

# Lapatinib and Obatoclox Kill Breast Cancer Cells through Reactive Oxygen Species-Dependent Endoplasmic Reticulum Stress<sup>[S]</sup>

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## ABSTRACT

Previous studies showed that lapatinib and obatoclox interact in a greater-than-additive fashion to cause cell death and do so through a toxic form of autophagy. The present studies sought to extend our analyses. Lapatinib and obatoclox killed multiple tumor cell types, and cells lacking phosphatase and tensin homolog (PTEN) function were relatively resistant to drug combination lethality; expression of PTEN in PTEN-null breast cancer cells restored drug sensitivity. Co-administration of lapatinib with obatoclox elicited autophagic cell death that was attributable to the actions of mitochondrial reactive oxygen species. Wild-type cells but not mitochondria-deficient rho-zero cells were radiosensitized by lapatinib and obatoclox treatment. Activation of p38 mitogen-activated protein kinase (MAPK) and c-Jun NH<sub>2</sub>-terminal kinase 1/2 (JNK1/2) by the drug combination was enhanced

by radiation, and signaling by p38 MAPK and JNK1/2 promoted cell killing. In immunohistochemical analyses, the autophagosome protein p62 was determined to be associated with protein kinase-like endoplasmic reticulum kinase (PERK) and inositol-requiring enzyme 1, as well as with binding immunoglobulin protein/78-kDa glucose-regulated protein, in drug combination-treated cells. Knockdown of PERK suppressed drug-induced autophagy and protected tumor cells from the drug combination. Knockdown of PERK suppressed the reduction in Mcl-1 expression after drug combination exposure, and overexpression of Mcl-1 protected cells. Our data indicate that mitochondrial function plays an essential role in cell killing by lapatinib and obatoclox, as well as radiosensitization by this drug combination.

## Introduction

Tumor cells frequently are addicted to signaling through growth factor receptors. Inhibitors of these receptors, such as gefitinib and lapatinib, have shown antitumor effects that

sometimes are genuinely cytotoxic but more frequently are cytostatic. To achieve greater effects on survival rates, multiple growth factor receptors and intracellular pathways generally need to be targeted for inhibition.

Lapatinib, a dual ErbB1/ErbB2 inhibitor, has been approved for clinical use in combination with capecitabine for ErbB2-overexpressing metastatic breast cancer (Geyer et al., 2006; Kong et al., 2008; Tao and Maruyama, 2008; Awada et al., 2011). Resistance to ErbB-inhibiting therapeutic agents develops with time, through secondary mutations within ErbB receptors, initiation of alternative receptor tyrosine kinase signaling pathways, or up-regulation of prosurvival proteins of the Bcl-2 family (Miller, 2004; Martin et al., 2009; Ware et al., 2010). It has been noted that tumors that present

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**ABBREVIATIONS:** BAX, Bcl-2-associated X protein; S6K, S6 kinase; siRNA, small interfering RNA; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH<sub>2</sub>-terminal kinase; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; PERK, protein kinase-like endoplasmic reticulum kinase; ASK1, apoptosis signaling kinase 1; BiP, binding immunoglobulin protein; GRP78, 78-kDa glucose-regulated protein; IRE1, inositol-requiring enzyme 1; BAK, Bcl-2-homologous antagonist/killer; BH, Bcl-2 homology; mTOR, mammalian target of rapamycin; DMSO, dimethylsulfoxide; ER, endoplasmic reticulum; ROS, reactive oxygen species; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; BEZ235, 2-methyl-2-{4-[3-methyl-2-oxo-8-(quinolin-3-yl)-2,3-dihydro-1H-imidazo[4,5-c]quinolin-1-yl]phenyl}propanenitrile.

with alterations in ErbB receptors often are more aggressive and are associated poorer clinical outcomes (Hynes and Lane, 2005; Parkin et al., 2005; Martin et al., 2008).

The Bcl-2 family of proteins includes protective proteins such as Bcl-2, Bcl-XL, and Mcl-1 and proapoptotic proteins such as BAX, BAK, p53-up-regulated modulator of apoptosis, and Noxa (PUMA) (van Delft and Huang, 2006; Martin et al., 2009; Mitchell et al., 2010; Cruickshanks et al., 2012; Tang et al., 2012). As noted frequently in the literature, the release of BAK and BAX from protective Bcl-2 proteins results in pore formation and mitochondrial stress with ROS generation, which leads to the release of cytochrome *c* and the activation of apoptosis effectors. These effects also can be induced by target-specific therapeutic agents, such as obatoclax (GX15-070), that act by inhibiting interactions between protective Bcl-2 family members and toxic Bcl-2 family members. Theoretically, this approach might increase the toxicity of other therapies that act to promote mitochondrial dysfunction (Martin et al., 2009). In our previous studies on the combination of lapatinib and obatoclax, however, we demonstrated that cell killing was attributable to a toxic form of autophagy, despite activation of BAX and BAK, and caspase inhibitors (such as *N*-benzyloxycarbonyl-valine-alanine-aspartate) had little or no effect in suppressing the cell-killing effects (Martin et al., 2009; Mitchell et al., 2010; Cruickshanks et al., 2012; Tang et al., 2012).

Autophagy is an evolutionarily conserved catabolic pathway that recycles or removes damaged or excess membranes and organelles and breaks down proteins into their amino acid constituents, to maintain viability (Gewirtz, 2007; Yoshimori and Noda, 2008; Graf et al., 2009; Mehrpour et al., 2010). Cancer cells often display reduced levels of autophagy, which allows continuing malignant progression and proliferation (Alva et al., 2004; Mathew et al., 2007) but also provides an anticancer role by limiting tumor size and growth (Hippert et al., 2006). This raises the question of whether autophagy in tumor cells is a cytoprotective or cytotoxic event (Amaravadi, 2009; Jia et al., 2010). We found that the Bcl-2 inhibitor obatoclax, either alone or in combination with the ErbB1/2/4 inhibitor lapatinib, kills cells through a toxic form of autophagy that is correlated with activation of toxic BH3 domain proteins such as BAX, BAK, Noxa, and p53-up-regulated modulator of apoptosis (Martin et al., 2009; Mitchell et al., 2010; Cruickshanks et al., 2012; Tang et al., 2012).

The present study aimed to establish additional mechanisms for lapatinib and obatoclax toxicity, the importance of PTEN status in drug toxicity, and the mechanisms through which the drug combination radiosensitized tumor cells. Lapatinib/obatoclax treatment radiosensitized cells through activation of p38 MAPK and increased expression of Noxa.

## Materials and Methods

**Materials.** Lapatinib was provided by GlaxoSmithKline (King of Prussia, PA). Obatoclax was provided by GeminX Pharmaceuticals (King of Prussia, PA). Other drugs were purchased from Selleck Chemicals (Houston, TX). Trypsin-EDTA, Dulbecco's modified Eagle's medium, minimal essential medium, RPMI 1640 medium, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). All established tumor cell lines were purchased from the American Type Culture Collection (Manassas, VA). Short hairpin PTEN, plasmids expressing green fluorescent protein (GFP)-tagged PTEN and mTOR, and a plasmid expressing

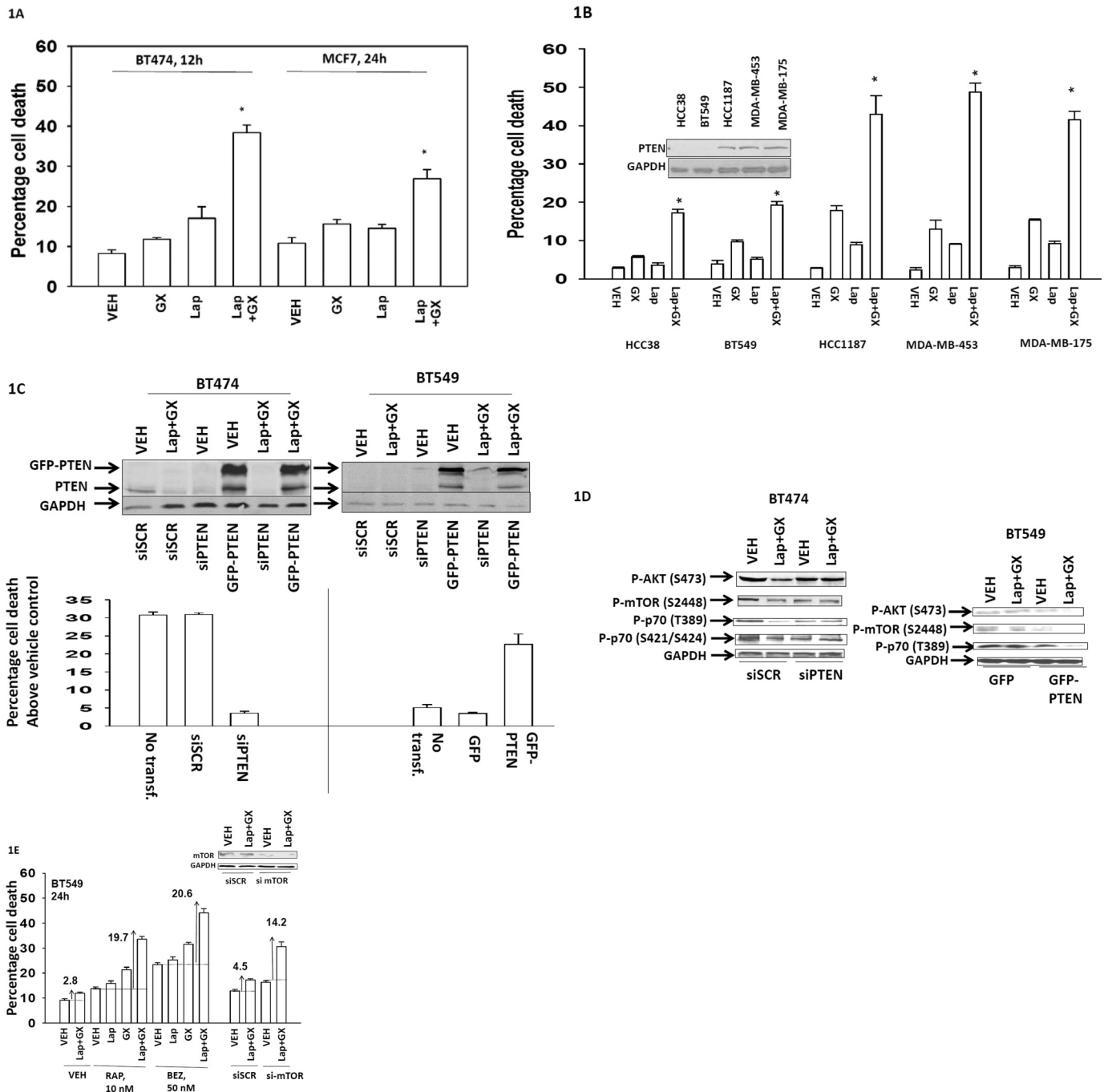
luciferase were purchased from Addgene (Cambridge, MA). A plasmid expressing GFP-tagged human LC3 was kindly provided by Dr. S. Spiegel (Virginia Commonwealth University). Validated siRNAs were purchased from QIAGEN (Valencia, CA). Antibodies were purchased from Cell Signaling Technology (Worcester, MA). All secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Reagents and experimental procedures were described previously (Rahmani et al., 2005, 2007; Park et al., 2008, 2010; Zhang et al., 2008; Martin et al., 2009; Eulitt et al., 2011; Cruickshanks et al., 2012; Tang et al., 2012).

