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Lapatinib Resistance in HCT116 Cells Is Mediated by Elevated MCL-1 Expression and Decreased BAK Activation and Not by ERBB Receptor Kinase Mutation[S]

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ABSTRACT

We have defined some of the mechanisms by which the kinase inhibitor lapatinib kills HCT116 cells. Lapatinib inhibited radiation-induced activation of ERBB1/2, extracellular signal-regulated kinases 1/2, and AKT, and radiosensitized HCT116 cells. Prolonged incubation of HCT116 cells with lapatinib caused cell killing followed by outgrowth of lapatinib-adapted cells. Adapted cells were resistant to serum starvation-induced cell killing and were cross-resistant to multiple therapeutic drugs. Lapatinib was competent to inhibit basal and epidermal growth factor (EGF)-stimulated ERBB1 phosphorylation in adapted cells. Coexpression of dominant-negative ERBB1 and dominant-negative ERBB2 inhibited basal and EGF-stimulated ERBB1 and ERBB2 phosphorylation in parental and adapted cells. However, in neither parental nor adapted cells did expression of dominant-negative ERBB1 and dominant-negative ERBB2 recapitulate the cell death-promoting effects of lapatinib. Adapted cells had increased expression of MCL-1, decreased expression of BAX, and decreased activation of BAX and BAK. Overexpression of BCL-XL protected parental cells from lapatinib toxicity. Knockdown of MCL-1 expression enhanced lapatinib toxicity in adapted cells that was reverted by knockdown of BAK expression. Inhibition of caspase function modestly reduced lapatinib toxicity in parental cells, whereas knockdown of apoptosis-inducing factor expression suppressed lapatinib toxicity. Thus, in HCT116 cells, lapatinib adaptation can be mediated by altered expression of pro- and antiapoptotic proteins that maintain mitochondrial function.

The ERBB receptor family consists of four members: ERBB1, ERBB2, ERBB3, and ERBB4 (Olayioye et al., 2000; Yarden and Sliwkowski, 2001). These receptors are present on the cell surface as monomers, and upon binding ligand, they can homo- or heterodimerize and autophosphorylate specific tyrosine residues on the cytosolic domain of the receptors, which then serve as docking sites for molecules that can mediate downstream intracellular signaling (Lin and Winer, 2004; Hynes and Lane, 2005; Nelson and Dolder, 2006). Many cancer types have been shown to have deranged ERBB receptor signaling via various mechanisms such as constitutive receptor activation, impaired receptor down-regulation, and increased receptor stimulation via an autocrine loop, leading to constitutive downstream prosurvival signaling, resulting in aggressive tumors (Sizeland and Burgess, 1992; Salomon et al., 1995; Peschard and Park, 2003; Hynes

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ABBREVIATIONS: siRNA, small interfering RNA; Lap, lapatinib; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated extracellular signal-regulated kinase; EGF, epidermal growth factor; PI3K, phosphatidyl inositol 3 kinase; dn, dominant negative; ca, constitutively active; WT, wild type; AIF, apoptosis-inducing factor; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; NFκB, nuclear factor κB; Ab, antibody; IGF-1R, insulin-like growth factor-1 receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; zVAD, *N*-benzyloxycarbonyl-valyl-alanyl-aspartyl; SCR, scrambled; PI, phosphatidylinositol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; PPP, picropodophyllin; UCN-01, 7-hydroxystaurosporine; VP-16, etoposide; ABT-737, (*R*)-4-(3-dimethylamino-1-phenylsulfanylmethyl-propylamino)-*N*-{4-[4-(4′-chloro-biphenyl-2-ylmethyl)-piperazin-1-yl]-benzoyl}-3-nitro-benzenesulfonamide.

and Lane, 2005; Sunpaweravong et al., 2005). Hence, studies have been performed to identify mechanisms via which ERBB receptors and their downstream signaling pathways can be inhibited in such tumor cells. Clinically used techniques to inhibit receptor activation include 1) monoclonal antibodies that prevent paracrine ligand binding to the receptors, 2) quinazoline-derived small-molecule tyrosine kinase inhibitors that prevent ATP binding in the kinase domain of the receptors, thereby preventing the receptor kinase activity required to activate downstream signaling; and 3) siRNA-mediated knockdown of ERBB receptor expression (Schmidt-Ullrich et al., 2003; Imai and Takaoka, 2006).

Lapatinib is a reversible small-molecule tyrosine kinase inhibitor developed by GlaxoSmithKline that inhibits ERBB1 and ERBB2 activation. The IC_{50} values for purified ERBB1 and EBB2 inhibition by lapatinib are 10.2 and 9.8 nM, respectively (Rusnak et al., 2001; Wood et al., 2004; Xia et al., 2006, 2007). Lapatinib showed a >300-fold selectivity for ERBB1 and ERBB2 inhibition compared with its ability to inhibit a panel of other kinases commonly found in cells (Rusnak et al., 2001). Lapatinib has been studied in Herceptin/trastuzumab-resistant breast cancer lines and induces apoptosis in such cells by inhibiting downstream prosurvival signaling mediated by ERBB1, ERBB2, and insulin-like growth factor-1 receptor (IGF-1R) (Konecny et al., 2006; Nahta et al., 2007). Combination studies involving lapatinib and BCL-2 inhibitors in certain cancer cells show synergistic antitumor effects (Reed et al., 1996; Witters et al., 2007). Lapatinib (Tykerb) was recently approved by the Food and Drug Administration to be used in combination with capecitabine to treat patients with advanced or metastatic breast cancer that overexpresses ERBB2 and has been treated previously with other drugs (available at http://www.cancer.gov/cancertopics/druginfo/fda-lapatinib).

It is generally accepted that cancer patients treated with chemotherapeutic agents usually respond well to the treatment initially, resulting in the reduction of tumor size and death of cancer cells. However, months or years later, the cancer can reappear as an aggressive and therapeutically refractory malignancy that may also be cross-resistant to many other therapeutic drug treatments, making such cancers very difficult to manage (Kobayashi et al., 2005). Resistance to trastuzumab has been suggested to be mediated via IGF-1R and/or c-Met signaling that can activate downstream prosurvival pathways (Lu et al., 2001; Camirand et al., 2002). Src and the estrogen receptor have also been implicated in mediating resistance to tyrosine kinase inhibitors, including lapatinib, by activating and/or reactivating prosurvival signaling pathways (Qin et al., 2006a; Xia et al., 2006). Resistance to ERBB-targeted drugs can also occur because of mutations in ERBB receptor kinase domains, resulting in the inability of the drug to inhibit the receptor kinase domain or ligand-independent constitutive activation of the receptor (Pao et al., 2005; Sok et al., 2006). Multidrug resistance pumps, overexpression of antiapoptotic molecules belonging to the BCL-2 family, and constitutive activity of NFκB have also been linked to drug resistance in a wide variety of cancer cell types (Raffo et al., 1995; Cabannes et al., 1999; Sumitomo et al., 1999; Baldwin, 2001; Szakács et al., 2006). Single-agent lapatinib activity has not been noted in the treatment of colon cancer, although various colon cancer cell lines have been reported to express ERBB1 and ERBB2 receptors, and lapatinib can cause apoptosis and inhibition of cell proliferation in vitro and in vivo in colon cancer cell lines (Cunningham et al., 2006; Zhou et al., 2006).

Growth factor signals, via guanine nucleotide exchange factors, activate RAS proteins (Sklar, 1988; Cox and Der, 2003). There are three widely recognized isoforms of RAS: Harvey (H), Kirsten (K), and Neuroblastoma (N). Mutation of RAS in cancer results in a loss of GTPase activity, generating a constitutively active RAS molecule downstream of growth factor receptors that can lead to elevated activity within intracellular signaling pathways. Thus, expression of a mutated active RAS protein has the potential to circumvent the antiproliferative and tumoricidal impact of inhibiting ERBB receptor function. Approximately one third of human cancers have RAS mutations, primarily the K-RAS isoform, that leads to a radioprotected phenotype (Sklar, 1988; Goldkorn et al., 1997; Dent et al., 1999, 2003; Ellis and Clark, 2000;). It is noteworthy that some studies suggest that K-RAS and H-RAS have different but overlapping signaling specificities to downstream pathways, as judged by in vitro cell-based studies and in animal knockout models (Ross et al., 2001; Yan et al., 1998; Liebmann, 2001; Chuang et al., 1994; Joneson et al., 1996; Dent et al., 1999; Lüdde et al., 2001; Moriuchi et al., 2001; Carón et al., 2005a,b).

HCT116 colon cancer cells express a mutated active K-RAS Asp13 protein but are also noted to be dependent for their in vitro growth on an ERBB1-transforming growth factor-α/epiregulin paracrine loop and totally dependent for their in vivo tumorigenic potential on both an ERBB1-epiregulin paracrine loop and K-RAS Asp13 expression (Shirasawa et al., 1993; Sizemore et al., 1999; Baba et al., 2000). The studies described in this article were initiated to determine some of the molecular mechanisms by which HCT116 cells survived prolonged exposure to the RTK inhibitor lapatinib.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium, penicillin/streptomycin, and 0.25% trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA). HCT116 cells were originally purchased from American Type Culture Collection (Manassas, VA) before multiple transfection procedures. Fetal bovine serum was purchased from Hyclone (Logan, UT). Trypan blue dye and crystal violet for colony-formation assays were purchased from Sigma-Aldrich (St. Louis, MO). For Western blot analysis, 8 to 16% Tris-HCl gels were used (Bio-Rad Laboratories, Hercules, CA). CMV control virus, ERBB1-CD533, and ERBB2-CD572 were obtained from Dr. Kristoffer Valerie (Virginia Commonwealth University, Richmond, VA). BCL-XL recombinant adenovirus was obtained from Dr. J. Moltken (University of Cincinnati, Cincinnati, OH). Dominant-negative (dn) dnIκB (S32A) and dnSTAT3 recombinant adenoviruses purchased from Cell Biolabs (Philadelphia, PA). Control siRNA and siRNA to knockdown apoptosis-inducing factor (AIF) (SI02662114, SI02662653), BCL-XL (SI03025141, SI03068352, SI03112018, SI00023191), MCL-1 (SI02781205, SI00131768), and BAK (SI00299376, SI02654512) were purchased from QIAGEN (Valencia, CA). Lapatinib was obtained from GlaxoSmithKline (Boston, MA). The IGF-1R inhibitor PPP, the Src family kinase inhibitor PP2, 4-hydroxy tamoxifen, and epidermal growth factor were purchased from Calbiochem (San Diego, CA). Primary antibodies against MCL-1, BCL-XL, BAX, BAK, AIF, and cytochrome *c* were purchased from Cell Signaling Technology Inc. (Danvers, MA). ERBB1 (Ab-5) antibody for fluorescence microscopy, primary antibody for active BAK (Ab-1), caspase 8 inhibitor LEHD, caspase 9 inhibitor IETD, and pan-caspase inhibitor zVAD were purchased from Calbiochem. EGF receptor (Ab-13 cock-

tail) and c-ERBB2 (Ab-11 cocktail) to immunoprecipitate ERBB1 and ERBB2 were purchased from NeoMarkers (Freemont, CA). Anti-PhosphoTyr 4G10 antibody was purchased from Millipore Bioscience Research Reagents (Temecula, CA). Primary antibodies for GAPDH, wild-type p53 (FL-393), mutant p53 (Pab 240), ERK2, active BAX (6A7), and protein A/G Plus agarose beads for immunoprecipitation were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary mouse antibody (Alexa Fluor 680 goat anti-mouse IgG) was purchased from Invitrogen, and secondary rabbit antibody (anti-rabbit IgG) was purchased from Rockland Immunochemicals (Gilbertsville, PA). UCN-01 was kindly supplied by was provided by the Cancer Treatment and Evaluation Program of the National Cancer Institute. VP-16 was purchased from Sigma. All other materials and basic methods of approach were as described elsewhere (Dent et al., 1999; Carón et al., 2005a,b).

