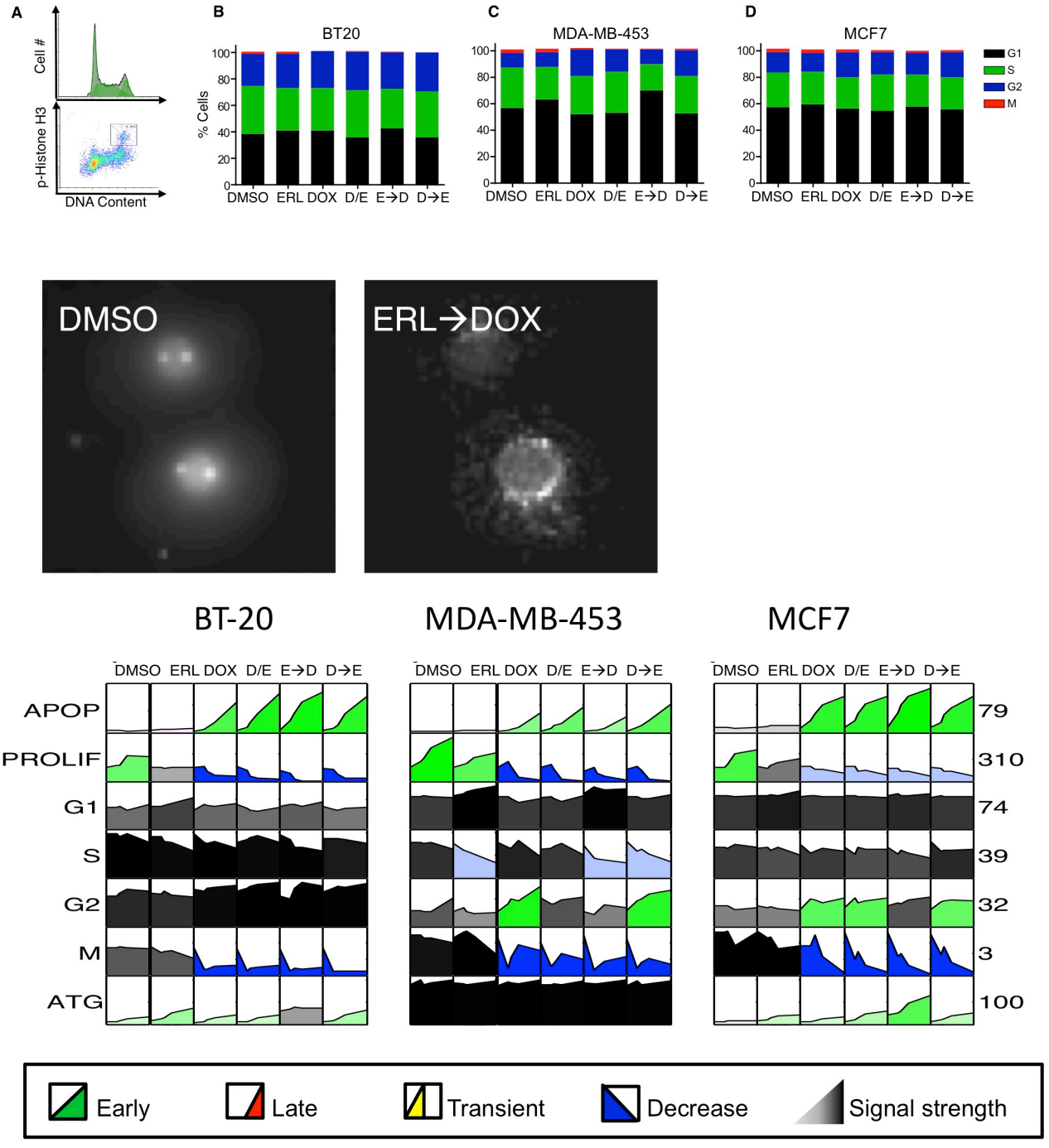


	ERL EC_{50}	DOX EC_{50}	D/E EC_{50}	E→D EC_{50}	D/E CI	E→D CI
BT-20	n/a	63.68	74.94	13.33	**1.18**	**0.21**
M-453	n/a	18.15	17.78	62.36	**0.98**	**3.34**
MCF7	n/a	9.90	9.05	8.08	**0.91**	**0.82**

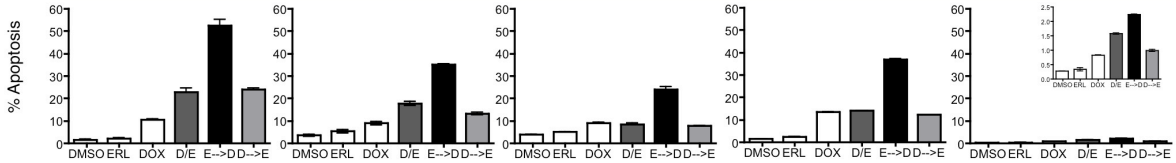

GENOTOXIN
INHIBITOR
DMSO
INHIB.
GENOTOX.
COMBO
PRE
POST
IR (10 GY)
ERLOTINIB
LAPATINIB
PD-98059
RAPAMYCIN
NVP-BEX-235
WORTMANNIN
CAMPTOTHECIN
ERLOTINIB
LAPATINIB
CISPLATIN
ERLOTINIB
LAPATINIB
ETOPOSIDE
ERLOTINIB
LAPATINIB
DOXORUBICIN
ERLOTINIB
GEFITINIB
LAPATINIB
PD-98059
BMS-345541
RAPAMYCIN
NVP-BEZ-235
WORTMANNIN
TEMOZOLOMIDE
ERLOTINIB
LAPATINIB
TAXOL
ERLOTINIB
IRESSA
LAPATINIB
RAPAMYCIN
NVP-BEZ-235
WORTMANNIN
% Apoptosis
50
40
30
20
10
0