**Cell Culture and In Vitro Drug Exposure.** All established cell lines were cultured at 37°C in 5% (v/v) CO<sub>2</sub> by using RPMI 1640 medium supplemented with 5% (v/v) fetal calf serum and 10% (v/v) nonessential amino acids. For short-term cell-killing assays and immunoblotting studies, cells were plated at  $\sim 2 \times 10^5$  cells/well in 12-well plates and, 48 h after plating, were treated with various drugs as indicated. In vitro drug treatments were from 100 mM stock solutions of each drug, and the maximal concentration of vehicle (DMSO) in the medium was 0.02% (v/v). Cells were not cultured in reduced-serum medium in any assays in this study.

**In Vitro Cell Treatments, Microscopy, SDS-PAGE, and Western Blotting.** For in vitro analyses of short-term cell death effects, cells were treated with vehicle, the combination of lapatinib and obatoclax, or the combination of lapatinib and obatoclax with the addition of either rapamycin or 2-methyl-2-[4-[3-methyl-2-oxo-8-(quinolin-3-yl)-2,3-dihydro-1*H*-imidazo[4,5-*c*]quinolin-1-yl]phenyl]propanenitrile (BEZ235), for the times indicated in the figure legends. For apoptosis assays as indicated, cells were isolated at the indicated times and subjected to trypan blue cell viability assays, with counting with a light microscope. For SDS-PAGE and immunoblotting studies, cells were plated at  $5 \times 10^5$  cells/cm<sup>2</sup>, treated with drugs at the indicated concentrations for the indicated times, and lysed in whole-cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1%  $\beta$ -mercaptoethanol, 0.02% bromophenol blue), and the samples were boiled for 30 min. The boiled samples were loaded onto 8 to 14% SDS-polyacrylamide gels, and electrophoresis was performed for approximately 1.5 h. Proteins were electrophoretically transferred to 0.22- $\mu$ m nitrocellulose membranes, and immunoblotting was performed with primary antibodies against various proteins. Blots were observed by using an Odyssey IR imaging system (LI-COR Biosciences, Lincoln, NE).

**Transfection of Cells with Plasmids or siRNA.** For transfection with plasmids, cells were plated as described above and were transfected 24 h after plating. For all cell types (0.5  $\mu$ g), plasmid expressing a specific mRNA (or siRNA) or appropriate vector control plasmid DNA was diluted in 50  $\mu$ l of serum-free and antibiotic-free medium (one portion for each sample). Concurrently, 2  $\mu$ l of Lipofectamine 2000 (Invitrogen) was diluted with 50  $\mu$ l of serum-free and antibiotic-free medium (one portion for each sample). Diluted DNA was added to the diluted Lipofectamine 2000 for each sample, and the mixture was incubated at room temperature for 30 min. This mixture was added to each well containing cells in 200  $\mu$ l of serum-free and antibiotic-free medium, for a total volume of 300  $\mu$ l, and the cells were incubated for 4 h at 37°C. An equal volume of 2 $\times$  medium was then added to each well. Cells were incubated for 48 h and then treated with drugs.

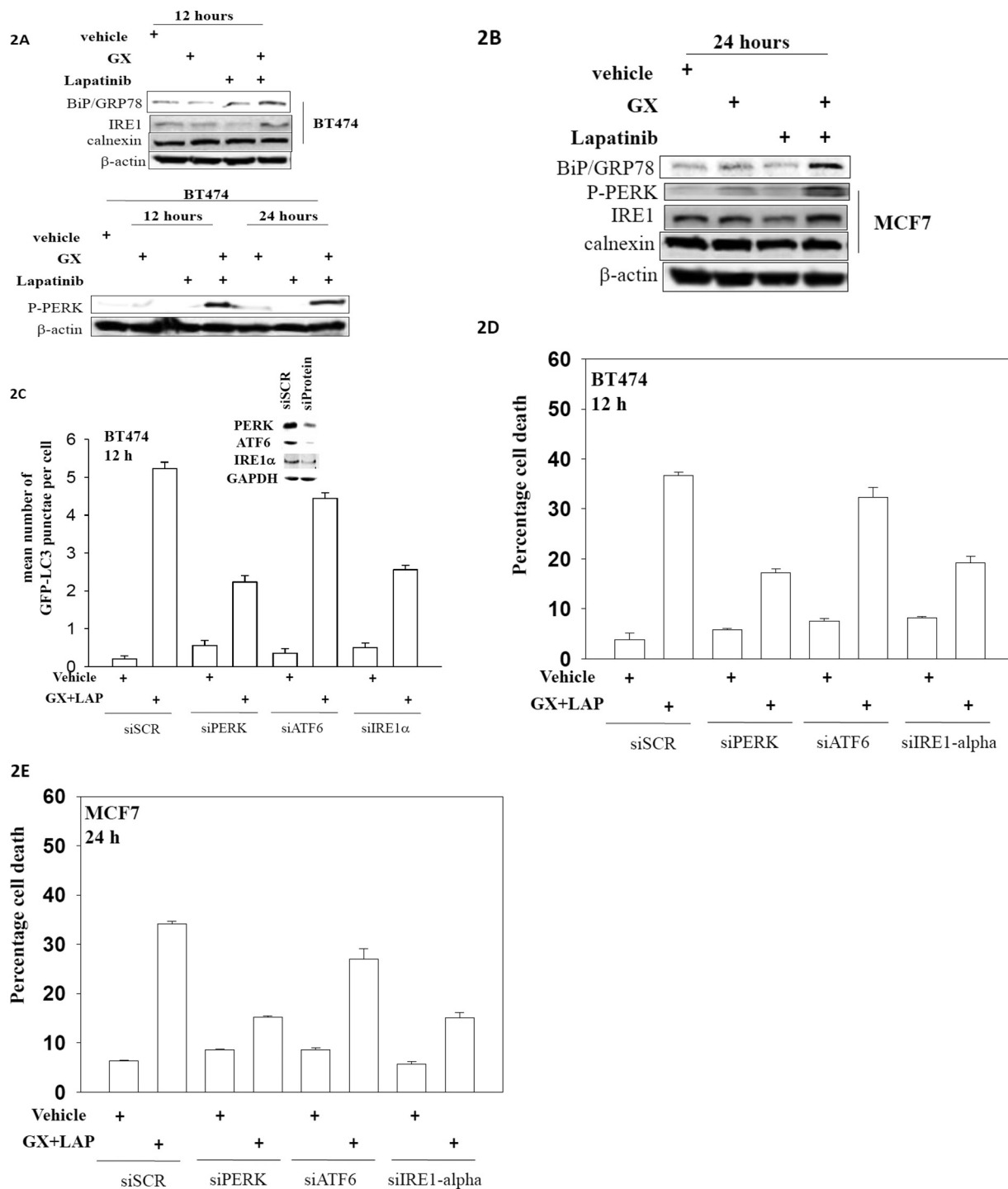
For transfection with siRNA, cells from a fresh culture growing in the logarithmic phase were plated in 60-mm dishes, as described above, and were transfected 24 h after plating. Before transfection, the medium was aspirated and 1 ml of serum-free medium was added to each plate. For transfection, 10 nM levels of the annealed siRNA, the positive sense control (double-stranded siRNA targeting glyceraldehyde-3-phosphate dehydrogenase), or the negative control (a "scrambled" sequence with no significant homology to any known gene sequences in mouse, rat, or human