Detection of Cell Death by Trypan Blue Assay. After treatment, medium was removed, and cells were washed in 1× phosphate-buffered saline. Cells were then harvested by trypsinization with trypsin/EDTA for ~5 min at 37°C. Because some apoptotic cells detached from the culture substratum into the medium, these cells were also collected by centrifugation of the medium at 1400 rpm for 5 min. The pooled cell pellets were resuspended and mixed with trypan blue dye. Trypan blue stain, in which blue dye-incorporating cells were scored as being dead, was performed by counting of cells using a light microscope and a hemacytometer. The number of dead cells was counted and expressed as a percentage of the total number of cells counted.

Culture of Cells and Drugs Treatments for Colony Formation Assays. Cells were plated (250–1000 cells/well of a six-well plate). Twelve hours after plating, medium was removed, and serum-free medium was added to the cells for 24 or 48 h as indicated. After this, the serum-free media were carefully removed, and fresh media (with serum) were added. Colony formation assays were cultured for an additional 8 to 10 days, after which the media were removed, cells were fixed with methanol, stained with crystal violet, and counted manually.

Immunoprecipitation and Western Blotting. Twelve hours after plating cells, they were either infected with ERBB1-CD533 and ERBB2-CD572 or control virus for 24 h or serum-starved and treated with indicated concentrations of lapatinib or dimethyl sulfoxide (DMSO) for 2 h. After either of these treatments, cells were treated with 20 ng/ml EGF or vehicle for 10 min. Cells were then scraped using radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM β-glycerophosphate, 0.5 mM EGTA, 0.5 mM EDTA, and protease inhibitor cocktail purchased from Roche, Indianapolis, IN) and ERBB1 or ERBB2 was immunoprecipitated as indicated, after which samples were boiled for 10 min in whole-cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, and 0.02% bromphenol blue). Twelve hours after plating cells, they were also scraped using a nondenaturing lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, protease, and phosphatase inhibitor cocktails; Roche) and mutant p53 was immunoprecipitated, after which samples were boiled for 10 min in whole-cell lysis buffer.

Cells were also scraped in CHAPS buffer (10 mM HEPES, 140 mM NaCl, and 1% CHAPS), and then active BAK or active BAX was immunoprecipitated. Samples were boiled for 10 min in whole-cell lysis buffer. All samples were then loaded on 8 to 16% Criterion precast gels (Bio-Rad Laboratories) after normalizing total protein and was run for approximately 2 h. Proteins were then electrophoretically transferred onto 0.22 μm nitrocellulose membranes and immunoblotted with various primary antibodies as indicated.

Virus Infections. Cells were infected 12 h after plating with adenoviruses at an approximate multiplicity of infection of 30 for 4 h with gentle rocking, after which time the media was replaced. Cells were further incubated for 24 h to ensure adequate expression of transduced gene products before drug exposures.

Transfection of Cells with Small Interfering RNA Molecules. RNA interference for down-regulating the expression of AIF, MCL-1, BCL-XL, and BAK was performed using validated target sequences designed by QIAGEN. For transfection, a 20 nM concentration of the annealed siRNA-targeting AIF, MCL-1, BCL-XL, or BAK or the negative control, a "scrambled" (SCR) sequence with no significant homology to any known gene sequences from mouse, rat, or human cell lines, was used. The siRNA molecules were transfected into cells according to the manufacturer's instructions. Cells were cultured for 48 h after transfection before any additional experimentation.

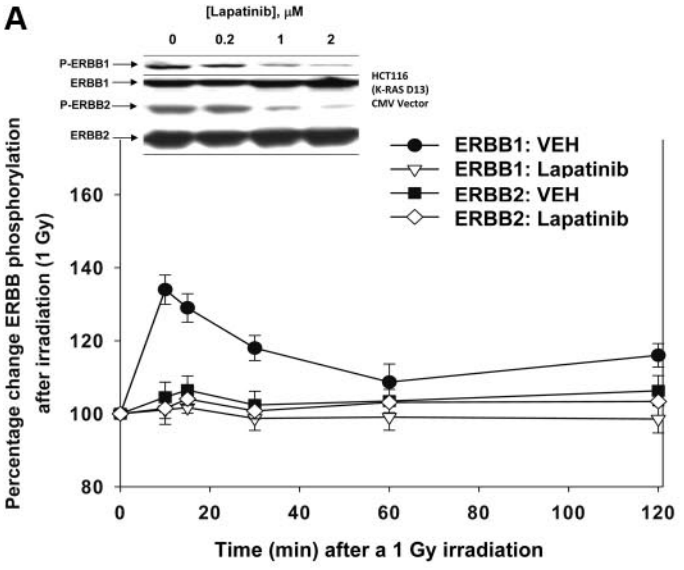
Cell Fractionation. Twelve hours after plating cells, they were serum-starved and treated with 2 μM lapatinib or DMSO for 36 h. This experiment was performed on ice at all times. Medium from plates was then aspirated, and cells were scraped in buffer (75 mM NaCl + 8 mM Na_2HPO_4 + 1 mM $Na_2H_2PO_4$ + 0.5 mM EDTA + 0.5 mM EGTA with freshly added 350 μg/ml digitonin, 250 mM sucrose, protease, and phosphatase inhibitor cocktails; Roche) and passed 12 times through a 25-gauge needle. After 15 to 30 min on ice, cells were spun down at 5000 rpm for 1.5 min at 4°C to remove cell debris. Pellet was discarded, and supernatant was transferred to a new tube and spun down at 13,000 rpm for 25 min at 4°C. The supernatant obtained is the cytosolic fraction, whereas the pellet is the mitochondrial fraction. Whole-cell lysis buffer was added to the supernatant and the pellet, boiled for 10 min, and then Western blot analysis was performed. This protocol was adapted from Leist et al. (1998).

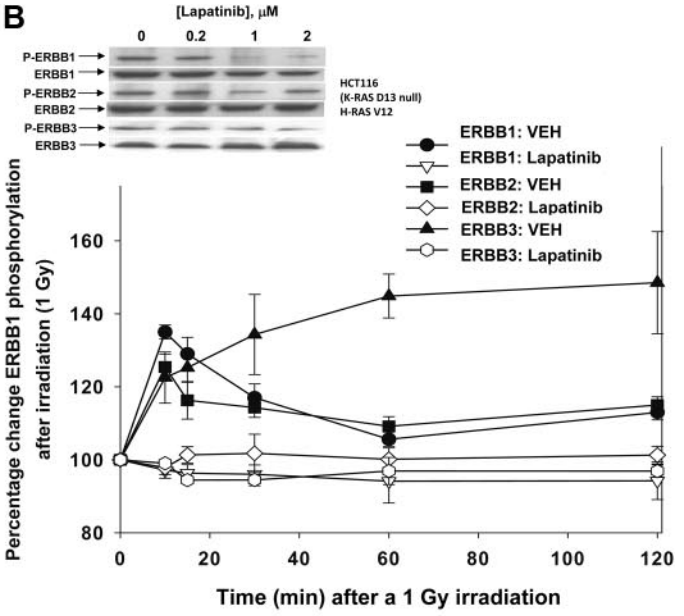
Flow Cytometry. Flow cytometric analysis of cells was performed after staining by the Annexin V-FITC kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions and read on a FACScan (BD Biosciences, San Jose, CA). In brief, media containing cells were collected. Cells were isolated by brief trypsinization, and this together with the growth media was centrifuged to isolate a cell pellet. The cell pellet was resuspended in phosphate-buffered saline on ice containing an fluorescein isothiocyanate-conjugated anti-Annexin V antibody followed by addition to this suspension of propidium iodide. The suspension was then within ~5 min subjected to flow cytometry to determine early apoptosis (annexin+ cells); early necrosis (propidium+ cells); or later forms of cell death (Annexin+ propidium+). Unless otherwise stated, where the percentage of cell death was calculated using this method, the percentage cell death included all registering PI+ and all annexin+ cells.

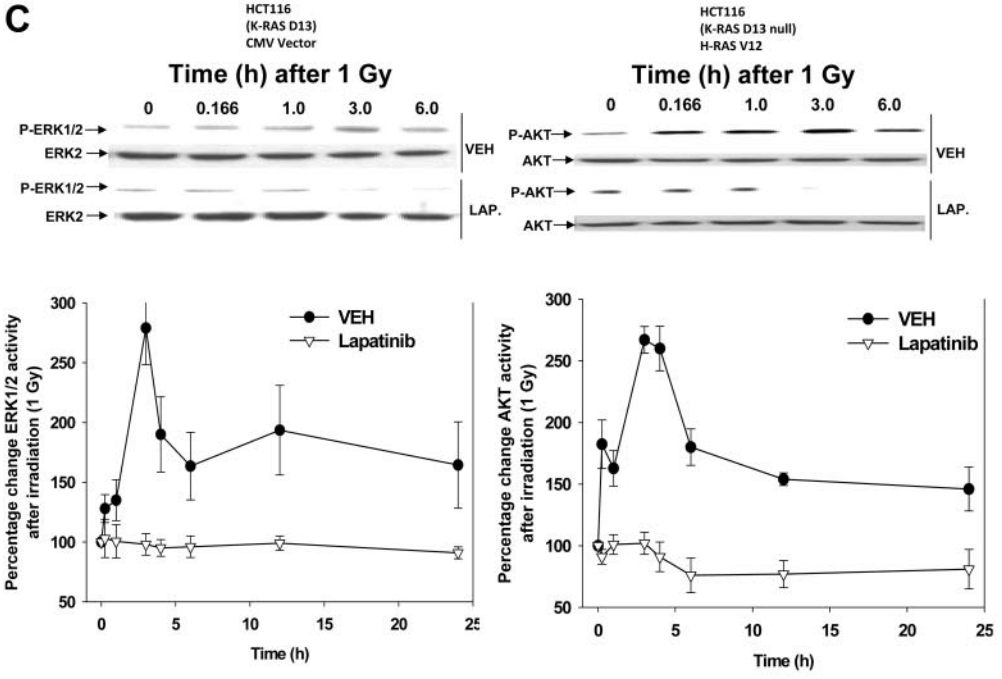
Data Analysis. Comparison of the effects of various treatments was performed after analysis of variance using the Student's t test. Differences with a p value of < 0.05 were considered statistically significant. Experiments shown are the means of multiple individual points (± S.E.M.).

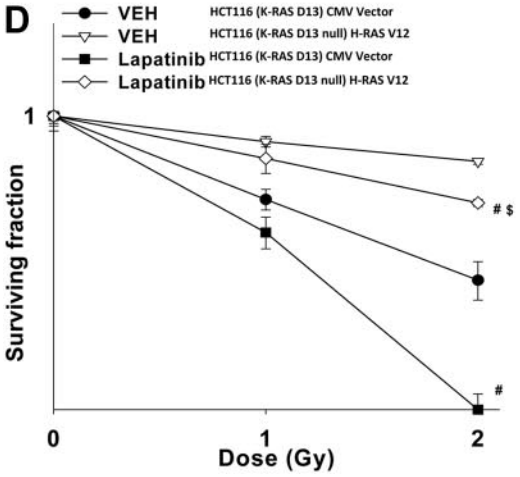
Results

Lapatinib is a clinically relevant receptor tyrosine kinase inhibitor that binds to the kinase domains of ERBB1 and ERBB2. ERBB1 and ERBB2 have been shown previously to act upstream of RAS proteins in radiation-induced signal transduction pathways and to play a role in protecting tumor cells from the toxic effects of ionizing radiation. As indicated, lapatinib blocked radiation-induced tyrosine phosphorylation of ERBB1, ERBB2, and ERBB3 in parental HCT116 cells and in HCT116 cells expressing H-RAS Val12 (Figs. 1, A and B, and S1) (Carón et al., 2005a,b). Inhibition of ERBB family receptor function correlated with lapatinib inhibiting radiation-induced activation of ERK1/2 and AKT (Fig. 1C). Lapatinib radiosensitized parental HCT116 cells expressing K-RAS Asp13 and HCT116 cells expressing H-RAS Val12 (Fig. 1D). These findings demonstrate that in the presence of expressed mutated active K-RAS and H-RAS proteins, the









pan-ERBB receptor inhibitor lapatinib can act as a radiosensitizer in HCT116 cells.