No RNA
Scramble
siRNA 1
siRNA 2
EGFR
actin
1
no RNA
Scr.
#1
#2
Control siRNA
EGFR knockdown
% Apoptotic Cells
50
40
30
20
10
0
DMSO
ERL
DOX
D/E
E→D
D→E
DMSO
ERL
DOX
D/E
E→D
D→E

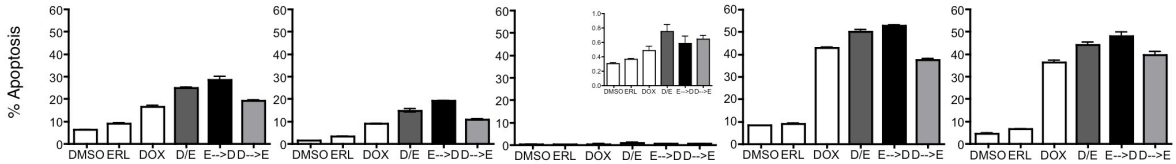

ERLOTINIB
(EGFR)
LAPATINIB
(HER2/EGFR)
BT-20
(TNBC)
MBA-MB-453
(HER2)
BT-474
(HER2)
% Apoptosis
DMSO ERL DOX D/E E--D D--E
DMSO LAP DOX L/D L--D D--L
DMSO ERL DOX D/E E--D D--T
DMSO LAP DOX L/D L--D D--L
DMSO ERL DOX D/E E--D D--E
DMSO LAP DOX D/L L--D D--L


A
Cell #
p-Histone H3
DNA Content
B
BT20
% Cells
DMSO ERL DOX D/E E→D D→E
C
MDA-MB-453
DMSO ERL DOX D/E E→D D→E
D
MCF7
DMSO ERL DOX D/E E→D D→E
G1
S
G2
M
DMSO
ERL→DOX
BT-20
MDA-MB-453
MCF7
DMSO ERL DOX D/E E→D D→E
APOP
PROLIF
G1
S
G2
M
ATG
79
310
74
39
32
3
100
Early
Late
Transient
Decrease
Signal strength

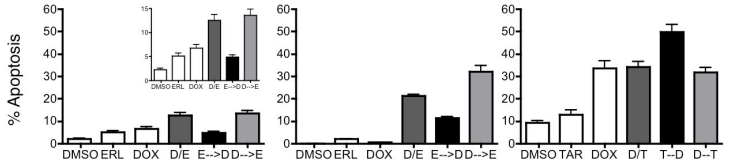
	MDA-MB-468	BT-20	HCC-1143	Hs-578T	MDA-MB-231
SUBTYPE	TNBC (Basal A)	TNBC (Basal A)	TNBC (Basal A)	TNBC (Basal B)	TNBC (Basal B)
p53 (protein)	+/+ (R273H)	+/+ (WT)	+/+ (WT)	+/m (V157F)	+/GOF (R280K)
EGFR	100 (n/a)	25.09 (3011)	59.79 (7175)	28.78 (3454)	49.95 (5994)
p-EGFR	100	67.85	28.18	17.68	17.13