**Fig. 1.** Lapatinib and obatoclox interact to kill multiple tumor cell types but not cells lacking PTEN function/expression. A, BT474 and MCF7 cells were treated with vehicle (VEH) (DMSO), lapatinib (Lap) (1  $\mu$ M), and/or obatoclox (GX) (50 nM) as indicated. Cells were isolated 12 to 24 h after exposure, and viability was determined through trypan blue exclusion ( $n = 3$ ; mean  $\pm$  S.E.M.). \*,  $p < 0.05$ , greater than vehicle control value. B, mammary carcinoma cells, as indicated, were treated with vehicle (DMSO), lapatinib (1  $\mu$ M), and/or obatoclox (50 nM). Cells were isolated 24 h after exposure, and viability was determined through trypan blue exclusion ( $n = 3$ ; mean  $\pm$  S.E.M.). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. \*,  $p < 0.05$ , greater than vehicle control value. C, lower, BT549 cells were transfected with either control plasmid (GFP) or plasmid expressing PTEN (GFP-PTEN). BT474 cells were transfected with either control plasmid siRNA or plasmid expressing a siRNA to knock down PTEN. Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or with lapatinib (1  $\mu$ M) and obatoclox (50 nM). Cells were isolated 12 to 24 h after exposure, and viability was determined through trypan blue exclusion, with data being expressed as the true percentage of cell death above the matched vehicle control value ( $n = 3$ ; mean  $\pm$  S.E.M.). Upper, expression and knockdown of PTEN in breast cancer cells. No transf., no transfection; siSCR, scrambled siRNA. D, in BT474 cells (left), lapatinib (1  $\mu$ M) and obatoclox (50 nM) treatment decreased Akt, mTOR, and p70 S6K activity that had been reduced through knockdown of PTEN. In BT549 cells (right), expression of PTEN restored lapatinib (1  $\mu$ M) and obatoclox (50 nM) treatment-induced loss of Akt, mTOR, and p70 S6K activity. E, BT549 cells were treated with vehicle (DMSO) or with lapatinib (1  $\mu$ M) and obatoclox (50 nM) in the presence or absence of rapamycin (RAP) (10 nM) or BEZ235 (BEZ) (50 nM). Parallel sets of cells were transfected to knock down mTOR expression. Cells were isolated 24 h after exposure, and viability was determined through trypan blue exclusion ( $n = 3$ ; mean  $\pm$  S.E.M.).

cell lines) were used. siRNA (scrambled or experimental) at 10 nM was diluted in serum-free medium; 4  $\mu$ l of Lipofectamine (QIAGEN) was added to this mixture, and the solution was mixed by being pipetted up and down several times. The solution was incubated at room temperature for 10 min and then added dropwise to

each dish. The medium in each dish was swirled gently to mix the contents, and the cells were incubated for 2 h at 37°C. One milliliter of 10% (v/v) serum-containing medium was added to each plate, and the cells were incubated for 48 h at 37°C before being replated (at  $50 \times 10^3$  cells/well) in 12-well plates. Cells were



**Fig. 2.** Lapatinib and obatoclax treatment induces an ER stress response that regulates drug lethality. A and B, BT474 cells (A) and MCF7 cells (B) were treated with vehicle (DMSO), lapatinib (1  $\mu$ M), and/or obatoclax (GX) (50 nM) as indicated. Cells were isolated 12 to 24 h after exposure, and IRE1 and GRP78/BiP expression and PERK phosphorylation levels were determined. C, BT474 cells were transfected with LC3-GFP and the indicated siRNA molecules. Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or with lapatinib (LAP) (1  $\mu$ M) and obatoclax (50 nM). Twelve hours after drug treatment, the numbers of LC3-GFP punctae were determined ( $n = 3$ ; mean  $\pm$  S.E.M.). siSCR, scrambled siRNA; siATF6, activating transcription factor 6 siRNA. D and E, BT474 cells (D) and MCF7 cells (E) were transfected with the indicated siRNA molecules. Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or with lapatinib (1  $\mu$ M) and obatoclax (50 nM). Twelve hours (BT474 cells) or 24 h (MCF7 cells) after drug treatment, cells were isolated and viability was determined through trypan blue exclusion ( $n = 3$ ; mean  $\pm$  S.E.M.).



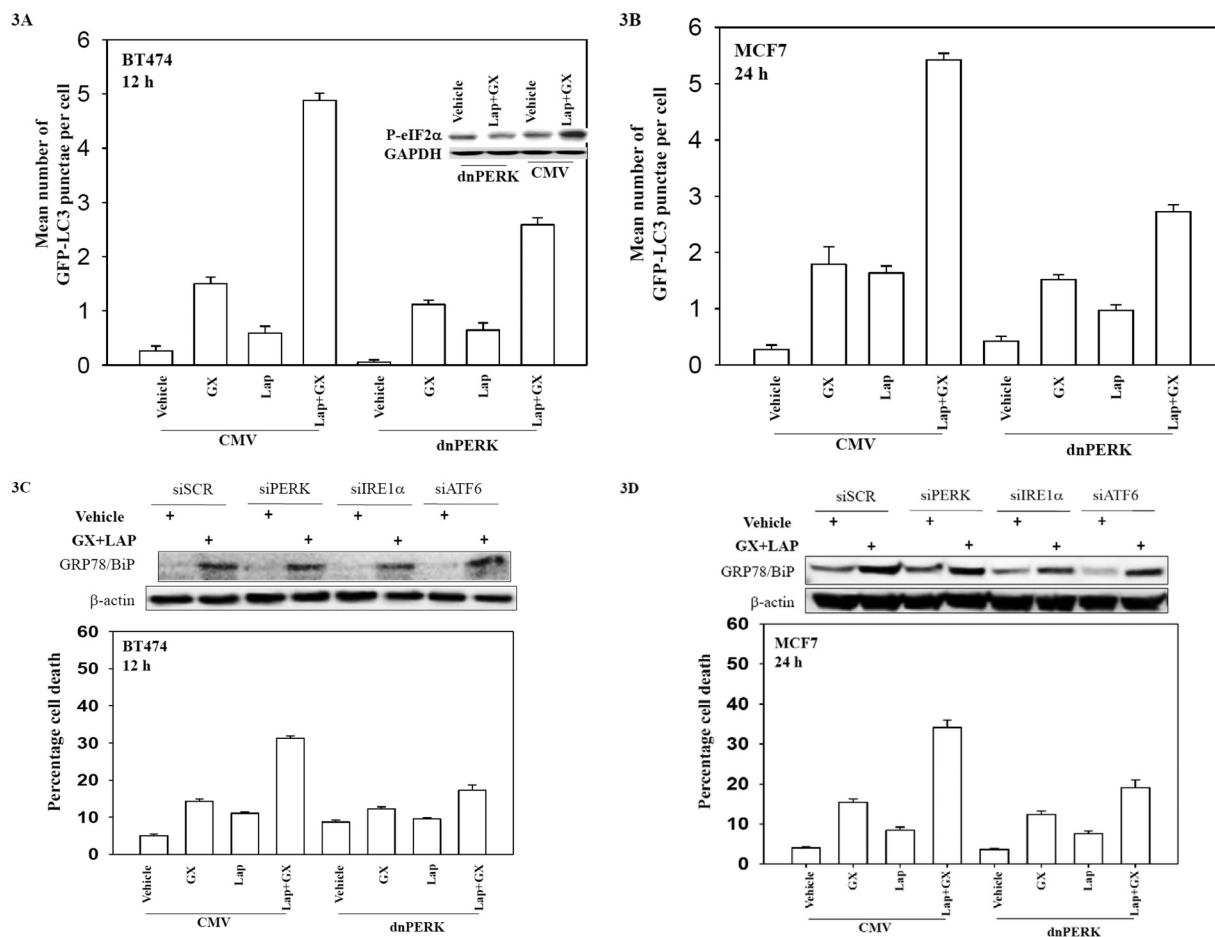
allowed to attach overnight and then were treated with drugs (0–48 h). Trypan blue exclusion/terminal deoxynucleotidyl transferase dUTP nick end-labeling and SDS-PAGE/immunoblotting analyses were performed at the indicated time points.

**Microscopic Analyses of LC3-GFP Expression.** Cells were transfected with a plasmid expressing a LC3-GFP fusion protein and then were cultured for 24 h. Cells were treated with drugs as indicated. LC3-GFP-transfected cells were observed at the indicated times by using a Zeiss Axiovert 200 microscope (Carl Zeiss Inc., Thornwood, NY) with a fluorescein isothiocyanate filter.