The development of resistance to ERBB receptor inhibitors has been observed clinically. In many of these studies, resistance to the ERBB tyrosine kinase inhibitor has been due to mutation of the receptor within its catalytic domain so that the kinase inhibitor no longer can bind and inhibit receptor tyrosine kinase activity. We cultured parental HCT116 cells in 10 μM lapatinib, a concentration that is lower than the C_{max} for this drug in patients, although the average plasma profile of a 1500-mg daily dose peaks at ~2.5 μM; within 72 h, many cells (>95%) became detached and died from this drug exposure (data not shown). Cells were cultured in the presence of lapatinib for a further ~3 months until a homogeneous population of cells grew out from the survivors that were adapted to lapatinib.

In assays to determine cell survival in the absence of serum with a lapatinib challenge, lapatinib-adapted cells survived to a significantly greater extent than parental cells (Figs. 2, A and B, and S2, in the presence of serum). Lapatinib-adapted cells grew more quickly than parental cells in the presence or absence of lapatinib (Fig. S3). In general agreement with these findings, lapatinib-resistant cells had a greater level of survival than parental cells in colony-formation assays (Fig. 2C). When lapatinib-adapted cells were cultured in the absence of lapatinib for >10 flask passages (~2 months), no reversion of the resistant phenotype was observed back to the parental phenotype (Fig. 2D). Lapatinib-adapted cells were cross-resistant to multiple chemotherapeutic agents, including VP-16, UCN-01, Taxotere, oxaliplatin, and doxorubicin (Figs. S4–S6). Resistance to Taxotere seemed to be somewhat less than to the other agents. Because drug efflux could represent a mechanism of lapatinib adaptation, particularly as we observed cross-resistance to multiple cytotoxic therapeutic drugs, we performed flow cytometric and immunoblotting analyses to determine the expression of ATP-binding cassette and multidrug-resistant plasma membrane drug transporters. Little change in the protein levels of any membrane drug transporter was observed, however, comparing wild-type and lapatinib-adapted HCT116 cells, arguing that changes in drug efflux was unlikely to be a major component of the lapatinib-resistance mechanism under investigation (Fig. S7).

Based on the above findings, we examined in molecular detail the role of ERBB receptors in lapatinib resistance. Coexpression of dominant-negative ERBB1 (CD533) and dominant-negative ERBB2 (CD572) proteins suppressed basal and EGF-stimulated tyrosine phosphorylation of ERBB1/ERBB2 in immunoprecipitates from parental and adapted HCT116 cells (Fig. 3A, top, inset). These findings are in general agreement with multiple studies performed previously in this laboratory (Dent et al., 1999). To our surprise, however, whereas coexpression of ERBB1 (CD533) and ERBB2 (CD572) acted in a manner very similar to lapatinib to inhibit ERBB receptor tyrosine phosphorylation, the dominant-negative receptors did not recapitulate the toxic effects of lapatinib in serum-starved parental or adapted cells (Fig. 3A, bottom). Further analyses revealed that parental and lapatinib-adapted cells expressed similar total cellular amounts of ERBB1 as judged by immunoblotting of whole-cell lysate, and that stimulated ERBB1 phosphorylation in response to EGF was inhibited equally well by lapatinib in both parental and adapted cells (Fig. 3B). However, the plasma membrane-associated levels of ERBB1 in adapted cells were considerably lower in adapted than those in parental cells (Fig. 3C). These findings were reflected also in a reduced ability of EGF to stimulate ERK1/2 signaling in adapted cells compared with parental cells (Fig. 3B). Together, these findings strongly argue that an ERBB1 kinase domain mutation point has not occurred in lapatinib-adapted HCT116 cells to make these cells resistant to lapatinib, modifying the toxicity of lapatinib in these cells.

We then examined/screened in an unbiased fashion the activities of known signaling pathways whose activities could become altered in the adapted HCT116 cell line. However, almost no difference in basal activities of any established signal transduction pathway or in the basal activity of any pathway in the presence of lapatinib could be obviously observed between parental and adapted cells (data not shown). In HCT116 cells expressing H-RAS Val12 and effector mutants of H-RAS Val12 that had been characterized to specifically activate the Raf-MEK-ERK pathway (Ser35), the RAL-GDS pathway (Gly37), and the PI3K-AKT pathway (Cys40), H-RAS Val12 expression, but not the expression of any H-RAS Val12 single point mutant that activated a single signaling pathway, suppressed lapatinib toxicity (Fig. 4A). In contrast to our findings with lapatinib, for example, expression of H-RAS Val12, H-RAS Val12 Ser35, and H-RAS Val12 Cys40, but not H-RAS Val12 Gly37, acted to protect HCT116 cells from the toxic effects of radiation in colony-formation

Fig. 1. Lapatinib blocks radiation-induced activation of ERBB1, ERBB2, and ERBB3 and activation of ERK1/2 and AKT in HCT116 cells. A and B, bottom, parental HCT116 cells and HCT116 (K-RAS Asp13-null) cells stably transfected with a plasmid to express H-RAS Val12 were plated and 24 h after plating were grown in serum-depleted medium. Two hours after serum withdrawal, cells were treated with either vehicle (DMSO) or lapatinib (2 μM) and 30 min later were irradiated (1 Gy). Cells were isolated at the indicated time points and subjected to SDS-PAGE to determine the phosphorylation and expression of ERBB1, ERBB2, and ERBB3 as indicated, as performed previously (Carón et al., 2005b). The -fold alteration in the phosphorylation of ERBB1, ERBB2, and ERBB3 in the presence or absence of lapatinib after irradiation is presented from three separate experiments ± S.E.M. each performed in duplicate for parental HCT116 cells and HCT116 (K-RAS Asp13-null) cells stably transfected with a plasmid to express H-RAS Val12. Top, inset, parental HCT116 cells and HCT116 (K-RAS Asp13-null) cells stably transfected with a plasmid to express H-RAS Val12 were plated and 24 h after plating were grown in serum-depleted medium. Two hours after serum withdrawal, cells were treated with either vehicle (DMSO) or lapatinib (0–2 μM), and 30 min later, cells were isolated. The phosphorylation of ERBB1, ERBB2, and ERBB2 and the expression of ERBB1, ERBB2, and ERBB3 were determined by immunoblotting (n = 3). C, bottom, parental HCT116 cells and HCT116 (K-RAS Asp13-null) cells stably transfected with a plasmid to express H-RAS Val12 were plated and 24 h after plating were grown in serum-depleted medium. Two hours after serum withdrawal, cells were treated with either vehicle (DMSO) or lapatinib (2 μM), and 30 min later were irradiated (1 Gy). Cells were isolated at the indicated time points and subjected to SDS-PAGE and immunoblotting to determine the phosphorylation and expression of ERK1/2 and AKT (S473), as indicated (± S.E.M., n = 3). Top, inset, a representative immunoblotting data set corresponding to the bottom graphs is shown. D, parental HCT116 and HCT116 (K-RAS Asp13-null) cells stably transfected with a plasmid to express H-RAS Val12 were plated as single cells in sextuplicate and 12 h after plating were treated with either vehicle (DMSO) or lapatinib (2 μM) and 30 min later irradiated (1 and 2 Gy). Lapatinib was removed 96 h after irradiation. Colonies were permitted to form over the following 10 to 14 days, after which the media were removed, the colonies were fixed and stained with crystal violet, and the colonies of >50 cells were counted. Data are the means of six separate plates from a representative study ± S.E.M. (n = 2). #, $p < 0.05$ less than corresponding HCT116 cell value without lapatinib.

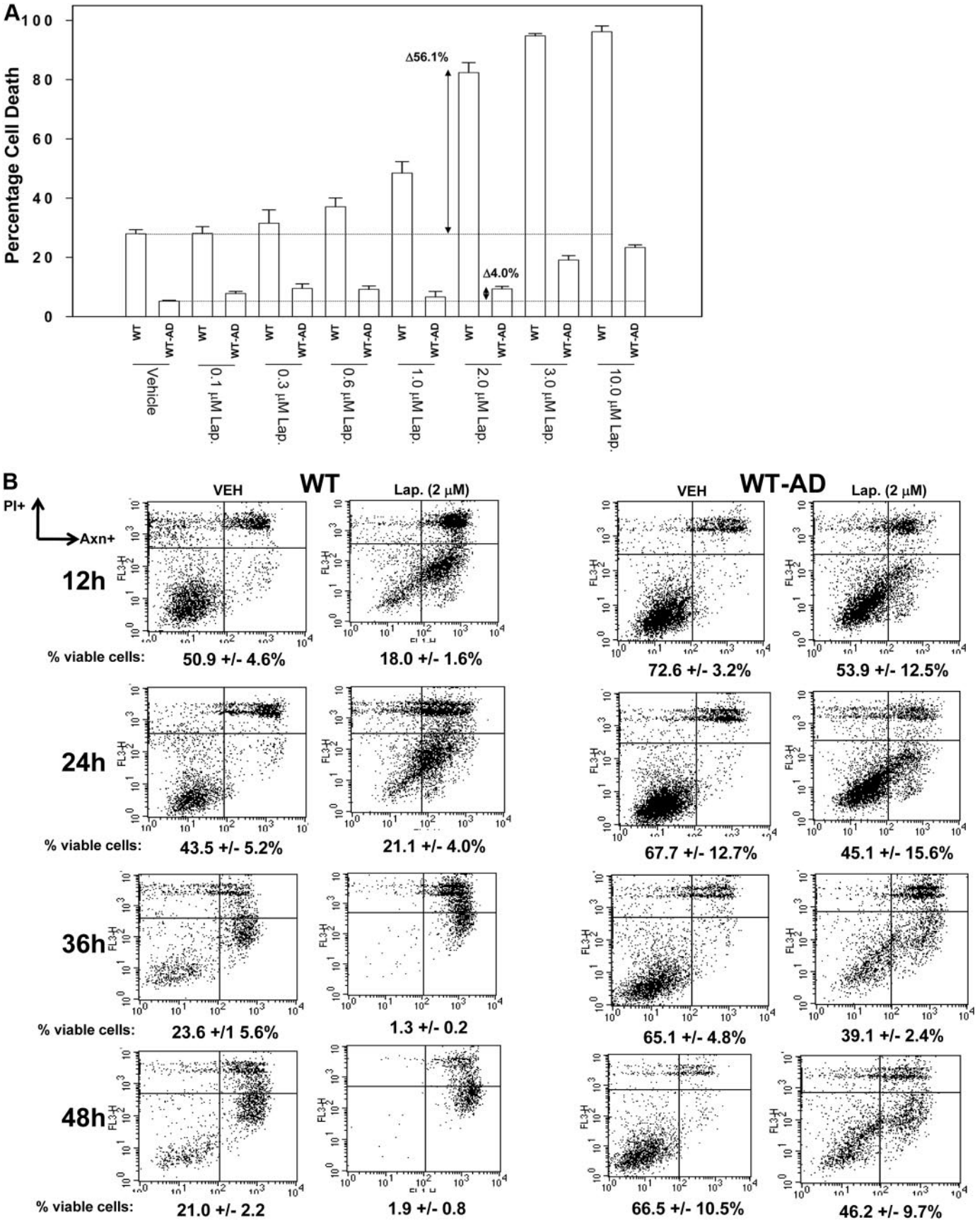


Fig. 2. The generation of lapatinib-resistant HCT116 cells. A, parental HCT116 cells (WT) and HCT116 lapatinib-adapted cells (WT-AD) were plated and 24 h after plating were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (0–10 μM). Cells were isolated 48 h after serum-starvation/lapatinib addition, and cell viability was determined in triplicate by trypan blue exclusion assay ± S.E.M. The data shown are a representative study from three separate studies. B, parental HCT116 cells (WT) and HCT116 lapatinib-adapted cells (WT-AD) were plated and 24 h after plating were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (0–10 μM). Cells were isolated at the indicated times, and cell viability was determined by annexin-PI flow cytometric analysis in triplicate ± S.E.M. (n = 2).

assays (Fig. 4B). After a 1 Gy radiation exposure, approximating to the shoulder of the survival curve, no statistically significant difference between cell survival for cells expressing H-RAS Val12 and H-RAS Val12 Cys40 was observed. Cells expressing H-RAS Val12 Ser35 had a greater level of survival than vector control transfected cells; however, these cells had significantly less survival than cells expressing H-RAS Val12 Cys40 (After a 1 Gy exposure: vector survival, 0.70 ± 0.04; H-RAS Val12 Ser35 survival, 0.82 ± 0.04; H-RAS Val12 Cys40 survival, 0.90 ± 0.03; $p < 0.05$ difference between each value). The survival of cells expressing H-RAS Val12 Ser35 was not significantly different from wild-type HCT116 cells expressing K-RAS Asp13.