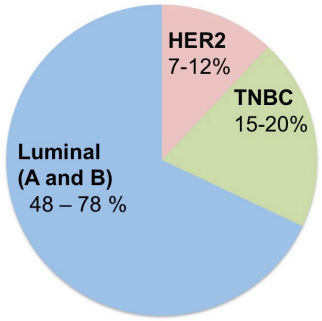


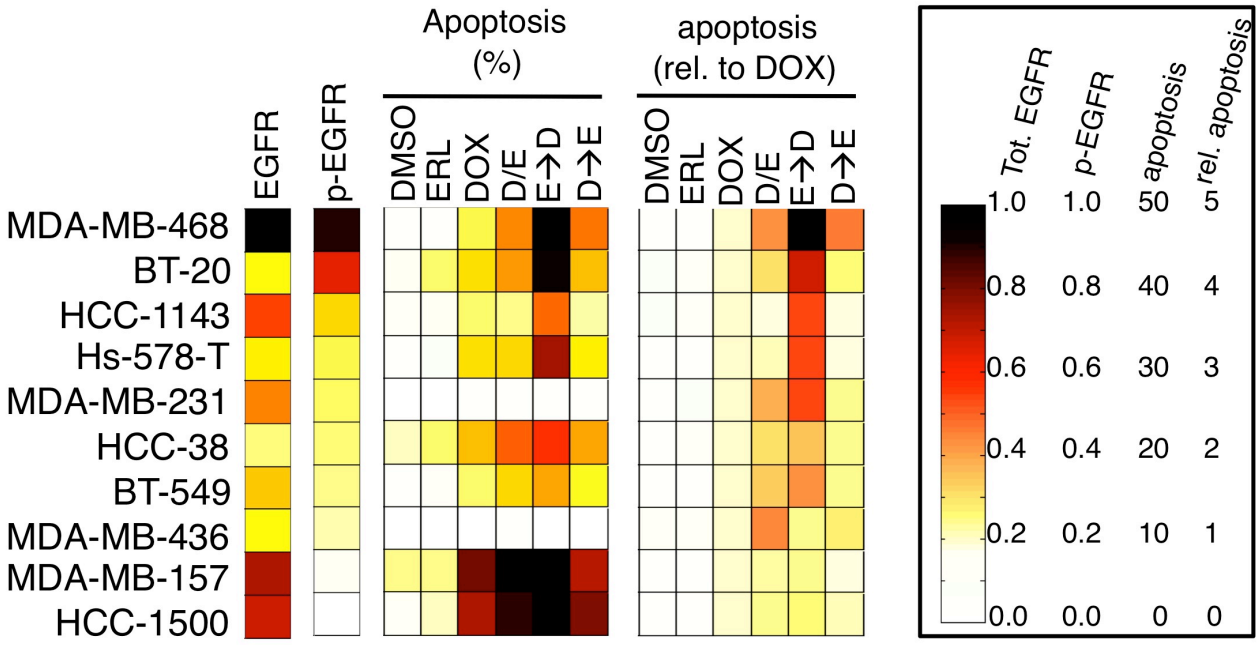
	HCC-38	BT-549	MDA-MB-436	MDA-MB-157	HCC-1500
SUBTYPE	TNBC (Basal B)	TNBC (Basal B)	TNBC (Basal B)	TNBC (Basal B)	TNBC (Basal B)
p53 (protein)	+/m (R273L)	+/m (R249S)	-/-	-/m	+/- (WT)
EGFR	18.68 (2241)	36.21 (4345)	25.50 (n/a)	75.28 (9034)	35.56 (8737)
p-EGFR	15.47	12.71	8.84	2.76	0.00

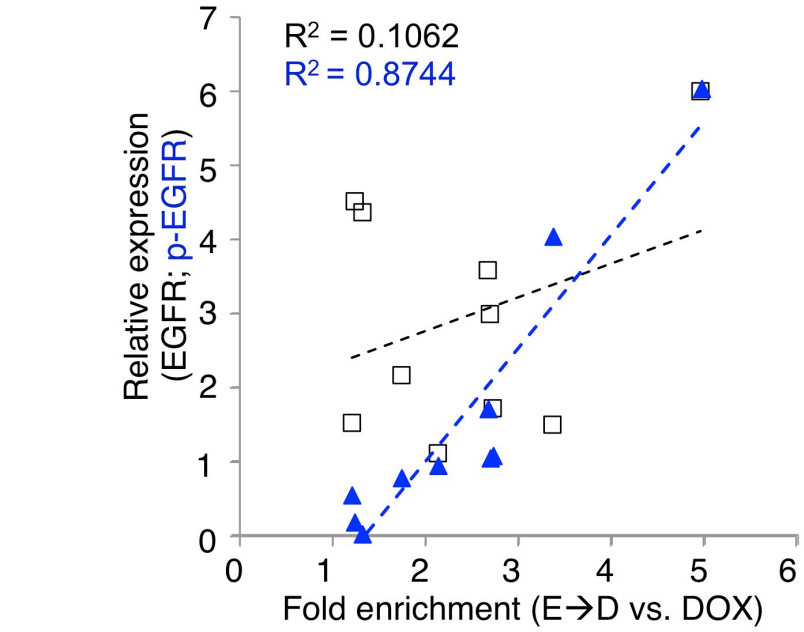


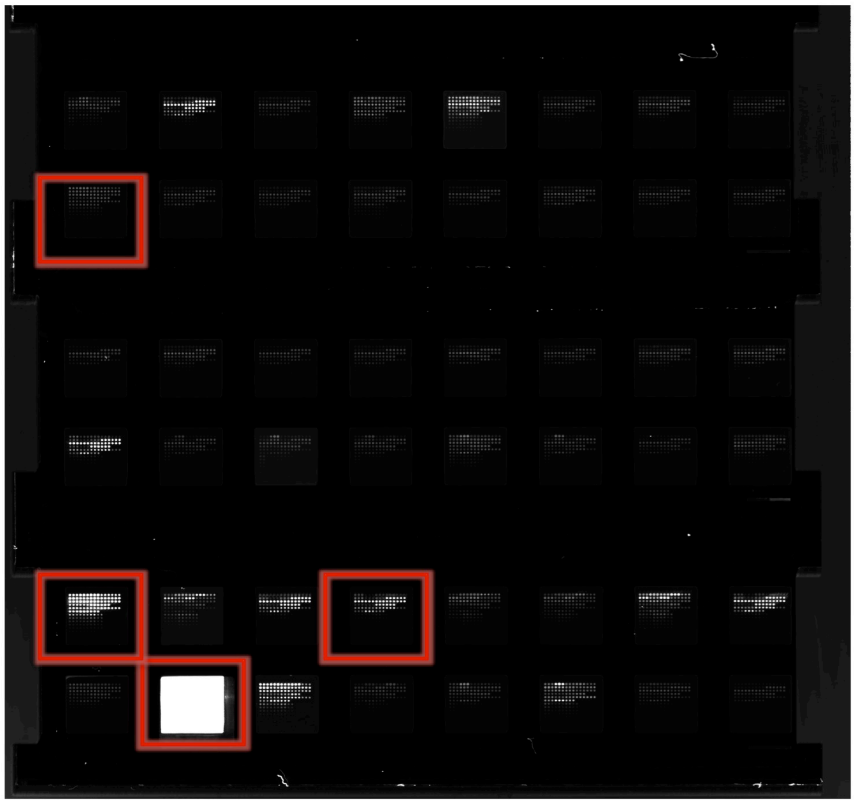
	MDA-MB-453	BT-474	MCF-7
SUBTYPE	HER2	HER2	Luminal
p53 (protein)	+/- (WT)	+/+ (E285K)	+/- (WT)
EGFR	0.75 (239)	34.23 (4107)	1.99 (90)
p-EGFR	0.00	0.00	0.00