**Intracerebral Inoculation of BT474 Cells.** Athymic, female, NCr-*nu/nu* mice (National Cancer Institute, Frederick, MD) weighing ~20 g were used for this study. Mice were maintained under pathogen-free conditions in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care, in accordance with current regulations and standards of the U.S. Department of Agriculture (Washington, DC), the U.S. Department of Health and Human Services (Washington, DC), and the National Institutes of Health (Bethesda, MD). Mice were anesthetized through intraperitoneal administration of ketamine (40 mg/kg) and xylazine (3 mg/kg) and were immobilized in a stereotactic

frame (David Kopf Instruments, Tujunga, CA). A 24-gauge needle attached to a Hamilton syringe was inserted into the right basal ganglia to a depth of 3.5 mm and then was withdrawn 0.5 mm, to make space for tumor cell accumulation. The entry point in the skull was 2 mm lateral and 1 mm dorsal to bregma. Intracerebral injection of BT474 cells in 2  $\mu$ l of PBS was performed over 10 min. The skull opening was closed with sterile bone wax, and the skin incision was closed with sterile surgical staples. Two to 4 weeks after tumor cell implantation, animals were divided into treatment groups. For administration to animals, lapatinib and obatoclox were dissolved in DMSO, and an equal volume of Cremophor A25/ethanol (50:50; Sigma-Aldrich, St. Louis, MO) was added. After mixing, a 1:10 dilution with sterile PBS was prepared. Animals were treated with vehicle (PBS/Cremophor/ethanol/DMSO), lapatinib, obatoclox, or a combination of lapatinib and obatoclox through oral gavage, to final concentrations of 5 mg/kg b.wt. daily for obatoclox and 100 mg/kg b.i.d. for lapatinib. Immunohistochemical analyses were performed as described previously (Mitchell et al., 2010).

**Data Analyses.** Comparisons of the effects of various treatments were performed by using analyses of variance and Student's *t* tests.



**Fig. 3.** Dominant negative PERK (dnPERK) suppresses the toxic interaction between lapatinib and obatoclox. A and B, BT474 cells (A) and MCF7 cells (B) were transfected with plasmid expressing LC3-GFP and, as indicated, with empty vector [cytomegalovirus (CMV)] or plasmid expressing dominant negative PERK. Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or with lapatinib (Lap) (1  $\mu$ M) and obatoclox (GX) (50 nM). Twelve hours (BT474 cells) or 24 h (MCF7 cells) after drug treatment, the numbers of LC3-GFP punctae were determined ( $n = 3$ ; mean  $\pm$  S.E.M.). \*,  $p < 0.05$ , less with dominant negative PERK, compared with the cytomegalovirus value. C and D, lower, BT474 cells (C) and MCF7 cells (D) were transfected with empty vector (cytomegalovirus) or a plasmid expressing dominant negative PERK. Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or with lapatinib (1  $\mu$ M) and obatoclox (50 nM). Twelve hours (BT474 cells) or 24 h (MCF7 cells) after drug treatment, cells were isolated and viability was determined through trypan blue exclusion ( $n = 3$ ; mean  $\pm$  S.E.M.). \*,  $p < 0.05$ , less with dominant negative PERK, compared with the cytomegalovirus value. Upper, cells were transfected with the indicated siRNA molecules. Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or with lapatinib (1  $\mu$ M) and obatoclox (50 nM). Twelve hours (BT474 cells) or 24 h (MCF7 cells) after drug treatment, cells were isolated and blotting analyses were performed to determine GRP78/BiP levels. siSCR, scrambled siRNA; siATF6, activating transcription factor 6 siRNA.

Differences with  $p$  values of  $<0.05$  were considered statistically significant. Results shown are the mean  $\pm$  S.E.M. of multiple individual points.

## Results

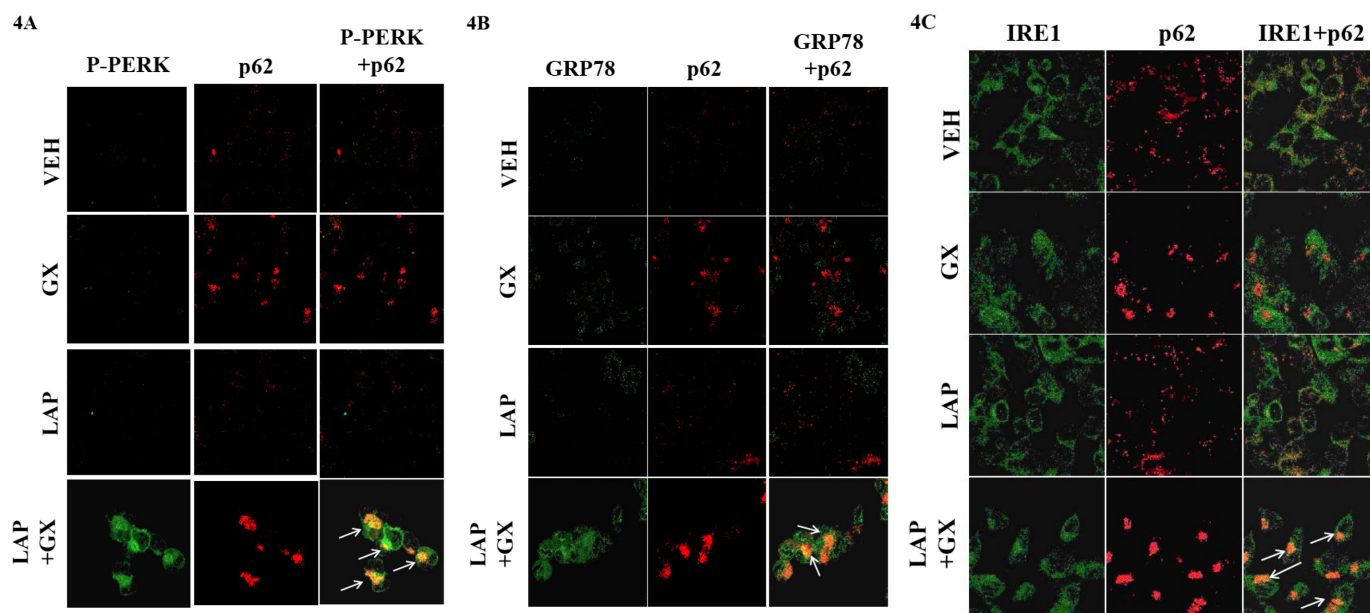
Lapatinib and obatoclastax interacted to kill a wide variety of mammary tumor cell lines (Fig. 1, A and B). Of note was the fact that cell lines that lacked PTEN (HCC38 and BT549) were relatively resistant to the drug combination, compared with cells that expressed PTEN (HCC1187, MDA-MB-453, and MDA-MB-175). Because expression of PTEN is often lost in breast cancers (Depowski et al., 2001; Höland et al., 2011), we determined whether manipulation of PTEN function altered the lethality of the drug combination. Re-expression of PTEN in PTEN-null BT549 cells facilitated lapatinib and obatoclastax lethality, whereas knockdown of PTEN in BT474 cells suppressed killing by the drug combination (Fig. 1C). Knockdown of PTEN suppressed drug-induced dephosphorylation of Akt, mTOR, and p70 S6K (Fig. 1D), and expression of PTEN facilitated drug-induced dephosphorylation of Akt, mTOR, and p70 S6K. Because loss of PTEN caused resistance to the drug combination, we hypothesized that inhibition of a downstream effector of PTEN, i.e., mTOR, might restore the lethal effects of lapatinib/obatoclastax treatment. In agreement with our hypothesis, treatment of BT549 cells with either rapamycin or BEZ235 caused significant enhancement of lapatinib/obatoclastax lethality, compared with vehicle-treated cells (Fig. 1E). Knockdown of mTOR expression caused effects similar to those obtained with either rapamycin or BEZ235.

Previous studies in our laboratory explored the roles of endoplasmic reticulum stress proteins in the responses of tumor cells to a variety of drug combinations. Treatment of cells with lapatinib and obatoclastax increased the expression of BiP/GRP78 and IRE1 and enhanced the phosphorylation of PERK, which is general evidence that an ER stress response was being induced (Fig. 2, A and B). We then defined the

roles of ER-resident proteins in the autophagic and survival responses to the drug combination. Knockdown of PERK or IRE1 suppressed the induction of autophagic vesicles by lapatinib and obatoclastax (Fig. 2C). Knockdown of PERK or IRE1 also suppressed the increase in cell death rates after treatment with the drug combination (Fig. 2, D and E). In agreement with our knockdown data, expression of dominant negative PERK suppressed drug-induced vesicle formation and the induction of cell killing (Fig. 3).

Because PERK played a role in the induction of cell killing, we also examined the effects of the drugs on the expression of the PERK chaperone BiP/GRP78. Drug exposure increased BiP/GRP78 levels, an effect that was not altered by knockdown of PERK, IRE1 $\alpha$ , or activating transcription factor 6 (Figs. 2, A and B, and 3, C and D, insets). We performed immunohistochemical analyses to examine the colocalization of autophagy and ER stress proteins in drug-treated cells. Drug treatment stimulated partial colocalization of phosphorylated PERK with the autophagy protein p62 (Fig. 4A). BiP/GRP78 and IRE1 $\alpha$  also partially colocalized with p62 after drug treatment (Fig. 4, B and C).