In general agreement with our short-term cell-killing data using lapatinib exposure and serum-starvation, expression of constitutively active MEK1 EE and constitutively active AKT, to a greater extent than the individual activated kinases, suppressed lapatinib toxicity in parental cells (Fig. 4C). In contrast to the use of activated proteins, expression of dominant-negative AKT and/or dominant-negative MEK1 did not restore lapatinib sensitivity in adapted cells (Fig. S8). Because inhibition of ERK1/2 and AKT did not restore lapatinib sensitivity, we explored whether other mechanisms of lapatinib resistance were present in HCT116 cells.

Lapatinib resistance has been linked to reactivation of the estrogen receptor in breast cancer cells, and the estrogen receptor is known to be expressed in some colon cancer cells (Xia et al., 2006; Cho et al., 2007). However, incubation of adapted cells with the ER inhibitor tamoxifen did not restore lapatinib sensitivity (Fig. 4D). Likewise, inhibition of NFκB

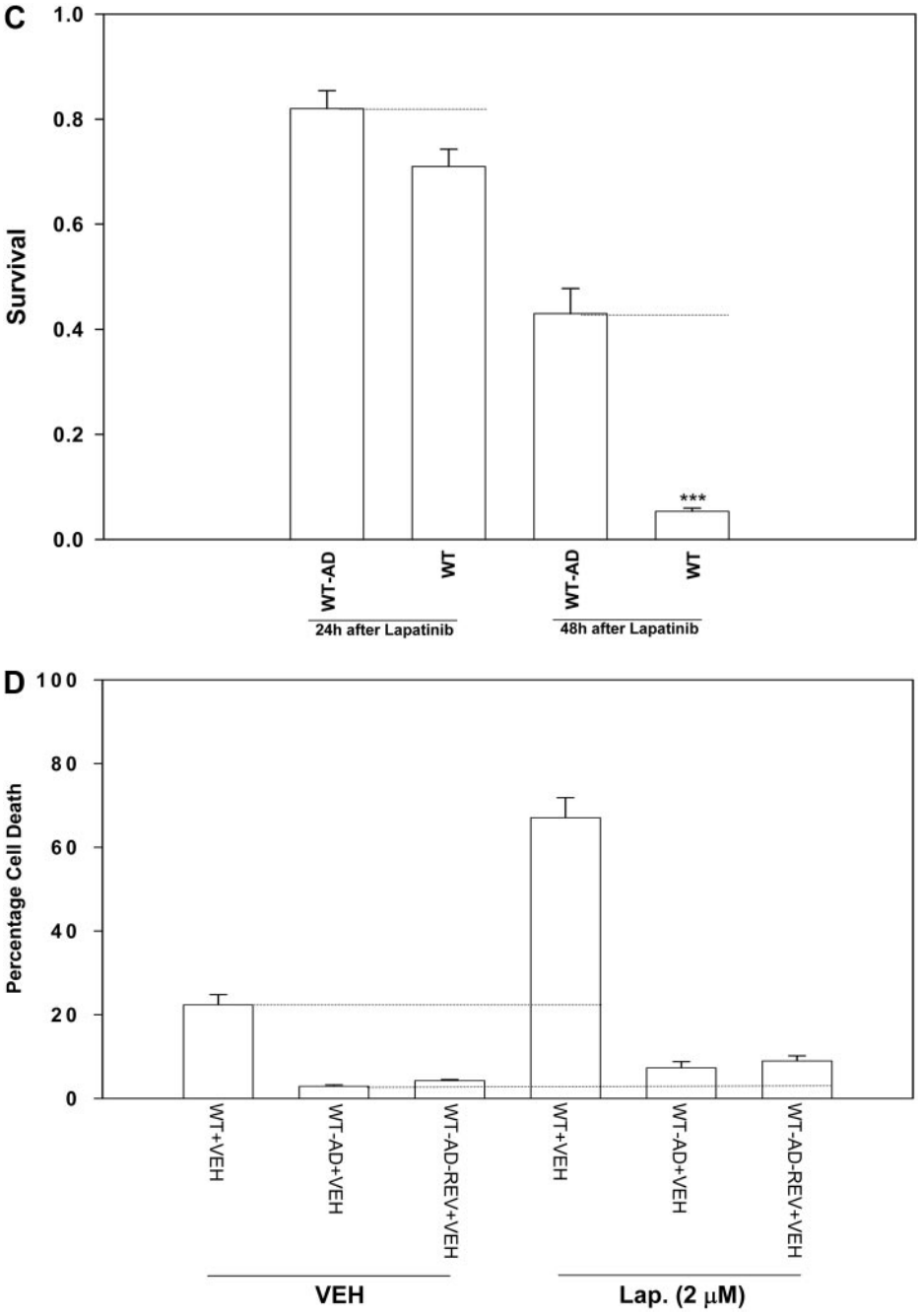


Fig. 2. (*continued*) C, parental HCT116 cells (WT) and HCT116 lapatinib-adapted cells (WT-AD) were plated as single cells in sextuplicate and 12 h after plating were placed into serum-depleted medium for either 24 or 48 h in the presence of lapatinib (2 μM). Media containing serum were resupplemented back to the cells 24 or 48 h after the initiation of serum starvation as indicated with the removal of lapatinib from the growth medium. Colonies were permitted to form over the following 10 to 14 days, after which the media were removed, the colonies fixed and stained with crystal violet, and the colonies of >50 cells were counted. Data are the means of four separate plates from a representative study ± S.E.M. ($n = 2$). *, $p <$ 0.05 less than corresponding HCT116 parental cell value. D, parental HCT116 cells (WT), HCT116 lapatinib-adapted cells (WT-AD), and HCT116 lapatinib-adapted cells that had been grown for >2 months in the absence of lapatinib (WT-AD-REV) were plated and 24 h after plating were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2 μM). Cells were isolated 48 h after serum starvation/lapatinib addition, and cell viability was determined in triplicate by trypan blue exclusion assay ± S.E.M. The data shown are a representative study from two separate studies.

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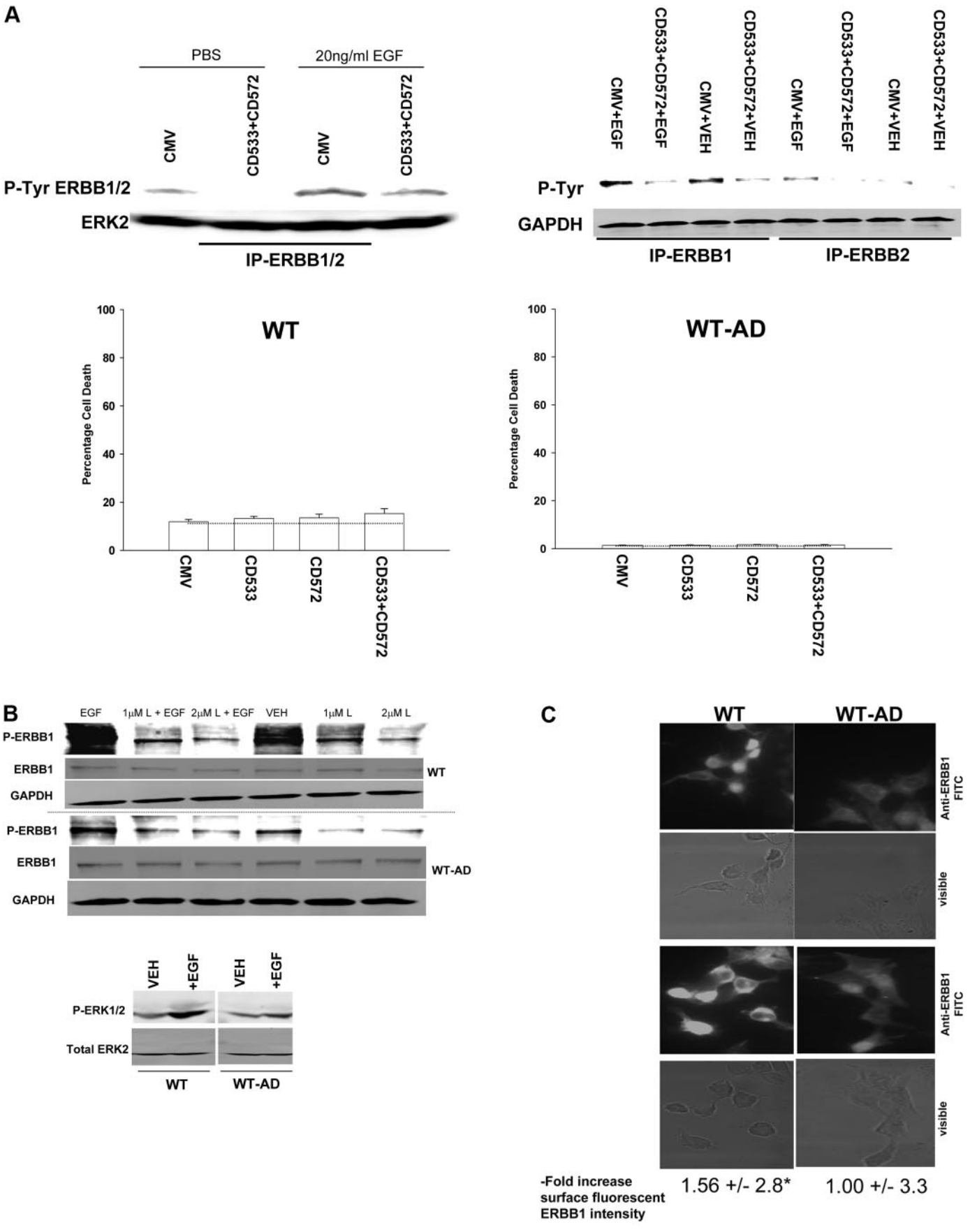

function by overexpression of the IκB super repressor (dominant-negative IκB) or inhibition of STAT1 and STAT3 function by expression of a dominant-negative STAT3 protein did not restore lapatinib sensitivity in adapted cells. In control studies, we noted that expression of dominant-negative IκB or dominant-negative STAT3 suppressed reporter construct activity in parental and adapted cells; basal NFκB activity was lower in adapted cells than in parental cells (Figs. S9 and S10). In phenol red-free media, basal estrogen receptor activity was almost undetectable in HCT116 cells; however, this activity was weakly reduced by tamoxifen treatment (Fig. S9).

In some cell types, including colon cancer cells, Src family nonreceptor tyrosine kinases and the insulin-like growth factor receptor tyrosine kinase have been linked to the transformed phenotype and tumor cell invasion. However, in our nonbiased screening approach, we noted that inhibition of neither Src family kinases using the inhibitor PP2 nor IGF1 receptor function using the inhibitor PPP restored lapatinib sensitivity (Fig. 4E). It is noteworthy that inhibition of the IGF1 receptor with PPP caused significant toxicity in parental cells that was abolished in lapatinib-adapted cells, arguing that adapted cells were also cross-resistant to agents that inhibit the function of other receptor tyrosine kinases that are known to compensate for ERBB1 survival signaling.