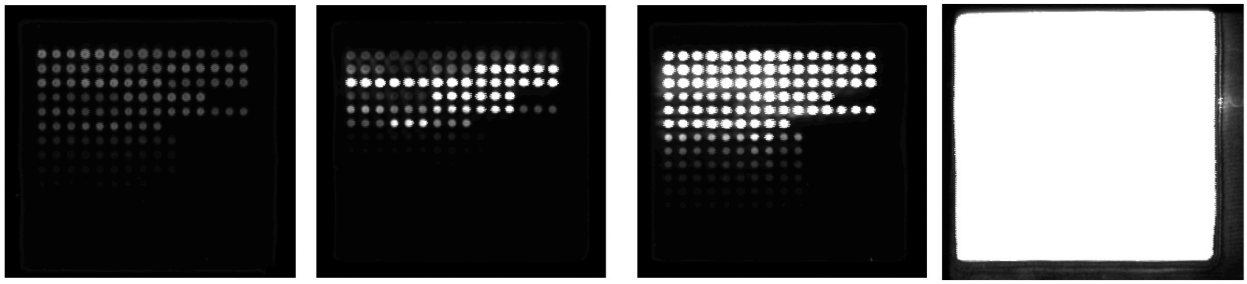


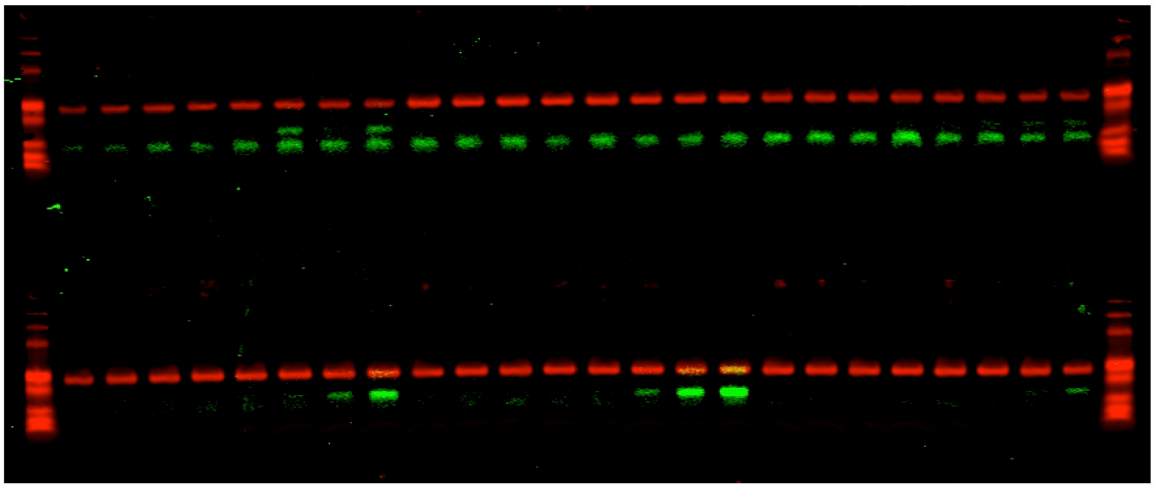


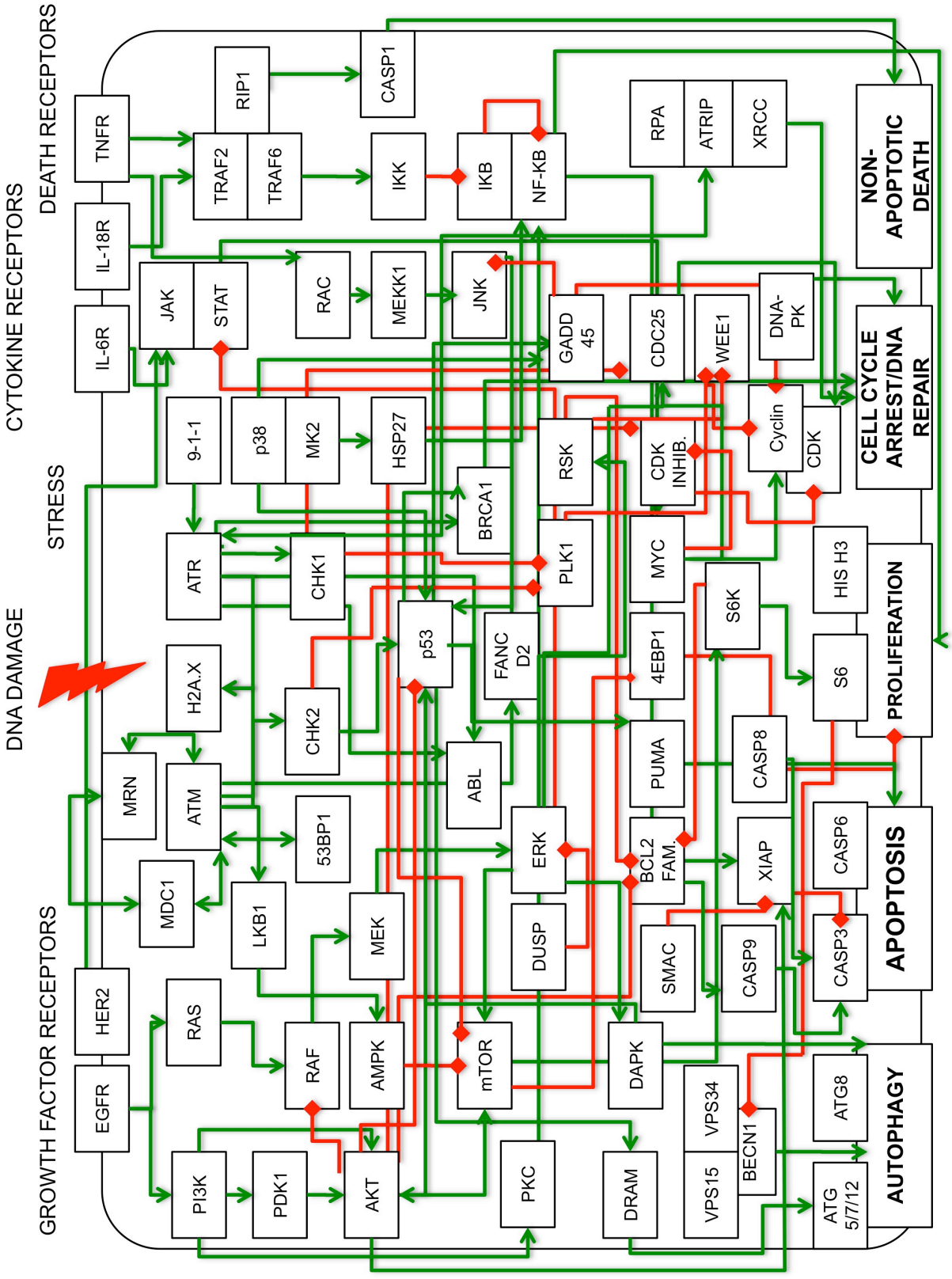

Apoptosis (%)
apoptosis (rel. to DOX)
EGFR
p-EGFR
DMSO
ERL
DOX
D/E
E→D
D→E
MDA-MB-468
BT-20
HCC-1143
Hs-578-T
MDA-MB-231
HCC-38
BT-549
MDA-MB-436
MDA-MB-157