We reported that lapatinib and obatoclastax treatment increased the expression of autophagy-related proteins through conversion of LC3-I to LC3-II, altered expression of p62, and phosphorylation of histone  $\gamma$ H2AX, which was inversely correlated with the lack of reactive oxygen species generation in mitochondria-deficient rho-zero cells (Cruickshanks et al., 2012; Tang et al., 2012). Treatment with lapatinib and obatoclastax caused initial increases in LC3-II and p62 levels, which subsequently decreased and were correlated with tumor cell killing (Cruickshanks et al., 2012; Tang et al., 2012). Therefore, we determined whether the drug combination altered the levels of DNA damage, as judged on the basis of the generation of oxidized guanine and the formation of single- and double-strand DNA breaks. Drug combination treatment increased the levels of oxidized deoxyguanine residues in cells (Fig. 5A). Knockdown of Beclin 1 (or parallel



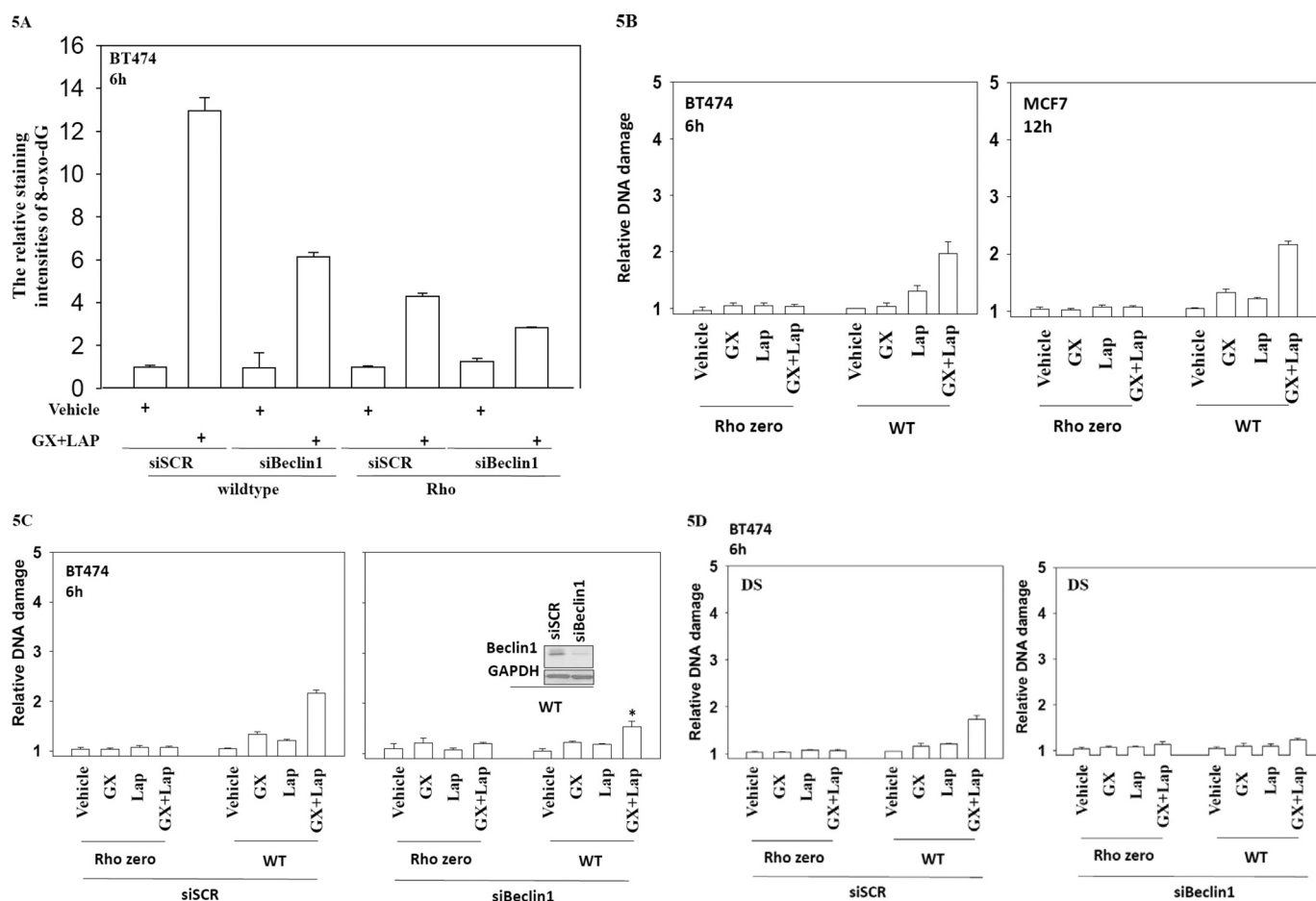
**Fig. 4.** p62 is associated with PERK, IRE1, and GRP78/BiP. BT474 cells were treated with vehicle (VEH) (DMSO) or with lapatinib (LAP) (1  $\mu$ M) and obatoclastax (GX) (50 nM). Twelve hours after drug treatment, cells were fixed and stained for p62 and phospho-PERK (A), for p62 and GRP78/BiP (B), or for p62 and IRE1 (C).

experiments with rho-zero cells) suppressed drug-induced DNA damage. Drug treatment increased single-strand DNA breaks (determined by using an alkaline comet assay) and double-strand DNA breaks (determined by using a neutral comet assay), effects that were suppressed in rho-zero cells or cells in which Beclin 1 expression was knocked down (Fig. 5, B–D). Therefore, drug-induced autophagy was downstream of ROS generation (data not shown) (Tang et al., 2012).

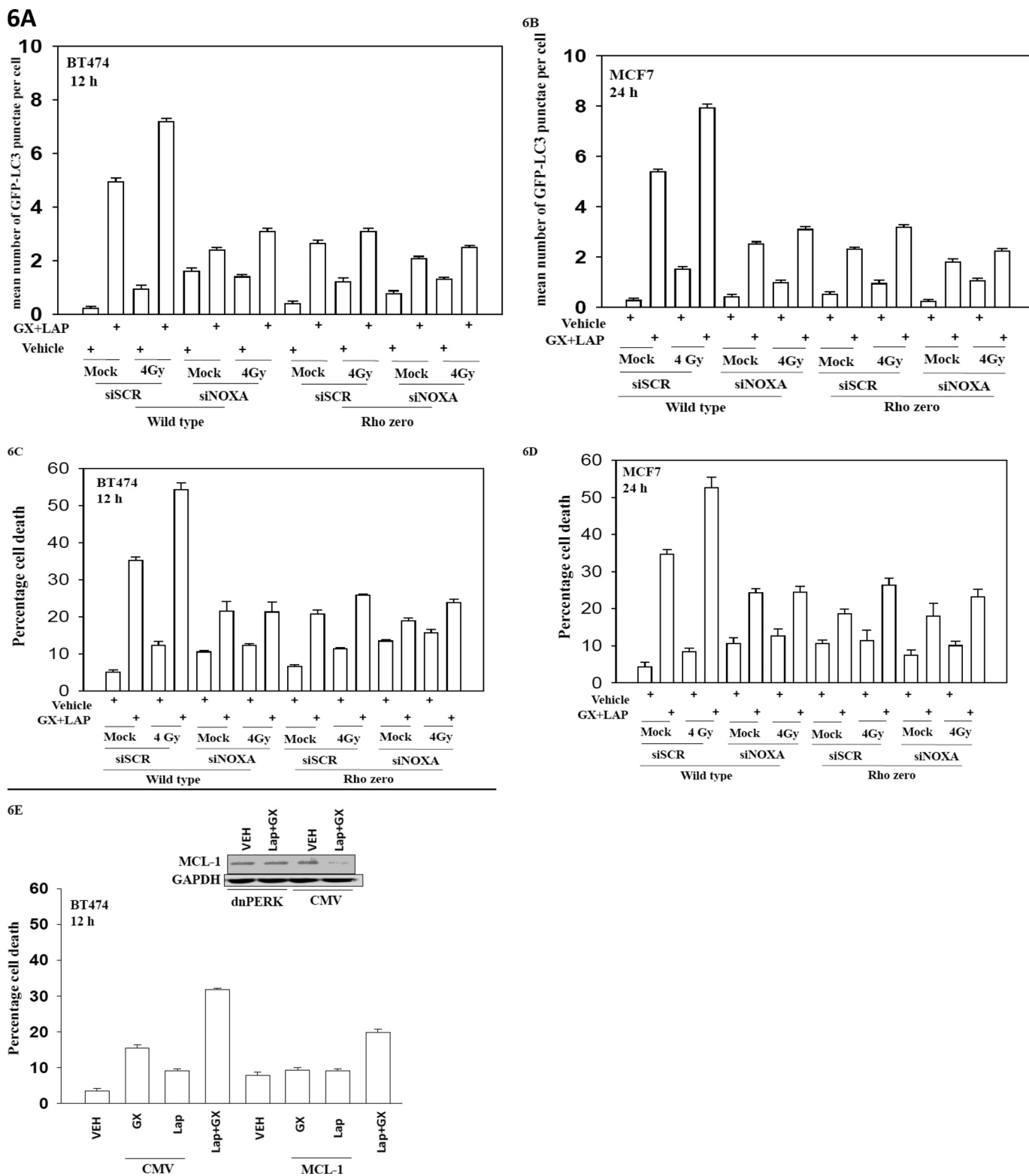
In addition to ROS, the release of  $\text{Ca}^{2+}$  from intracellular stores has been linked to the regulation of autophagy and DNA damage in other systems. Treatment of cells with lapatinib and obatoclox increased cytosolic  $\text{Ca}^{2+}$  levels in cells, an effect that was quenched with expression of calbindin D28 (Supplemental Fig. 1A). The generation of ROS and the induction of autophagy were independent of  $\text{Ca}^{2+}$  levels (Supplemental Fig. 1, B and C). Quenching of  $\text{Ca}^{2+}$  also did not alter drug combination-induced cell killing or the expression of DNA damage and autophagy regulatory proteins (Supplemental Fig. 1, D and E).