Based on our relative lack of success at precisely defining the signaling pathways downstream of ERBB1 and ERBB2 that could be mediating lapatinib adaptation, we next determined the proximal downstream molecular mechanisms by which serum-starved and lapatinib-treated cells die and the mechanisms by which adaptation was gained. Adapted HCT116 cells expressed higher levels of MCL-1 (duplicate presentation), BCL-XL, and p53 than parental cells; these cells did not express lower BAK than parental cells but had abolished BAX expression, which will in all likelihood profoundly reduce the apoptotic threshold (Fig. 5A, top blotting section). Again, based on an unbiased screening approach, we found that no obvious changes in the protein expression of CD95, FAS ligand, pro-caspase 8, pro-caspase 9, pro-caspase 3, Apaf-1, A10, Smac/DIABLO, c-FLIP-s, XIAP, BCL-2, BID, BIM, NOXA, or PUMA were noted based on immunoblotting analyses (data not shown). Based on the established concept of the so-called "apoptotic rheostat," in which BCL-2 family proteins act in a dynamic balance to suppress the proapoptotic signals generated by BH3 domain proteins such as BAX and BAK, our data suggest that adapted cells could be more resistant to lapatinib than parental cells because they express more of the mitochondrial protective proteins BCL-XL and MCL-1 and that they express less of the mitochondrial toxic protein BAX.

Because we observed changes in the expression of proteins that act at the mitochondrion to modulate mitochondrial stability, we next determined whether activation of caspase proteases, and specifically pro-caspase 9, played a role in lapatinib toxicity. To our surprise, pan-inhibition of caspase function only modestly suppressed lapatinib toxicity in serum-starved parental cells treated with lapatinib (Fig. 5A, bottom). In contrast, inhibition of caspases significantly reduced serum withdrawal-induced cell killing (Fig. S11). Inhibition of cathepsin, calpain, and serine protease function also caused similar very modest effects on promoting cell survival in lapatinib-treated cells (data not shown). Overexpression of a mitochondrial protective protein, BCL-XL, abolished lapatinib toxicity in parental cells (Fig. 5B). Finally, we tested whether AIF played a role in lapatinib toxicity. Knockdown of AIF expression reduced lapatinib toxicity in parental HCT116 cells, and knockdown of AIF expression combined with pan-caspase inhibition almost eliminated lapatinib toxicity (Fig. 5C).

Knockdown of MCL-1 expression, to a greater extent than that of BCL-XL, partially reverted lapatinib sensitivity in adapted cells by ~50% (Fig. 5D). In Fig. 5A, we note that the expression levels of pro- and antiapoptotic proteins were altered comparing parental and adapted HCT116 cells. In parental cells, lapatinib treatment caused a release of AIF into the cytosol, whereas in adapted cells, no AIF release was observed (Fig. 5E). Thus, the induction of cell killing by lapatinib in parental cells correlated with activation of BAK and BAX. In adapted cells, knockdown of BAK activation significantly reduced, by ~50%, the reversion of their resistant phenotype by reduced MCL-1 expression (Fig. 6A). Thus, the adaptation mechanism seems to be in part due to the loss of BAX expression and loss of BAK activation due to overexpression of MCL-1.

Fig. 3. Molecular inhibition of ERBB1 and ERBB2 does not recapitulate the effects of lapatinib in HCT116 cells. Lapatinib-resistant cells express less cell surface ERBB1 than parental cells. A, parental HCT116 cells (WT) and HCT116 lapatinib-adapted cells (WT-AD) were plated and 12 h after plating were infected with either a control empty vector adenovirus, a recombinant adenovirus to express dominant-negative ERBB1-CD533, a recombinant adenovirus to express dominant-negative ERBB2-CD572, or both viruses to express ERBB1-CD533 and ERBB2-CD572. Twenty-four hours after virus infection, cells were grown in serum-depleted medium in a similar manner to our prior studies using lapatinib. Cells were isolated 48 h after serum starvation, and cell viability was determined in triplicate by trypan blue exclusion assay ± S.E.M. The data shown are a representative study from three separate studies. Top, left, parental HCT116 cells (WT) were plated and 12 h after plating were infected with either a control empty vector adenovirus or recombinant adenoviruses to express dominant-negative ERBB1-CD533 and to express dominant negative ERBB2-CD572. Twenty-four hours after virus infection, cells were grown in serum-depleted medium for 2 h and then treated with 20 ng/ml EGF as indicated for 10 min. Cells were isolated and subjected to immunoprecipitation of ERBB1 and ERBB2 (combined) followed by SDS-PAGE of the immunoprecipitate as indicated followed by anti-phospho-tyrosine blotting to determine the activation of ERBB1/2 proteins. A representative study is shown (n = 2–3). Right, adapted HCT116 cells (WT-AD) were plated and 12 h after plating were infected with either a control empty vector adenovirus or recombinant adenoviruses to express dominant-negative ERBB1-CD533 and to express dominant-negative ERBB2-CD572. Twenty-four hours after virus infection, cells were grown in serum-depleted medium for 2 h and then treated with 20 ng/ml EGF as indicated for 10 min. Cells were isolated and subjected to immunoprecipitation of ERBB1 or ERBB2 followed by SDS-PAGE of the immunoprecipitate, as indicated, followed by anti-phospho-tyrosine blotting to determine the activation of ERBB1/2 proteins. A representative study is shown (n = 2–3). B, parental HCT116 cells (WT) and HCT116 lapatinib-adapted cells (WT-AD) were plated and 24 h after plating were grown in serum-depleted medium for 2 h followed by treatment with vehicle (DMSO) or lapatinib (L, 0–2 μM), as indicated. Thirty minutes after lapatinib exposure, cells were treated as indicated with 20 ng/ml EGF. Ten minutes after EGF addition, cells were harvested and subjected to lysis, and portions of the lysate either were immunoprecipitated to isolate ERBB1 and determine ERBB1 tyrosine phosphorylation or were directly subjected to SDS-PAGE followed by SDS-PAGE to determine total ERBB1, ERK2, and GAPDH expression and ERK1/2 phosphorylation. A representative study is shown (n = 2–3). C, parental HCT116 cells (WT) and HCT116 lapatinib-adapted cells (WT-AD) were plated and 24 h after plating were grown in serum-depleted medium for 2 h. Cells were fixed but not permeabilized, and cell surface levels of ERBB1 were determined by immunostaining and examination under fluorescent light microscope. Two representative images are shown from one experiment (n = 2).

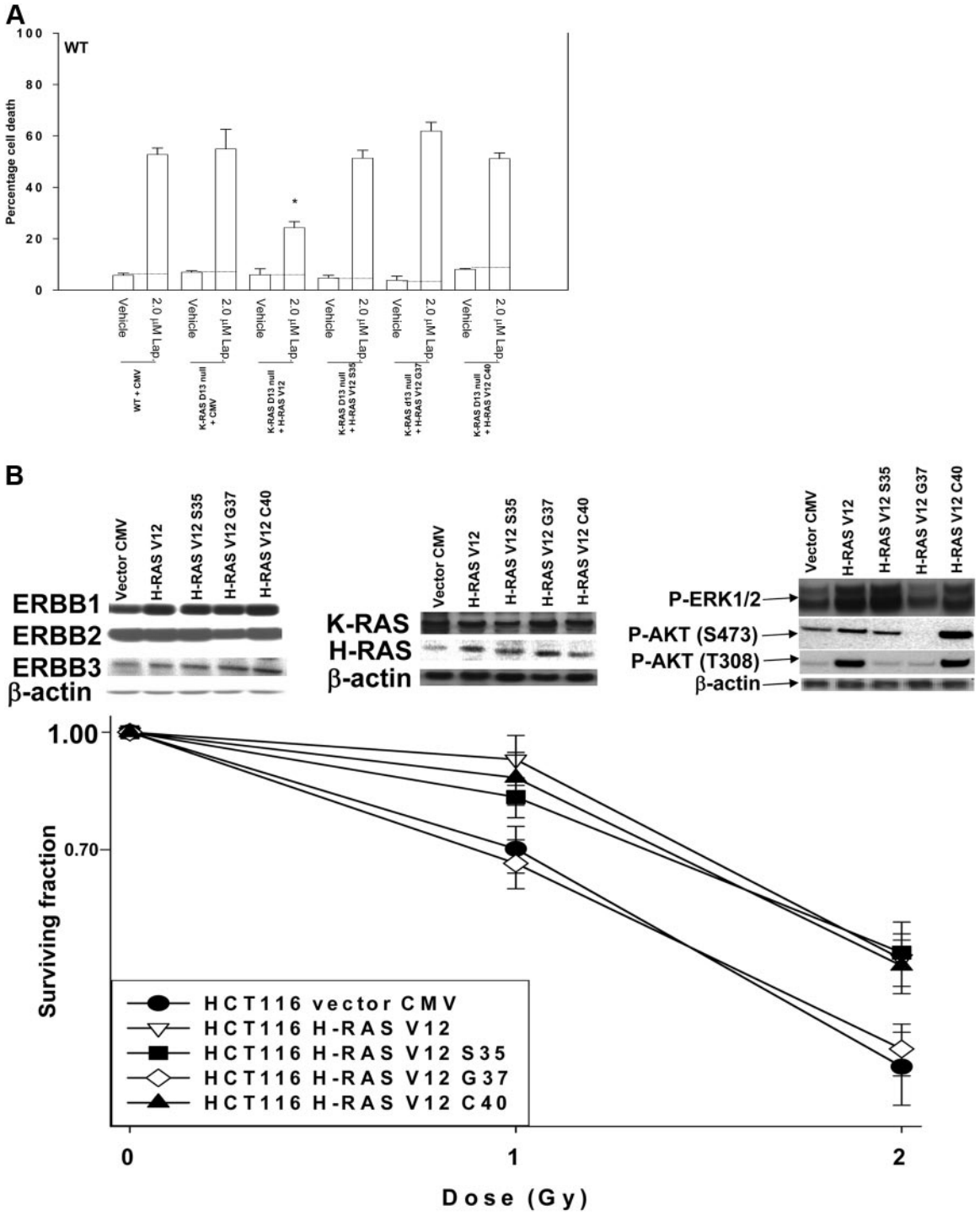

Fig. 4. Lapatinib resistance requires multiple pathways downstream of RAS: lack of involvement of classic effectors of resistance. A, parental HCT116 cells and HCT116 (K-RAS Asp13-null) cells were stably transfected with either empty vector control (CMV), H-RAS Val12, H-RAS Val12 Ser35, H-RAS Val12 Gly37, or H-RAS Val12 Cys40 and selected as described in under *Materials and Methods* (Carón et al., 2005b). Cells were plated and 24 h after plating were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2 μM). Cells were isolated 48 h after drug addition, and cell viability was determined in triplicate by trypan blue exclusion assay ± S.E.M. The data shown are a representative study from two to three separate studies. *, $p < 0.05$ less than corresponding value in other HCT116 cell isolates. B, top, HCT116 (K-RAS Asp13-null) cells stably transfected with plasmids either empty vector control (CMV), H-RAS Val12, H-RAS Val12 Ser35, H-RAS Val12 Gly37, or H-RAS Val12 Cys40 and selected as described under *Materials and Methods*. Cells were plated and 24 h after plating were grown in serum-depleted medium. Two hours after culture in serum-depleted medium, cells were harvested for SDS-PAGE and immunoblotting analyses. Data are representative of three to five separate studies. Right, phosphorylation (activity) of protein kinases was determined by immunoblotting using specific antibodies for the phosphorylated forms of ERK1/2, AKT Ser473, c-Jun NH_2-terminal kinase, and p38 in the HCT116 cell lines. Total β-actin protein expression was blotted in the same membrane as a loading control. Center, expression of K-RAS and H-RAS proteins determined by immunoblotting using RAS isoform-specific antibodies. Left, expression of ERBB1, ERBB2, and ERBB3 receptor proteins determined by immunoblotting using receptor-specific antibodies. Total β-actin protein expression was blotted in the same membrane as a loading control. Bottom colony-formation graph, parental HCT116 and HCT116 (K-RAS Asp13-null) cells stably transfected with plasmids either empty vector control (CMV), H-RAS Val12, H-RAS Val12 Ser35, H-RAS Val12 Gly37, or H-RAS Val12 Cys40 were plated as single cells in sextuplicate and 12 h after plating were irradiated (1 and 2 Gy). Colonies were permitted to form over the following 10 to 14 days, after which the media were removed, the colonies fixed and stained with crystal violet, and colonies of >50 cells were counted. Data are the means of six separate plates from a representative study ± S.E.M. ($n = 2$).