HCC-1500
Tot. EGFR
p-EGFR
apoptosis
rel. apoptosis
1.0 1.0 50 5
0.8 0.8 40 4
0.6 0.6 30 3
0.4 0.4 20 2
0.2 0.2 10 1
0.0 0.0 0 0

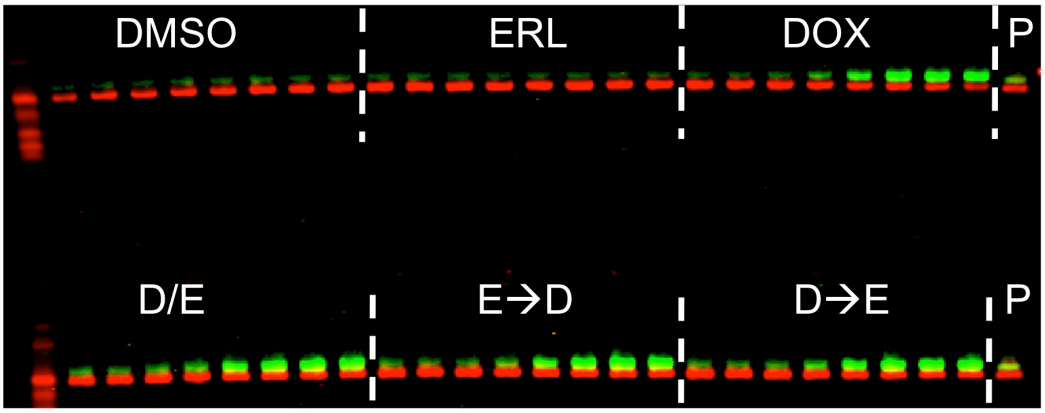

R² = 0.1062
R² = 0.8744
Relative expression (EGFR; p-EGFR)
Fold enrichment (E→D vs. DOX)
0
1
2
3
4
5
6
7