Ionizing radiation is a modality that is used frequently to treat breast cancer. On the basis of our previous data, we investigated whether lapatinib/obatoclox treatment radiosensitized breast cancer cells, as well as the mechanism of any effect. Radiation and lapatinib/obatoclox treatment interacted to enhance the formation of autophagic vesicles (Fig. 6, A and B). Knockdown of Noxa suppressed the formation of autophagic vesicles induced by the drug combination and the combination plus radiation. Radiation and lapatinib/obatoclox treatment interacted to kill tumor cells in a greater-than-additive fashion (Fig. 6, C and D). Knockdown of Noxa suppressed drug- and drug/radiation-induced killing. Treatment with lapatinib and obatoclox reduced expression of the protective Bcl-2 family member Mcl-1, which was blocked by expression of dominant negative PERK (Fig. 6E). Expression of Mcl-1 suppressed drug combination lethality.

Irradiation of tumor cells is known to generate significant amounts of ROS shortly after exposure. Treatment of breast cancer cells with lapatinib and obatoclox generated ROS 12



**Fig. 5.** Lapatinib and obatoclox interact to cause DNA damage in tumor cells that is dependent on ROS generation and autophagy. A, BT474 cells (wild-type and rho-zero) were transfected with the indicated siRNA molecules. Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or with lapatinib (LAP) (1  $\mu\text{M}$ ) and obatoclox (GX) (50 nM). Six hours after drug treatment, cells were isolated and stained for 8-oxo-dG; the levels of 8-oxo-dG were analyzed through flow cytometry ( $n = 3$ ; mean  $\pm$  S.E.M.). siSCR, scrambled siRNA. B, BT474 and MCF7 cells [wild-type (WT) and rho-zero] were treated with vehicle (DMSO) or with lapatinib (1  $\mu\text{M}$ ) and obatoclox (50 nM). Six hours (BT474 cells) or 12 h (MCF7 cells) after drug treatment, cells were isolated and subjected to an alkaline comet assay. The relative lengths of the comet tails were determined ( $n = 3$ ; mean  $\pm$  S.E.M.). C, BT474 cells (wild-type and rho-zero) were transfected with the indicated siRNA molecules. Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or with lapatinib (1  $\mu\text{M}$ ) and obatoclox (50 nM). Six hours after drug treatment, cells were isolated and subjected to an alkaline comet assay. The relative lengths of the comet tails were determined ( $n = 3$ ; mean  $\pm$  S.E.M.). \*,  $p < 0.05$ , less than the corresponding value for scrambled siRNA-treated cells. D, BT474 cells (wild-type and rho-zero) were treated with vehicle (DMSO) or with lapatinib (1  $\mu\text{M}$ ) and obatoclox (50 nM). Six hours after drug treatment, cells were isolated and subjected to a neutral comet assay. The relative lengths of the comet tails were determined ( $n = 3$ ; mean  $\pm$  S.E.M.). \*,  $p < 0.05$ , less than the corresponding value for scrambled siRNA-treated cells.



**Fig. 6.** Lapatinib and obatoclox radiosensitize breast cancer cells. A and B, BT474 cells (wild-type and rho-zero) (A) and MCF7 cells (wild-type and rho-zero) (B) were transfected with LC3-GFP and with the indicated siRNA molecules. Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or with lapatinib (Lap) (1  $\mu$ M) and obatoclox (GX) (50 nM). Thirty minutes later, cells were mock-exposed or irradiated (4 Gy). Twelve hours (BT474 cells) or 24 h (MCF7 cells) after drug treatment, the numbers of LC3-GFP punctae were determined ( $n = 3$ ; mean  $\pm$  S.E.M.). siSCR, scrambled siRNA. C and D, BT474 cells (wild-type and rho-zero) (C) and MCF7 cells (wild-type and rho-zero) (D) were transfected with the indicated siRNA molecules. Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or with lapatinib (1  $\mu$ M) and obatoclox (50 nM). Thirty minutes later, cells were mock-exposed or irradiated (4 Gy). Twelve hours (BT474 cells) or 24 h (MCF7 cells) after drug treatment, cells were isolated and viability was determined through trypan blue exclusion ( $n = 3$ ; mean  $\pm$  S.E.M.). E, lower, BT474 cells were transfected with an empty vector [cytomegalovirus (CMV)] or plasmid expressing MCL-1. Twenty-four hours after transfection, cells were treated with vehicle (VEH) (DMSO) or with lapatinib (1  $\mu$ M) and obatoclox (50 nM). Twelve hours after drug treatment, cells were isolated and viability was determined through trypan blue



to 24 h after treatment, an effect that was enhanced by irradiation of the cells (Fig. 7A). Quenching of ROS through expression of thioredoxin or use of *N*-acetylcysteine protected cells from lapatinib/obatoclox and radiation lethality (data not shown) (Fig. 7, B and C). This is in general agreement with the data in Fig. 6, C and D, for mitochondria-deficient rho-zero cells, which lack an ROS response (Tang et al., 2012).

Lapatinib/obatoclox treatment increased p38 MAPK and JNK1/2 phosphorylation and Noxa and LC3-II expression, effects that were enhanced by radiation exposure (Fig. 8, A and B). In rho-zero cells, the increases in JNK1/2 and p38 MAPK phosphorylation were blunted and the drug-induced increases in Noxa and LC3-II levels were decreased. Expression of dominant negative p38 MAPK or incubation with the JNK inhibitory peptide suppressed lapatinib/obatoclox toxicity and lapatinib/obatoclox plus radiation toxicity (Fig. 8, C and D). Upstream of p38 MAPK and JNK1/2 is the ROS-sensitive MAPK kinase apoptosis signaling kinase 1 (ASK1); ASK1 is regulated by ROS in part through its association with thioredoxin. Expression of dominant negative ASK1 suppressed drug-induced activation of p38 MAPK and JNK1/2 and inhibited induction of apoptosis (Fig. 8E) (data not shown). Data obtained with dominant negative ASK1 were confirmed through knockdown of ASK1 expression (Fig. 8F).

The metastatic spread of breast cancer is a major cause of patient morbidity; spread from the breast to the lungs, bones, and brain is frequently observed (Taskar et al., 2012). To assess whether lapatinib/obatoclox treatment could affect the growth of breast cancer cells in tissues other than the breast/mammary fat pad, we infused BT474 tumor cells into the brains of athymic mice, allowed tumors to form, and then treated the animals with the drug combination. Treatment of animals with lapatinib and obatoclox caused tumor growth inhibition, as indicated by reduced Ki67 levels that were correlated with prolonged animal survival beyond a simple tumor growth-delay effect (Fig. 9). Collectively, our data indicate that treatment with lapatinib and obatoclox might represent a useful strategy to control the growth of mammary tumors *in vivo*.

## Discussion

In the present study, we aimed to determine the mechanisms through which lapatinib and obatoclox interacted to kill tumor cells and whether the drug combination radiosensitized tumor cells. Lapatinib is an inhibitor of ErbB1/2/4 and is approved for treatment of advanced breast cancer in combination with capecitabine (Arteaga et al., 2012). Obatoclox is an inhibitor of the protective Bcl-2/Bcl-XL/Mcl-1 proteins and has undergone phase II evaluation (Ni Chonghaile and Letai, 2008). Our initial studies with this drug combination were focused on the fact that lapatinib-resistant tumor cells were shown to exhibit elevated Bcl-XL and Mcl-1 expression (Martin et al., 2008). Inhibition of Bcl-XL and Mcl-1 by using molecular tools or obatoclox but not the Bcl-2/Bcl-XL inhibi-

tor navitoclax reversed resistance and enhanced lapatinib toxicity in a greater-than-additive fashion (Cruickshanks et al., 2012; Tang et al., 2012).

Breast cancer and other tumor cell types can become resistant to chemotherapies through multiple mechanisms. Overexpression of growth factor receptors (e.g., ErbB2) and activation of the PI3K pathway through mutation of PI3K itself or loss of PTEN function have been considered as resistance factors (Seminario et al., 2003; Pattingre et al., 2008). We noted that, in tumor cell types that expressed a mutant active PI3K (e.g., MCF7 cells), lapatinib and obatoclox interacted to cause significant amounts of cell killing (Mitchell et al., 2010; Tang et al., 2012). Lapatinib and obatoclox lethality was reduced in breast cancer cells that lacked expression of PTEN. Knockdown of PTEN resulted in less toxicity after drug treatment, whereas expression of PTEN in a PTEN-null cell line restored sensitivity to the drug combination. Previously, we observed similar effects regarding PTEN expression in glioblastoma cells. Collectively, our findings indicate that, in any future clinical applications of this drug combination, PTEN functional status should be assessed before intervention/enrollment. These data also indicate that mutational activation of PI3K and loss of PTEN do not have the same biological effects in tumor cells, at least for the responses to this particular drug combination.