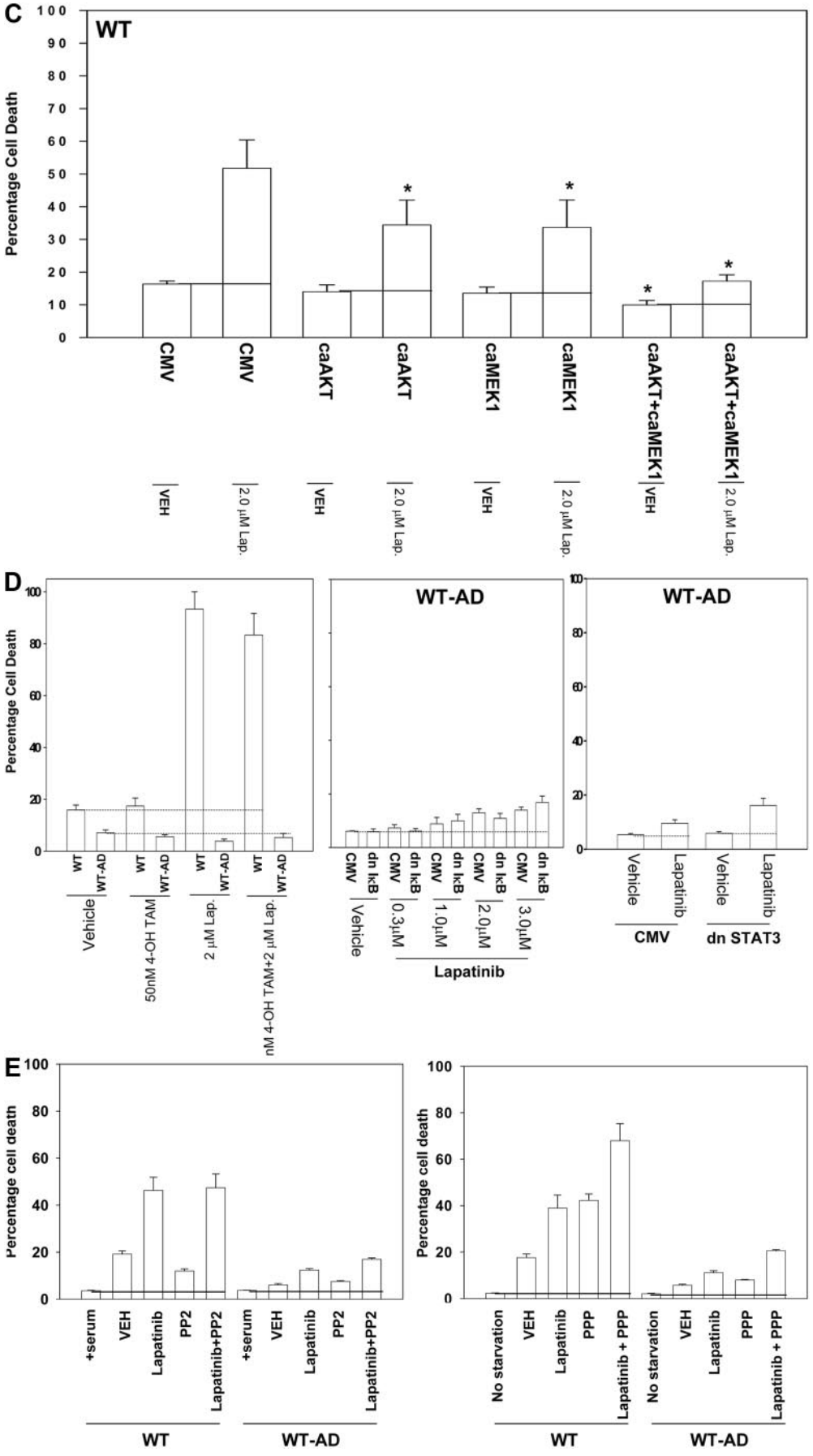

Fig. 4. (*continued*) C, parental HCT116 cells (WT) were plated and 12 h after plating were infected with either a control empty vector adenovirus (CMV), a recombinant adenovirus to express constitutively active AKT (caAKT), a recombinant adenovirus to express constitutively active MEK1 (caMEK1), or both viruses to express caAKT and caMEK1. Twenty-four hours after virus infection, cells were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2 μM). Cells were isolated 48 h after drug addition, and cell viability was determined in triplicate by trypan blue exclusion assay ± S.E.M. The data shown are a representative study from three separate studies. *, $p < 0.05$ less than corresponding HCT116 CMV-infected cell value. D, Left, parental HCT116 cells (WT) and HCT116 lapatinib-adapted cells (WT-AD) were plated and 24 h after plating were grown in serum-depleted, phenol red-free medium in the presence or absence of vehicle (DMSO), 4-hydroxytamoxifen (4-OH TAM, 50 nM) or lapatinib (2 μM); Center, HCT116 lapatinib-adapted cells (WT-AD) were plated and 12 h after plating were infected with either a control empty vector adenovirus or a recombinant adenovirus to express dominant-negative IκB (dnIκB). Twenty-four hours after virus infection, cells were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (0–3 μM). Cells were isolated 48 h after drug addition, and cell viability was determined in triplicate by trypan blue exclusion assay ± S.E.M. The data shown are a representative study from two separate studies; Right, HCT116 lapatinib-adapted cells (WT-AD) were plated and 12 h after plating were infected with either a control empty vector adenovirus or a recombinant adenovirus to express dominant-negative STAT3 (dnSTAT3). Twenty-four hours after virus infection, cells were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (3 μM). Cells were isolated 48 h after drug addition, and cell viability was determined in triplicate by trypan blue exclusion assay ± S.E.M. The data shown are a representative study from two separate studies. E, parental HCT116 cells (WT) and HCT116 lapatinib-adapted cells (WT-AD) were plated and 24 h later were grown in serum-depleted medium in the presence or absence of vehicle (DMSO), lapatinib (2 μM), PP2 (10 μM), PPP (250 nM), or the drug combinations indicated. Cells were isolated 48 h after serum starvation, and cell viability was determined in triplicate by trypan blue exclusion assay ± S.E.M. The data shown are a representative study from two separate studies.

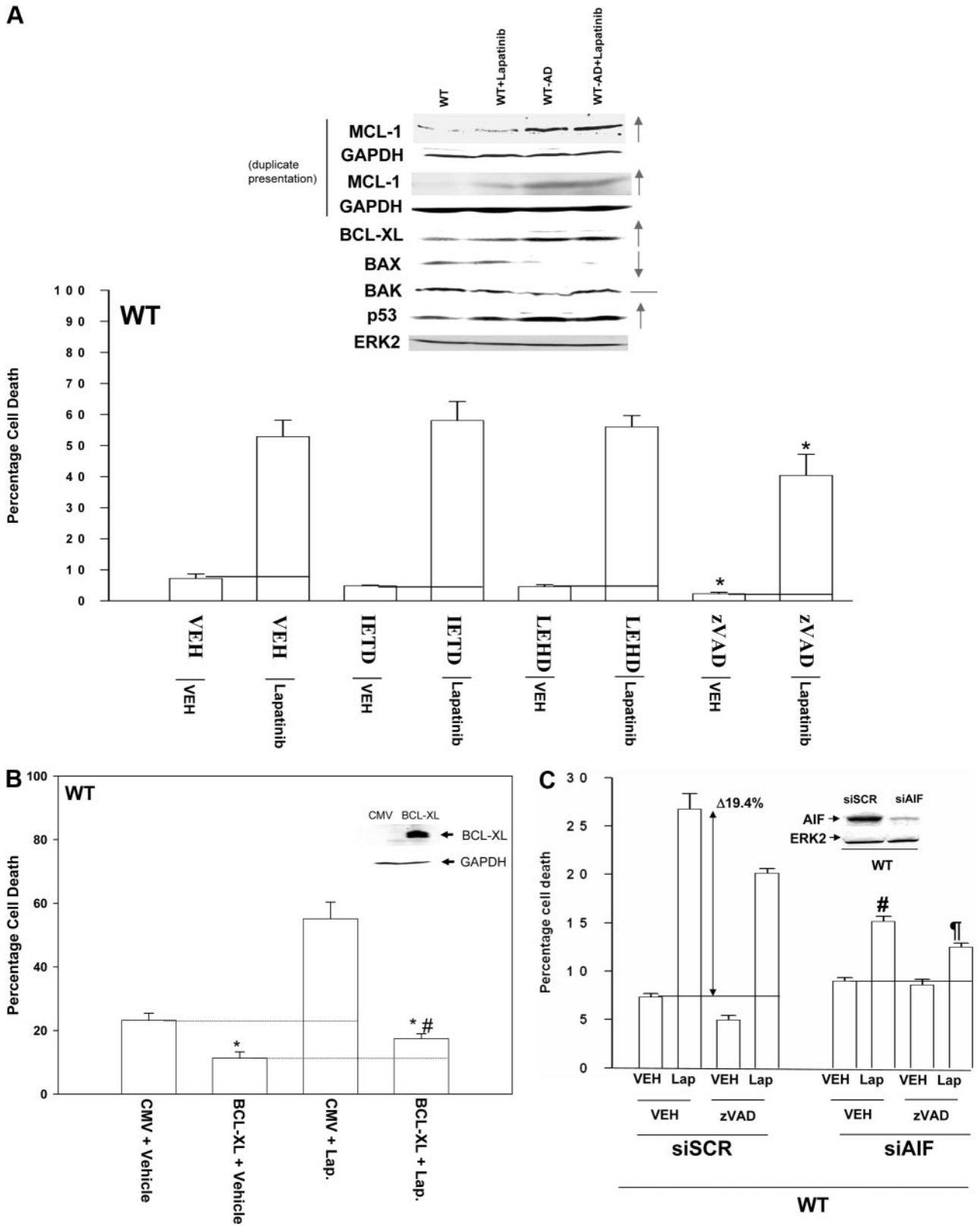

Fig. 5. Lapatinib resistance is mediated by increased expression of MCL-1, decreased activation of BAK and mutation of p53. A, top, parental HCT116 cells (WT) and HCT116 lapatinib-adapted cells (WT-AD) were plated and 24 h later were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2 μM). Cells were isolated 48 h after lapatinib addition and were subjected to SDS-PAGE to determine the expression of multiple proteins, which are shown: BCL-XL, MCL-1 (duplicate presentation), BAX, BAK, p53, ERK2, and GAPDH. Bottom, parental HCT116 cells (WT) were plated and 24 h later were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2 μM). In parallel, cells were grown in the presence or absence of vehicle (DMSO), the caspase 8 inhibitor IETD (50 μM), the caspase 9 inhibitor LEHD (50 μM) or the pan-caspase inhibitor zVAD (50 μM). Cells were isolated 36 h after serum starvation, and cell viability was determined in triplicate by trypan blue exclusion assay ± S.E.M. The data shown are a representative study from two separate studies. *, $p < 0.05$ less than corresponding HCT116 vehicle control cell values. B, parental HCT116 cells (WT) were plated and 12 h after plating were infected with either a control empty vector adenovirus or a recombinant adenovirus to express BCL-XL. Twenty-four