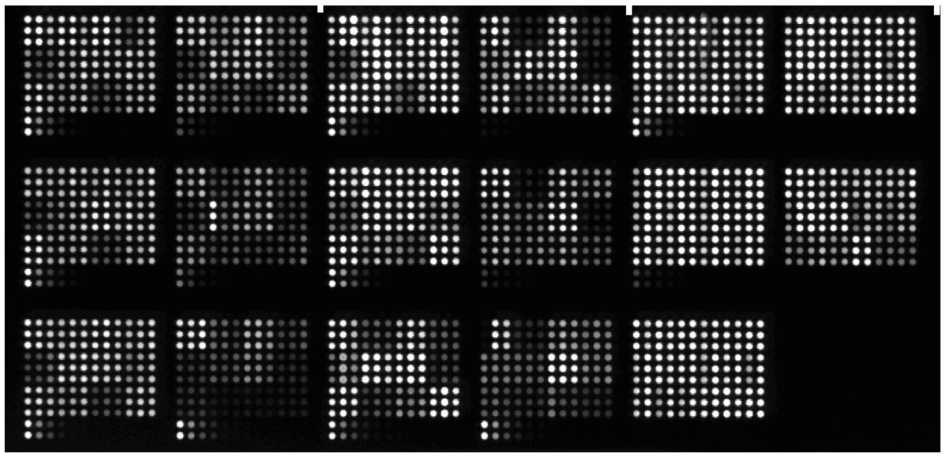






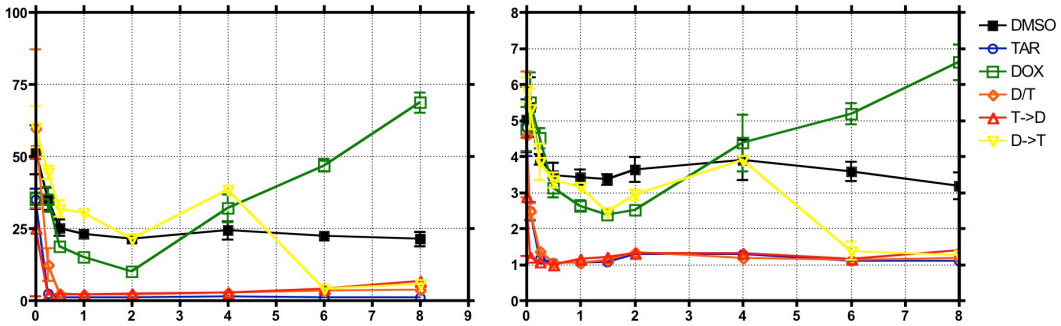

GROWTH FACTOR RECEPTORS
DNA DAMAGE
STRESS
CYTOKINE RECEPTORS
DEATH RECEPTORS
HER2
EGFR
IL-6R
IL-18R
TNFR
MRN
ATM
H2A.X
MDC1
LKB1
53BP1
CHK2
ATR
CHK1
9-1-1
p38
MK2
HSP27
JAK
STAT
RAC
MEKK1
JNK
TRAF2
TRAF6
RIP1
CASP1
IKK
IKB
NF-KB
RAS
RAF
MEK
AMPK
mTOR
PI3K
PDK1
AKT
PKC
DRAM
DAPK
ERK
DUSP
ABL
p53
FANC D2
BRCA1
PLK1
RSK
GADD 45
CDC25
WEE1
DNA-PK
CDK INHIB.
Cyclin
CDK
RPA
ATRIP
XRCC
MYC
4EBP1
S6K
HIS H3
S6
PUMA
BCL2 FAM.
SMAC
CASP9
CASP8
XIAP
CASP6
CASP3
VPS34
VPS15
BECN1
ATG8
ATG 5/7/12
NON-APOPTOTIC DEATH
CELL CYCLE ARREST/DNA REPAIR
PROLIFERATION
APOPTOSIS
AUTOPHAGY