Our previous studies showed that lapatinib and obatoclox treatment induced the formation of autophagic vesicles and cell killing proceeded through a toxic form of autophagy that involved mitochondria-associated proteins, i.e., mitophagy (Tang et al., 2012). Increased expression of toxic BH3 domain proteins, including Noxa and BAK, played a key role in this process. We observed similar levels of toxic autophagy in cells treated with melanoma differentiation-associated gene 7 protein/interleukin 24, in which other toxic BH3 domain proteins (BAX, BAK, Bad, and Bim) played roles in tumor cell death (Dent et al., 2010). In other studies, however, we linked increased autophagy levels to activation of the endoplasmic reticulum stress response, notably after activation of the death receptor CD95; this autophagy response was protective against CD95-induced apoptosis (Park et al., 2008). In the present study, knockdown of the ER sensor proteins PERK and IRE1 $\alpha$  suppressed the drug combination-induced formation of autophagic vesicles. Similar data with respect to survival rates and induction of autophagy were obtained with cells expressing dominant negative PERK. In agreement with the occurrence of an ER stress response, expression of GRP78/BiP was increased after drug treatment. PERK and IRE1 $\alpha$  partially colocalized with the autophagy-related protein p62, as judged through immunohistochemical analyses, in drug-treated cells. These findings indicate that portions of the ER, in addition to mitochondria, must play a role in the formation of toxic autophagic vesicles after lapatinib/obatoclox treatment.

Because p62 interacted with ER-localized proteins, we initially thought that release of Ca<sup>2+</sup> from the ER (or mitochondria) might play a role in regulation of the observed au-

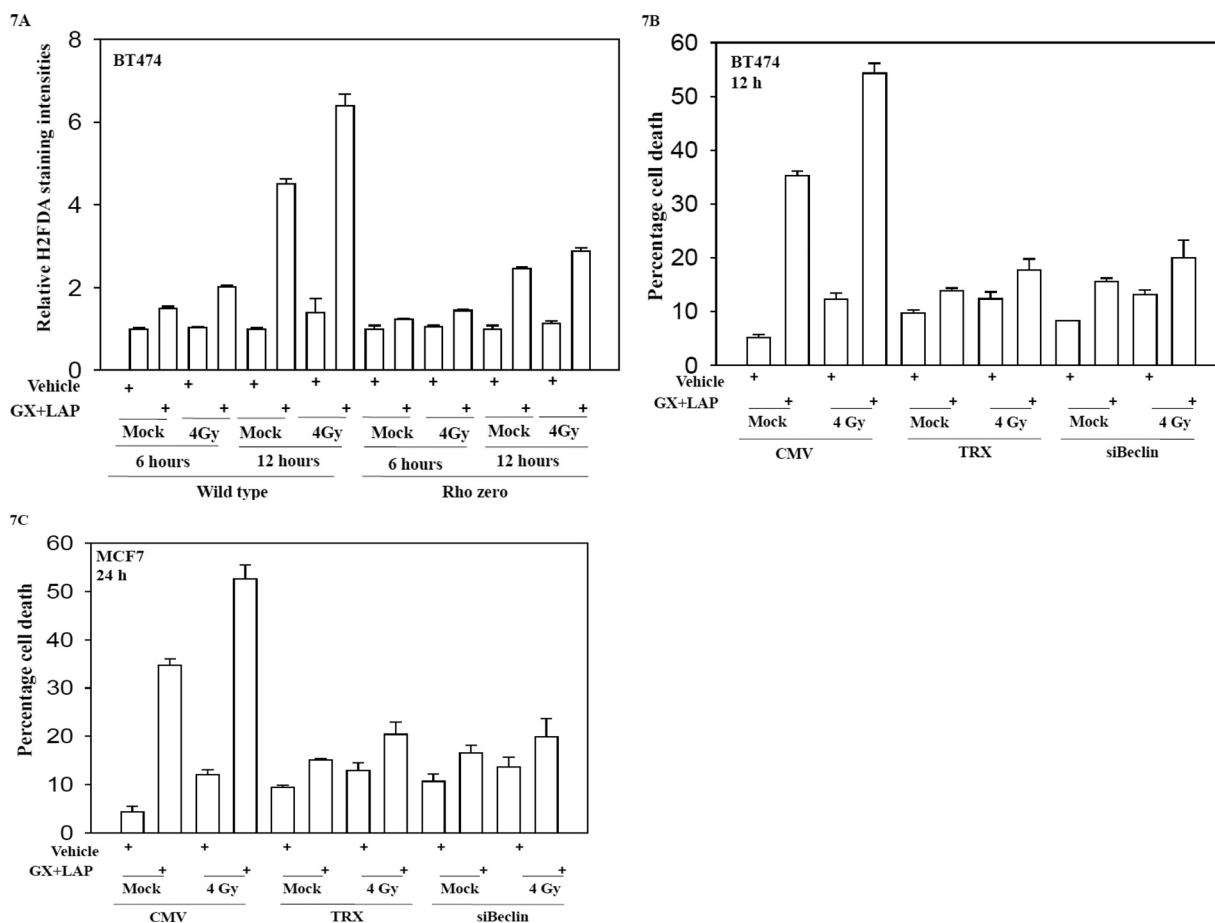
exclusion ( $n = 3$ ; mean  $\pm$  S.E.M.). Upper, BT474 cells were transfected with an empty vector (cytomegalovirus) or plasmid expressing dominant negative PERK (dnPERK). Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or with lapatinib (1  $\mu$ M) and obatoclox (50 nM). Twelve hours after drug treatment, cells were isolated and lysates were subjected to SDS-PAGE and blotting analyses, to determine expression of the indicated proteins. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

tophagy response. Lapatinib/obatoclax treatment did induce release of  $\text{Ca}^{2+}$  into the cytosol, which could be quenched by using the molecular tool calbindin. However, quenching of  $\text{Ca}^{2+}$  release did not block drug-induced ROS production, autophagy, or drug combination lethality. Quenching of  $\text{Ca}^{2+}$  release also did not alter drug-induced changes in the expression of lysosome-associated membrane protein 2, LC3-II, or p62. We conclude that  $\text{Ca}^{2+}$  release from the ER (or mitochondria) is not an important regulatory factor with respect to our drug combination.

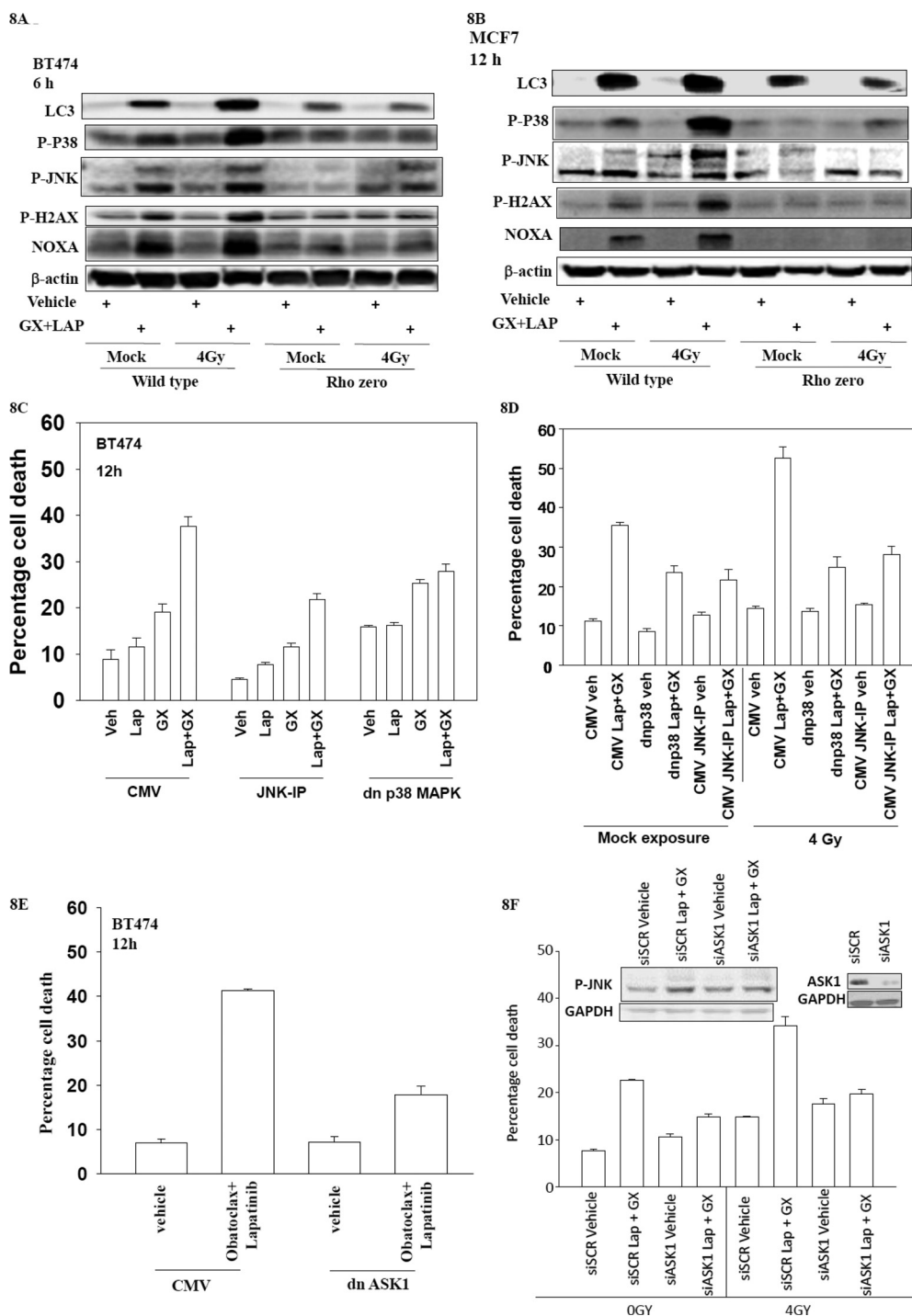
A multitude of drugs can induce various forms of DNA damage. Unlike drugs such as cisplatin, neither lapatinib nor obatoclax is an agent that would be expected to interact directly with DNA to cause DNA damage. Treatment of cells with lapatinib and obatoclax did increase DNA damage, however, as measured by using several assays. The phosphorylation of histone  $\gamma\text{H2AX}$  was unaltered in rho-zero cells, in comparison with wild-type cells in which phosphorylation was enhanced by lapatinib/obatoclax treatment, an effect that was further stimulated by radiation exposure. DNA damage was dependent on the generation of ROS and on the induction of autophagy. Our previous studies with lapatinib and obatoclax placed the generation of ROS upstream of drug combination-induced autophagy. ROS signaling was also up-