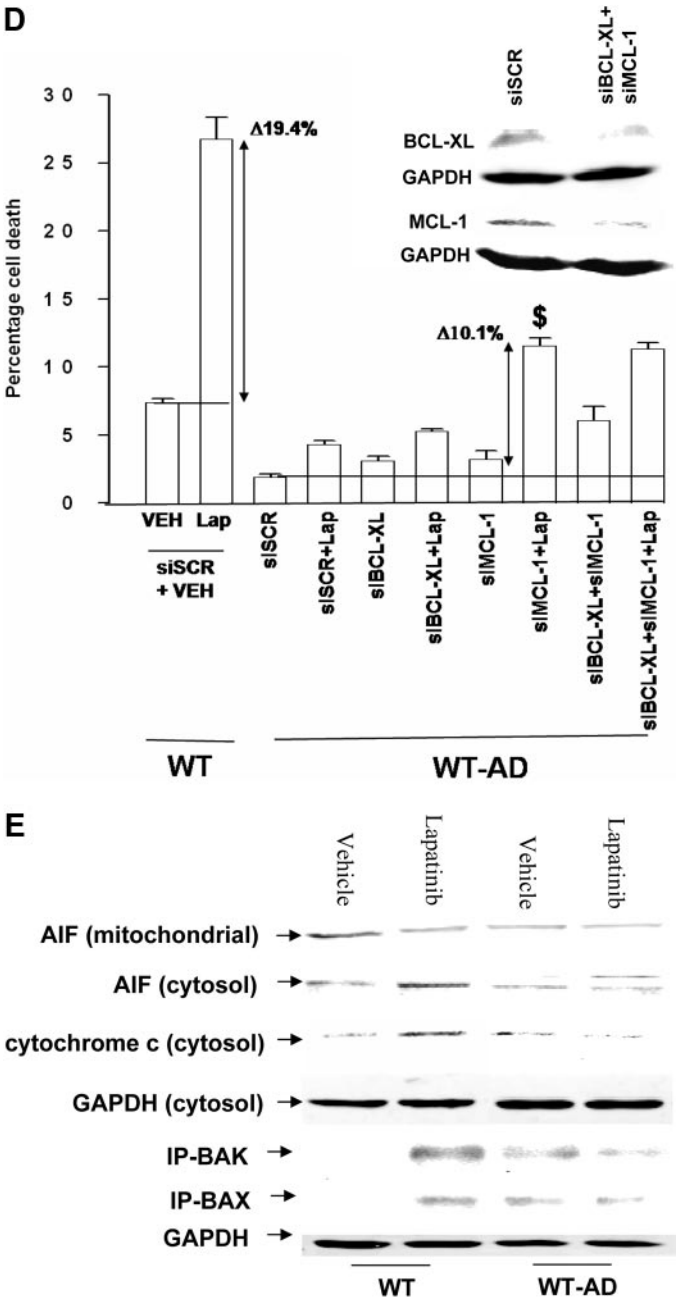

Fig. 5. (*continued*) hours after virus infection, cells were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2 μM). Cells were isolated 48 h after drug addition, and cell viability was determined in triplicate by trypan blue exclusion assay ± S.E.M. The data shown are representative from two separate studies. *, $p < 0.05$ less than corresponding HCT116 cell value. C, parental HCT116 cells (WT) and HCT116 lapatinib-adapted cells (WT-AD) were plated and 12 h later as indicated were transfected with siRNA molecules to reduce the expression of nothing/control (siSCR) or AIF (siAIF). Forty-eight hours after transfection, cells were grown in serum-depleted medium in the presence or absence of vehicle (DMSO), lapatinib (2 μM), or the pan-caspase inhibitor zVAD (50 μM), as indicated. Cells were isolated 36 h after serum starvation, and cell viability was determined in triplicate by trypan blue exclusion assay ± S.E.M. The data shown are representative from two separate studies. #, $p < 0.05$ less than corresponding HCT116 siSCR cell value; ¶, $p < 0.05$ less than parallelvehicle value. D, parental HCT116 cells (WT) and HCT116 lapatinib-adapted cells (WT-AD) were plated and 12 h later as indicated were transfected with siRNA molecules to reduce the expression of nothing/control (siSCR), BCL-XL (siBCL-XL), or MCL-1 (siMCL). Forty-eight hours after transfection, cells were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2 μM) as indicated. Cells were isolated 36 h after serum starvation, and cell viability was determined in triplicate by trypan blue exclusion assay ± S.E.M. The data shown are representative from two separate studies. $, $p < 0.05$ greater than corresponding HCT116 siSCR + Lap cell value. E, parental HCT116 cells (WT) and HCT116 lapatinib-adapted cells (WT-AD) were plated and 24 h after plating were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2 μM). Thirty-six hours after serum depletion/lapatinib addition, cells were isolated for the assay of AIF and cytochrome *c* release into the cytosol and by immunoprecipitation to determine the amount of the activated forms of BAX and BAK. A representative from two separate studies is shown.

In Fig. 5A, we note that the expression of p53 was elevated, even though the protein levels of a bona fide p53 target protein, BAX, were abolished. In cells that express a mutated p53 protein, the expression of total p53 within a cell is often noted to be elevated. Thus, parental HCT116 cells that express a wild type p53 protein may have in part survived and adapted to lapatinib exposure by mutating one of their p53 alleles.

Native p53 proteins were immunoprecipitated from parental and lapatinib-resistant HCT116 cells using an antibody that specifically recognizes mutated forms of p53, as judged by the recognition of mutant p53 tertiary structure within the DNA binding domain of p53. The p53 proteins were then separated on denaturing SDS-PAGE and immunoblotted; lapatinib-resistant cells, but not parental cells, immunoprecipitated a greater amount of "mutant" p53 (Fig. 6B). Total poly(A) mRNA was isolated from adapted HCT116 cells and amplified and sequenced using primers specific for the DNA binding domain of p53. We noted, however, that adapted HCT116 cells did not contain a mutation in p53, suggesting that either our antibody was recognizing an alteration in p53 tertiary conformation in adapted cells unrelated to p53 mutation or that p53 mutation had occurred in a domain unrelated to the DNA binding domain of p53 but that was affecting the tertiary conformation of the DNA binding domain. These findings suggest, but do not prove, that lapatinib adaptation in HCT116 cells is mediated by changes in the expression of multiple mitochondrial protective proteins rather than mutation of ERBB receptors.

Discussion

Previous studies from this group have demonstrated that mutated active forms of K-RAS and H-RAS differentially regulate ERK1/2 and AKT signaling after irradiation. Prior studies from multiple groups have also demonstrated that radiation-induced activation of ERBB1, ERBB2, and ERBB3 is a cytoprotective response. The present studies were proposed to determine the impact of the clinically relevant ERBB1/ERBB2 inhibitor lapatinib on tumor cell radiosensitivity and the mechanisms by which HCT116 tumor cells become resistant to the toxic effects of lapatinib in vitro.

HCT116 cells and the variant cell lines used in this study expressing a mutated active RAS protein were radiosensitized by lapatinib, even though a priori it would be predicted that activated RAS proteins would tend to overcome the impact of an inhibitor of an upstream receptor tyrosine kinase on radiosensitivity in any cell type. Furthermore, HCT116 cells were sensitive, in the presence or absence of serum, to being killed by doses of lapatinib that were within the C_{max} patient serum concentration of the drug. Multiple studies have argued that tumor cell resistance to therapeutic

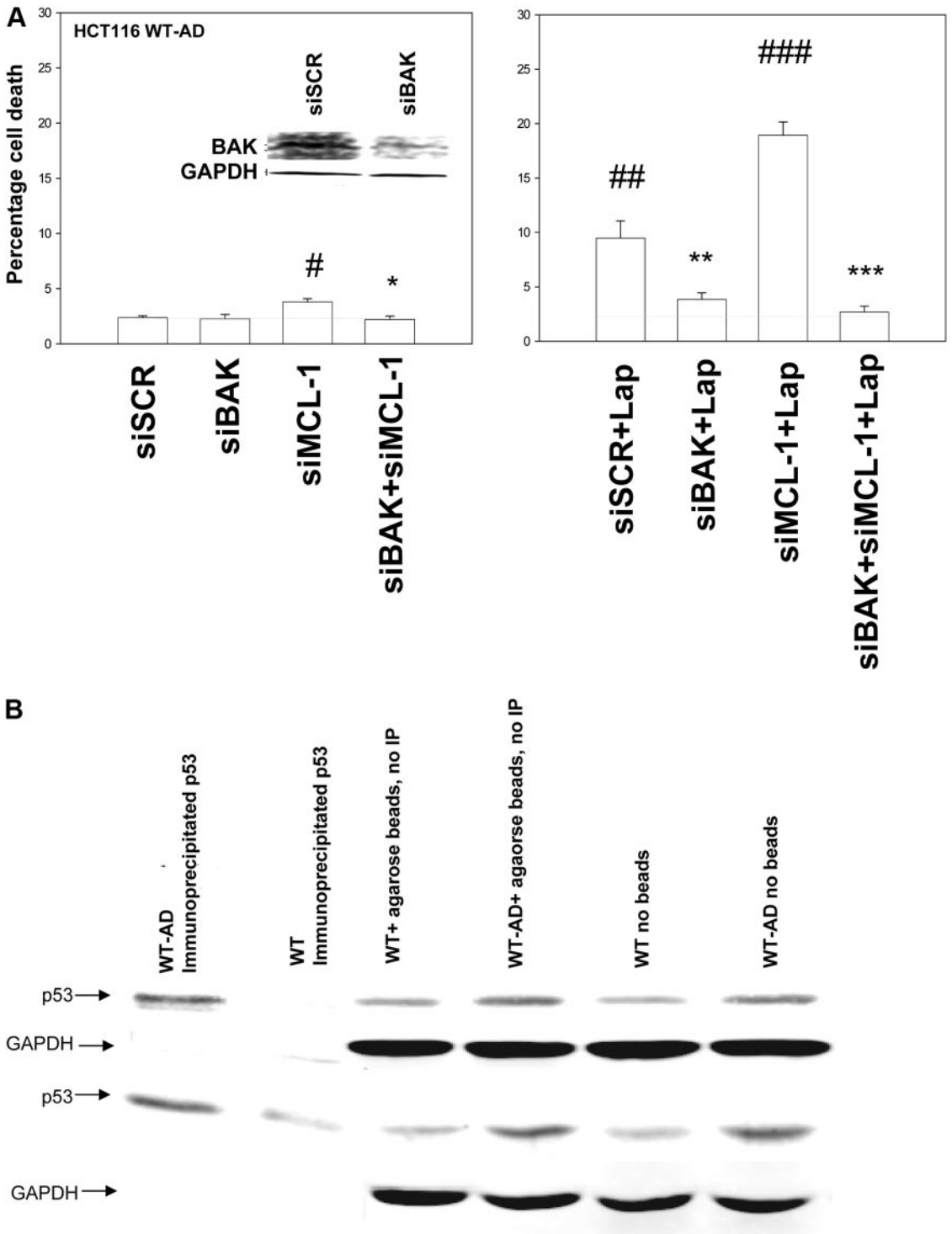

Fig. 6. Knockdown of BAK re-reverts lapatinib-adapted cells after siMCL-1 exposure. A, HCT116 lapatinib-adapted cells (WT-AD) were plated and 12 h later as indicated were transfected with siRNA molecules to reduce the expression of nothing/control (siSCR), MCL-1 (siMCL-1), BAK (siBAK), or MCL-1 and BAK (siMCL+siBAK). Forty-eight hours after transfection, cells were grown in serum-depleted medium in the presence or absence of vehicle (DMSO) or lapatinib (2 μM). Cells were isolated 48 h after serum starvation, and cell viability was determined in triplicate by trypan blue exclusion assay ± S.E.M. The data shown are representative from two separate studies. *, $p < 0.05$ less than HCT116 siMCL-1 cell value; **, $p < 0.05$ less than HCT116 siSCR + Lap cell value; ***, $p < 0.05$ less than HCT116 siMCL-1 + Lap cell value; #, $p < 0.05$ greater than HCT116 siSCR cell value; ##, $p < 0.05$ greater than HCT116 siSCR and siMCL-1 cell values; ###, $p < 0.05$ greater than corresponding HCT116 siSCR + Lap cell value. B, parental HCT116 cells (WT) and HCT116 lapatinib-adapted cells (WT-AD) were plated and 24 h after plating were lysed and prepared for immunoprecipitation and pseudoimmunoprecipitation with or without mixing with agarose beads against mutated inactive p53 followed by SDS-PAGE. The mixing with or without agarose beads was to ensure as a control that no spurious effects were observed on the SDS-PAGE mobility of p53 due to agarose bead inclusion in the loading of the gel. The SDS-PAGE was transferred to nitrocellulose and probed with an anti-p53 antibody and an anti-GAPDH antibody. Data shown are from two representative studies ($n = 3$).

agents is composed of the actions of multiple signal transduction pathways, and based on the expression of Ser35/Gly37/Cys40 effector domain mutants of H-RAS Val12 and expression of activated forms of MEK1 and AKT, we concluded that we could prevent lapatinib-induced cell killing by activating both PI3K-AKT and MEK-ERK1/2 signaling and but not by activating either pathway individually (Dent et al., 2003; Carón et al., 2005a,b). On the other hand, our data using point mutants of H-RAS Val12 demonstrated that mutant oncogenic RAS is a negative predictor of therapeutic response to lapatinib exposure.