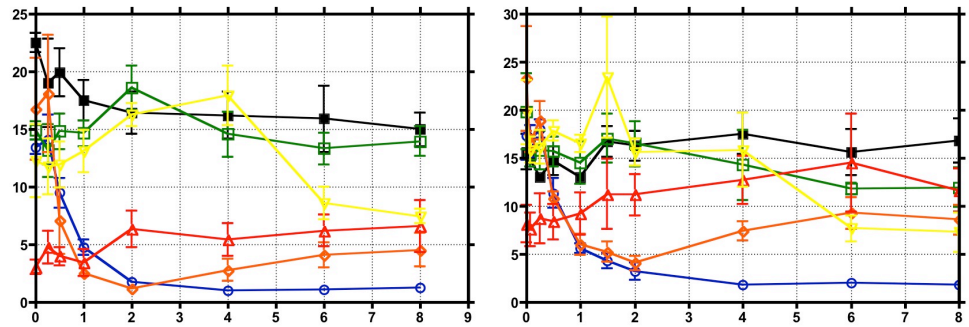


GEL ARRAY

pERK in BT20



pS6 in 453



ANTIBODY TARGET	ACQUISITION FORMAT	ANTIBODY TARGET	ACQUISITION FORMAT
B-RAF	RPMA	p-CyclinE	RPMA
Beclin1	WB	p-ERK	RPMA
BID	WB	p-H2AX	RPMA
BIM	WB	p-HistoneH3	RPMA
c-ABL	WB	p-HSP27	RPMA/WB
Cdc25C	WB	p-JNK	RPMA/WB
CIAP1	WB	p-MEK	RPMA
CIAP2	WB	p-MK2	RPMA
cleaved-CASPASE3	WB	p-p38	RPMA
cleaved-CASPASE6	WB	p-p53 (15)	WB
cleaved-CASPASE8	WB	p-p53 (20)	WB
cleaved-CASPASE9	WB	p-p90RSK	RPMA
CyclinD1	WB	p-S6 (235)	RPMA/WB
DAPK2	WB	p-S6 (240)	RPMA
DNA-PKcs	RPMA	p-S6K	RPMA
DUSP6	WB	p-SEK	RPMA
EGFR	WB	p-STAT3	WB
HER2	WB	p-Wee1	RPMA
IKBa	WB	p27	WB
mTOR	RPMA	p53	WB
p-4E-BP1	RPMA/WB	pDAPK1	WB
p-53BP1	RPMA	pEGFR	WB
p-AKT	RPMA/WB	pHER2	WB
p-BRCA1	RPMA	PUMA	WB
p-CDC25C	RPMA	RIP	WB
p-Chk1 (345)	WB	SMAC	WB
p-Chk2	RPMA/WB	XIAP	WB

FIGURE LEGENDS

FIGURE 1: Efficacy of drug combinations depends on timing of drug presentation. Analysis of apoptosis in BT-20 cells following various combinations of erlotinib (Tarceva) and doxorubicin (adriamycin). Cleaved-caspase 3/Cleaved-PARP double positive cells were quantified using flow cytometry (bottom panels). In cells treated with doxorubicin (DOX), apoptosis measurements were performed 8 hours after DOX exposure. In cells treated with DMSO or erlotinib (ERL) alone, apoptosis was measured either at 8 hours after exposure or at the indicated times. "D/E," ERL→DOX," and "DOX→ERL" refer to doxorubicin and erlotinib added at the same time, erlotinib given at the indicated times before DOX, and DOX given at the indicated times before ERL, respectively. In each of these combination treatment conditions, apoptotic measurements were made 8 hours after the addition of doxorubicin. Both erlotinib and doxorubicin were used at 10mM final concentration. Mean values ± S.D. of 3 independent experiments each performed in duplicate are shown (top panel).

FIGURE 2: Determining sensitivity of each cell line to various genotoxins and inhibitors. Before testing drugs in combinations, optimal doses were chosen for each genotoxin/inhibitor in each cell line. Intermediate doses were chosen such that both sensitization and de-sensitization could be seen. Shown here are examples of doxorubicin dose-response profiles in U2OS (A), BT-20 (B), MCF-7 (C), and MDA-MB-453 (D) cell lines, respectively. None of the inhibitors tested resulted in significant levels of cell death, even at very high doses. See also Figure 5.

FIGURE 3: A schematic of treatment combinations. 7 cytotoxic agents and 9 targeted signaling inhibitors were tested in pair-wise combinations, varying dose, order

of drug presentation, dose duration, and dosing schedule. Cell viability was assessed at various times after treatment using CellTiterGlo. See also Figure 5.

FIGURE 4: Combination efficacy varied by breast cancer subtype. Apoptosis in breast cancer cell lines representing different subtypes of breast cancer. (Top 4 panels) Apoptosis was measured 8 hours after DOX as in Figure 1. "ERL," "D/E," E→D," and "D→E" refer to erlotinib, doxorubicin and erlotinib added at the same time, erlotinib given 24 hours before DOX, and DOX given 4 hours before ERL, respectively. Both drugs were used at 10 μM. (Bottom 3 panels and table) Dose-response profiles of erlotinib/doxorubicin drug combinations. Apoptosis was measured as in Figure 1. In all cases, drugs were added at a fixed 1:1 ratio, and combination index (CI) was calculated according to the Chou-Talalay method (Chou and Talalay, 1984)(Tallarida, 2002). EC_{50} and combination index for each combination shown in table.

FIGURE 5: Combination screen for synergistic drug combinations in triple-negative BT-20 cells. An initial screen of various genotoxins combined with targeted inhibitors was performed in triple-negative BT-20 cells. Dose, time, and combination timing were first screened using the CellTiterGlo assay (Promega). Shown in heatmap form are apoptotic responses for each combination. For each, "PRE" refers to addition of the inhibitor 24 hours before genotoxin; "POST" refers to addition of the inhibitor 4 hours after the genotoxin; "COMBO" refers to the addition of 2 drugs at the same time. All data were collected 8 hours after genotoxin exposure as described in Figure 1.

FIGURE 6: EGFR knockdown using siRNA also causes sensitization to DNA damage in BT-20 cells. (Top panels) Knockdown efficiency of EGFR in BT-20 cells was measured 48 hours after addition of the indicated siRNA by immunoblotting (left).

EGFR expression relative to “no RNA” control is quantified on right. (Bottom panel) Apoptosis in BT-20 cells +/- EGFR knockdown was measured 8 hours after DOX exposure. Scrambled RNAi shown as control. Data shown are the mean ± S.D. of both siRNAs, each performed in biological duplicate. Doxorubicin sensitivity in cells in which EGFR was knocked down is very similar to that induced by chronic erlotinib exposure. Also, addition of erlotinib to cells in which EGFR was knocked down has little effect. These data suggest that the effect of chronic erlotinib exposure is mediated through inhibition of EGFR.