stream of the ER-sensing proteins PERK and IRE1 $\alpha$ . In the literature, DNA damage has most often been reported to induce autophagy, with this autophagy generally having a protective role (Robert et al., 2011; Rodriguez-Rocha et al., 2011; Yoon et al., 2012). This is opposite our findings of ROS/autophagy stimulating DNA damage and autophagy being a toxic event. Determination of whether other drug combinations cause autophagy upstream of DNA damage would require studies beyond the scope of the present work.

There are multiple signaling pathways in cells, with some (e.g., extracellular signal-regulated kinase 1/2) most often being associated with growth and survival and others (e.g., JNK1/2) usually being associated with tumor cell killing. Multiple studies have indicated that activation of JNK1/2 plays a central role in radiation-induced apoptosis (Valerie et al., 2007). We showed previously that expression of activated forms of p70 S6K and mTOR protected cells from lapatinib/obatoclax lethality (Tang et al., 2012). Drug combination exposure increased the activities of p38 MAPK and JNK1/2. Irradiation of drug-treated cells further increased the activities of both kinases. Activation of p38 MAPK and JNK1/2 was decreased in rho-zero cells, compared with wild-type cells, which indicates that ROS generation was required for activation. Expression of a dominant negative form of the



**Fig. 7.** Radiation interacts with lapatinib/obatoclax treatment through ROS generation to kill tumor cells. A, BT474 cells (wild-type and rho-zero) were treated with vehicle (DMSO) or with lapatinib (LAP) (1  $\mu\text{M}$ ) and obatoclax (GX) (50 nM). Thirty minutes later, cells were mock-exposed or irradiated (4 Gy). After 6 and 12 h, ROS levels were measured by using dihydrofluorescein diacetate (H2FDA) ( $n = 3$ ; mean  $\pm$  S.E.M.). B and C, BT474 cells (B) and MCF7 cells (C) were transfected with empty vector [cytomegalovirus (CMV)] or with plasmid expressing thioredoxin (TRX). Cells were transfected with the indicated siRNA molecules. Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or with lapatinib (1  $\mu\text{M}$ ) and obatoclax (50 nM). Twelve hours (BT474 cells) or 24 h (MCF7 cells) after drug treatment, cells were isolated and viability was determined through trypan blue exclusion ( $n = 3$ ; mean  $\pm$  S.E.M.).



**Fig. 8.** Increased Noxa expression and phosphorylation of histone  $\gamma$ H2AX, p38 MAPK, and JNK1/2 are dependent on ROS generation. A and B, BT474 cells (wild-type and rho-zero) (A) and MCF7 cells (wild-type and rho-zero) (B) were treated with vehicle (DMSO) or with lapatinib (LAP) (1  $\mu$ M) and obatoclox (GX) (50 nM). Thirty minutes later, cells were mock-exposed or irradiated (4 Gy). After 6 and 12 h, cells were isolated and subjected to SDS-PAGE and blotting analyses for the indicated proteins. C, BT474 cells were infected with recombinant adenovirus to express nothing [cytomegalovirus (CMV)] or to express dominant negative (dn) p38 MAPK. Twenty-four hours later, portions of cells were treated with DMSO or the JNK inhibitory peptide (JNK-IP) (10  $\mu$ M). Cells were then treated with vehicle (Veh) (DMSO) or with lapatinib (1  $\mu$ M) and obatoclox (50 nM). Twelve hours later, cells were isolated and viability was determined through trypan blue exclusion ( $n = 3$ ; mean  $\pm$  S.E.M.). D, BT474 cells were infected with recombinant adenovirus to express nothing (cytomegalovirus) or to express dominant negative p38 MAPK. Twenty-four hours later, portions of cells were treated with DMSO or the JNK inhibitory peptide (10  $\mu$ M). Cells were then treated with vehicle (DMSO) or with lapatinib (1  $\mu$ M) and obatoclox (50 nM). Thirty minutes later, cells were irradiated. Twelve hours later, cells were isolated and viability was determined through trypan blue exclusion ( $n = 3$ ; mean  $\pm$  S.E.M.). E, BT474 cells were transfected with empty vector (cytomegalovirus) or with a plasmid expressing dominant negative ASK1. Twenty-four hours later, cells were treated with vehicle (DMSO) or with lapatinib (1  $\mu$ M) and obatoclox (50 nM). Twelve hours later, cells were isolated and viability was determined through trypan blue exclusion ( $n = 3$ ; mean  $\pm$  S.E.M.). F, BT474 cells were transfected with scrambled siRNA (siSCR) or with a siRNA to knock down ASK1 (siASK1). Twenty-four hours later, cells were treated with vehicle (DMSO) or with lapatinib (1  $\mu$ M) and obatoclox (50 nM), with or without radiation exposure (4 Gy). Twelve hours later, cells were isolated and viability was determined through trypan blue exclusion ( $n = 3$ ; mean  $\pm$  S.E.M.). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.



ROS-sensitive kinase ASK1 or overexpression of thioredoxin prevented activation of both p38 MAPK and JNK1/2. Molecular inhibition of p38 MAPK and JNK1/2 suppressed cell killing by the drug combination alone or together with radiation. These data suggest that a separate ROS-dependent route, involving the p38 MAPK and JNK1/2 pathways, exists downstream of mitochondria to signal tumor cell death after lapatinib/obatoclax treatment.

The metastatic spread of breast cancer is invariably associated with poor long-term prognoses. One site of breast cancer spread, particularly for ErbB2<sup>+</sup> tumor cell types, is the brain. We found that treatment of established intracranial ErbB2<sup>+</sup> BT474 tumors with lapatinib and obatoclax prolonged animal survival beyond simple tumor growth delay. Previous studies with glioma cells indicated that tumor cells growing in the brains of athymic mice were sensitive to lapatinib/obatoclax treatment. If the lapatinib/obatoclax drug combination is used clinically, it will be of interest to determine whether patients with metastatic tumors in the brain exhibit a similar degree of tumor response, compared with the responses of tumors growing in the breast (Tang et al., 2012).

In conclusion, treatment with lapatinib plus obatoclax kills mammary tumor cells through a mechanism in which ER stress signaling stimulates a toxic form of autophagy. The DNA damage associated with this treatment occurs downstream of ROS generation and autophagy. Drug combination treatment radiosensitized tumor cells through the actions of the toxic BH3 domain protein Noxa and the actions of p38 MAPK and JNK1/2. Mammary tumors growing in the brains of mice were susceptible to lapatinib/obatoclax treatment.

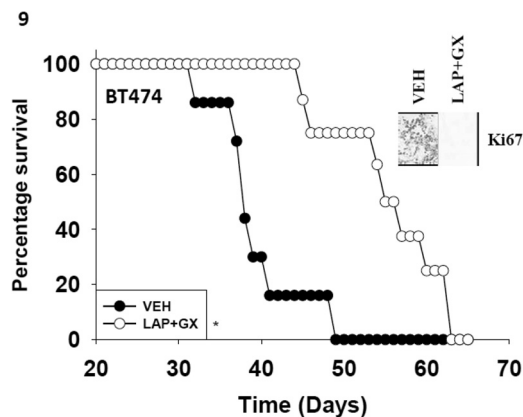
#### Authorship Contributions

Participated in research design: Grant and Dent.

Conducted experiments: Cruickshanks, Tang, Booth, and Hamed.

Performed data analysis: Cruickshanks and Dent.

Wrote or contributed to the writing of the manuscript: Cruickshanks and Dent.



**Fig. 9.** Lapatinib and obatoclax prolong survival of animals with intracranial mammary tumors. BT474 cells ( $1 \times 10^6$ ) were implanted into the brains of mice. Eight days after implantation, animals were treated for 5 days with vehicle (VEH) (Cremophor) or the combination of lapatinib (LAP) (100 mg/kg b.i.d.) and obatoclax (GX) (5 mg/kg daily). Animals were humanely sacrificed when near death because of tumor burden (two studies,  $n = 6$ ; mean  $\pm$  S.E.M.). \*,  $p < 0.05$ , greater than vehicle treatment value. Inset, 5 days after the start of treatment, brains were removed and sectioned (10  $\mu$ m), and immunohistochemical analyses were performed to assess levels of Ki67 staining.

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