FIGURE 7: Inhibition of a *bona fide* driving oncogene causes enhanced sensitivity to DNA damage. Time-staggered inhibition of HER2 in HER2 over-expressing breast cancer cells causes sensitization to doxorubicin. Apoptosis was measured 8 hours after doxorubicin exposure as described in Figure 1. Note that Lapatinib is a duel specificity inhibitor of HER2 and EGFR.

FIGURE 8: Quantitative cell fate analysis performed for various responses following treatment. (Top) Quantitative cell cycle analysis by flow cytometry. DNA content and the percentage of mitotic cells were measured by FACS using propidium iodide (PI) staining and anti-phospho-Histone H3 (Ser10) immunoreactivity, respectively. **(A)** Example FACS plots from untreated BT-20 cells. **(B-D)** Cell cycle stage was quantified from 3 experiments each performed in duplicate, using the Dean-Jett-Fox algorithm. Cells were treated as in Figure 1 and data were collected at 6, 8, 12, 24, and 48 hours after DOX treatment. 8 hour data is shown for each cell type. (Middle) Autophagy was monitored using automated fluorescence microscopy of cells expressing mCherry-EGFP-LC3B, and quantified using the CellProfiler image analysis software. (Bottom) The complete cellular response dataset for 3 different breast cancer

sub-types following combined EGFR inhibition and genotoxic chemotherapy treatments as in Figure 1. Each box represents an 8- or 12-point time course of biological triplicate experiments. Time course plots were generated using DataRail and are colored by response profile, with early sustained increases in signal colored green, late sustained increases colored red, and transient increases colored yellow. Decreases in signal are colored blue. Signals that are not significantly changed by treatment are shaded grey to black with darkness reflecting signal strength. The numbers to the right of each plot report fold change across all conditions/cells.

FIGURE 9: Apoptotic response across a panel of breast cancer cell lines reveals a correlation between EGFR activity and sensitivity to erlotinib-doxorubicin combinations in triple-negative cells. Apoptosis was measured by flow cytometry 8 hours after treatment as described in Figure 1. For each protein, basal subtype (A or B) and p53 status are reported (according to (Neve et al., 2006). For p53, protein status is shown in parentheses. EGFR protein levels and EGFR activity (p-EGFR) were determined by quantitative Western blot with an antibodies directed against EGFR or phospho-EGFR (pY1173). EGFR or p-EGFR values reported are relative to maximum in the cell line panel. For EGFR, shown in parentheses are data reported in Neve et al. when applicable.

FIGURE 10: Time-staggered inhibition of EGFR signaling enhances apoptotic response in a subset of TNBC cells. (Top) Panel of TNBC cell lines with a wide range of EGFR expression levels. Heatmaps shown for total EGFR expression, p-EGFR (Y1173), percent apoptosis, and apoptosis relative to doxorubicin alone. Apoptosis measurements were made by flow cytometry as described in Figure 1.

EGFR and p-EGFR expression was measured by Western blotting of normally growing, untreated cells. See also Figure 9. (Bottom) EGFR activity—but not total EGFR expression—is correlated with sensitivity to time-staggered erlotinib-doxorubicin combinations. Fold enrichment of "E→D" compared to DOX alone regressed against total EGFR or p-EGFR (pY1173) as measured in untreated cells.

FIGURE 11: Validation of antibodies for RPMA analysis. (Top) Sample validation for 48 antibodies. Shown are 3 slides, each slide has 16 nitrocellulose patches, each patch contains 90 control lysates. Each patch was probed with a different antibody. 4 antibodies are highlighted and expanded to show possible results. (Middle, left to right). Examples of uniformly low signal, candidate antibody, uniformly high signal, and an antibody that bound to the membrane. Positive candidate antibody in middle panel is p-S6. (Bottom) Candidate antibodies are then tested in Western blot format to insure that the changes reported in RPMA are quantitatively similar to that reported in Western format. Example on bottom gel is for p-S6.

FIGURE 12: Schematic of targets of interest. An expanded signaling-response network. This network includes canonical components of the DNA damage response, together with components in general stress response pathways, and growth factor, cytokine and cell death pathways. Specific targets selected for measurement were based on prior knowledge of the pathway. 1000 antibodies to over 200 targets of interest were tested in both reverse phase protein lysate array format and quantitative Western blot format using a panel of 90 control lysates. Antibodies to targets of interest that were validated to be high fidelity (band at appropriate size; report predicted changes in expression across control lysate panel) will be included for computational analysis if treatment-dependent or cell line dependent differences are observed.

Figure 13: Examples of Western blot and RPMA data. (Top) 48-sample Western blots will be used and analyzed using 2-color infrared detection. Each gel will contain an antibody-specific positive control (P) for blot-to-blot normalization. The example shown is total p53 in MCF7 cells (p53 in green; b-actin in red). (Middle) Reverse phase protein lysate microarrays will be used to analyze targets of interest when array-compatible antibodies were available. The slide shown contains ~2,500 lysate spots (experimental and technical triplicates of all of our experimental samples, and control samples used for antibody calibration), probed for phospho-S6. This portion of the data analysis has begun and should be completed within the following year. (Bottom) Comparison of quantitative data from Western (gel) and RPMA (array) formats.

TABLE 1: RPMA antibodies and other antibodies to be used in this study