In the clinic, resistance to the toxic and radio-/chemosensitizing effects of ERBB1 receptor inhibitors has been noted primarily with the development of mutations in the tyrosine kinase domain, rendering the receptor tyrosine kinase insensitive to the ATP binding site "competitive inhibitor" drug (Kobayashi et al., 2005; Pao et al., 2005). Resistance to lapatinib in breast cancer cells has been ascribed to reactivation of the estrogen receptor; in a variety of other tumor cell types, general resistance to chemotherapeutic drug toxicity has also been linked to hyperactivation of the transcription factor NFκB, the IGF-1R, STAT transcription factors, Src nonreceptor tyrosine kinases, the PI3K-AKT pathway, and to increased levels of drug export pumps. None of these factors seemed to play a primary role in the adaptive resistance of HCT116 cells to lapatinib. After many studies we determined that lapatinib-adapted cells expressed higher levels of MCL-1 and that knockdown of MCL-1 expression significantly reverted the lapatinib-adapted phenotype. That these cells also had abolished BAX expression will have also profoundly reduced the apoptotic threshold. Unlike parental cells, lapatinib-adapted cells did not exhibit activation of BAK after serum starvation and lapatinib treatment; in the absence of MCL-1 expression, knockdown of BAK expression restored lapatinib resistance.

Recent evidence has argued that BAK activation requires simultaneous disruption of its associations with MCL-1 (e.g., by proteins such as NOXA) and BCL-XL (e.g., by proteins such as BAD) (Willis et al., 2005). Moreover, increased production of NOXA can oppose MCL-1 antiapoptotic functions, leading to simultaneous activation of BAX and BAK (Qin et al., 2006b). In adapted cells, we did not observe altered levels of either NOXA or BAD, or altered BAD phosphorylation, arguing against changes in the functions of these proteins in the adaptation process (A. Yacoub, A. P. Martin, and P. Dent, unpublished observations). In a recent study, we noted that overexpression of BCL-2 or BCL-XL failed to protect leukemia cells from cotreatment with the BCL-2/BCL-XL inhibitor ABT-737 and the CDK inhibitor roscovitine, potentially reflecting an important contribution of MCL-1 down-regulation to the lethality of this drug regimen in leukemic cells (Chen et al., 2007). We found that ectopic expression of MCL-1 diminished the potentiation of ABT-737 lethality by roscovitine, and this probably highlights a central role of MCL-1 and its down-regulation in the synergism of interaction between these agents. This interpretation was further supported by results showing that roscovitine was unable to enhance ABT-737-mediated apoptosis in transformed MCL-1-null mouse embryonic fibroblasts. In these studies, overexpression of MCL-1, but not BCL-2 or BCL-XL, abrogated BAK activation after exposure to ABT-737 and roscovitine, arguing that MCL-1 plays a major role in regulating BAK function. This is consistent with data demonstrating that MCL-1 binds with greater affinity to BAK compared with BCL-XL ($IC_{50} < 10$ versus < 100 nM) (Willis et al., 2005).

Whether a strategy combining CDK inhibitors or other transcriptional repressors capable of down-regulating MCL-1 expression, with BCL-2/BCL-XL/MCL-1 antagonists such as ABT-737 or obatoclax will result in enhanced therapeutic efficacy in lapatinib-adapted HCT116 cells will depend on multiple other factors, including the capacity of such agents to diminish MCL-1 expression in vivo and whether the therapeutic index is enhanced. In this context, it is noteworthy that ABT-737 and obatoclax display in vivo antitumor selectivity in preclinical studies (Oltersdorf et al., 2005; Trudel et al., 2007). The present findings suggest that in addition to combining BCL-2/BCL-XL/MCL-1 antagonists with conventional cytotoxic drugs, combination strategies involving targeted agents that down-regulate MCL-1, a protein that can compensate for the loss of BCL-2/BCL-XL function, could represent a potentially useful alternative approach to subvert lapatinib resistance.

In our studies to determine the mechanism of lapatinib resistance, we noted that p53 was overexpressed in lapatinib-adapted cells and that the expression of a transcriptional target of p53, BAX, was significantly lower in adapted cells. The expression of p53 is often elevated when mutated. We also noted that on a per-molecule basis, the phosphorylation of p53 serine 15 was reduced (A. P. Martin and P. Dent, unpublished observation). Together, this suggested that HCT116 cells that express a wild-type p53 protein may have undergone a portion of their lapatinib-adaptation process by developing a p53 mutation. In agreement with this hypothesis, using an antibody that specifically immunoprecipitates mutant forms of p53 because of the conformation of the p53 DNA binding domain, we noted that adapted cells but not wild-type cells expressed a p53 protein that could be immunoprecipitated by an antibody that recognizes a mutant-specific form of p53. However, upon sequencing the coding regions in DNA binding domains of p53, no mutations were noted in the p53 sequences between parental and adapted cells. This suggests that either our antibody was recognizing an alteration in p53 tertiary conformation in adapted cells unrelated to a true p53 mutation but that was in all likelihood still suppressing p53 function (i.e., reduced BAX expression) or that p53 mutation had occurred in a domain unrelated to the DNA binding domain of p53 but that was affecting the tertiary conformation of the DNA binding domain. Further studies beyond the scope of this article are required to understand how p53 function, with respect to modulation of all p53 targets and functions [e.g., the extrinsic (CD95) and intrinsic (BAX) apoptosis pathways, senescence, autophagy, and metabolism], has been altered in lapatinib-adapted HCT116 cells.

References

Baba I, Shirasawa S, Iwamoto R, Okumura K, Tsunoda T, Nishioka M, Fukuyama K, Yamamoto K, Mekada E, and Sasazuki T (2000) Involvement of deregulated epiregulin expression in tumorigenesis in vivo through activated Ki-Ras signaling pathway in human colon cancer cells. *Cancer Res* **60:**6886–6889.

Baldwin AS (2001) Control of oncogenesis and cancer therapy resistance by the transcription factor NF-κB. *J Clin Invest* **107:**241–246.

Cabannes E, Khan G, Aillet F, Jarrett RF, and Hay RT (1999) Mutations in the IkBα gene in Hodgkin's disease suggest a tumour suppressor role for Ikappa Balpha. *Oncogene* **18:**3063–3070.

Camirand A, Lu Y, and Pollak M (2002) Co-targeting HER2/ErbB2 and insulin-like growth factor-1 receptors causes synergistic inhibition of growth in HER2-overexpressing breast cancer cells. *Med Sci Monit* **8:**BR521–BR526.

Carón RW, Yacoub A, Li M, Zhu X, Mitchell C, Hong Y, Hawkins W, Sasazuki T, Shirasawa S, Kozikowski AP, et al. (2005a) Activated forms of H-RAS and K-RAS differentially regulate membrane association of PI3K, PDK-1, and AKT and the effect of therapeutic kinase inhibitors on cell survival. *Mol Cancer Ther* **4:**257–270.
Carón RW, Yacoub A, Zhu X, Mitchell C, Han SI, Sasazuki T, Shirasawa S, Hagan MP, Grant S, and Dent P (2005b) H-RAS V12-induced radioresistance in HCT116 colon carcinoma cells is heregulin dependent. *Mol Cancer Ther* **4:**243–255.
Chen S, Dai Y, Harada H, Dent P, and Grant S (2007) Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation. *Cancer Res* **67:**782–791.
Cho NL, Javid SH, Carothers AM, Redston M, and Bertagnolli MM (2007) Estrogen receptors alpha and beta are inhibitory modifiers of Apc-dependent tumorigenesis in the proximal colon of Min/+ mice. *Cancer Res* **67:**2366–2372.
Chuang E, Barnard D, Hettich L, Zhang XF, Avruch J, and Marshall MS (1994) Critical binding and regulatory interactions between Ras and Raf occur through a small, stable N-terminal domain of Raf and specific Ras effector residues. *Mol Cell Biol* **14:**5318–5325.
Cox AD and Der CJ (2003) The dark side of Ras: regulation of apoptosis. *Oncogene* **22:**8999–9006.
Cunningham MP, Thomas H, Fan Z, and Modjtahedi H (2006) Responses of human colorectal tumor cells to treatment with the anti-epidermal growth factor receptor monoclonal antibody ICR62 used alone and in combination with the EGFR tyrosine kinase inhibitor gefitinib. *Cancer Res* **66:**7708–7715.
Dent P, Reardon DB, Park JS, Bowers G, Logsdon C, Valerie K, and Schmidt-Ullrich R (1999) Radiation-induced release of transforming growth factor alpha activates the epidermal growth factor receptor and mitogen-activated protein kinase pathway in carcinoma cells, leading to increased proliferation and protection from radiation-induced cell death. *Mol Biol Cell* **10:**2493–2506.
Dent P, Yacoub A, Fisher PB, Hagan MP, and Grant S (2003) MAPK pathways in radiation responses. *Oncogene* **22:**5885–5896.
Ellis CA and Clark G (2000) The importance of being K-RAS. *Cell Signal* **12:**425–434.
Goldkorn T, Balaban N, Shannon M, and Matsukuma K (1997) EGF receptor phosphorylation is affected by ionizing radiation. *Biochim Biophys Acta* **1358:**289–299.
Hynes NE and Lane HA (2005) ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* **5:**341–354.
Imai K and Takaoka A (2006) Comparing antibody and small-molecule therapies for cancer. *Nat Rev Cancer* **6:**714–727.
Joneson T, White MA, Wigler MH, and Bar-Sagi D (1996) Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of RAS. *Science* **271:**810–812.
Kobayashi S, Boggon TJ, Dayaram T, Jänne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG, and Halmos B (2005) EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* **352:**786–792.
Konecny GE, Pegram MD, Venkatesan N, Finn R, Yang G, Rahmeh M, Untch M, Rusnak DW, Spehar G, Mullin RJ, et al. (2006) Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. *Cancer Res* **66:**1630–1639.
Leist M, Volbracht C, Fava E, and Nicotera P (1998) 1-Methyl-4-phenylpyridinium induces autocrine excitotoxicity, protease activation, and neuronal apoptosis. *Mol Pharmacol* **54:**789–801.
Liebmann C (2001) Regulation of MAP kinase activity by peptide receptor signalling pathway: paradigms of multiplicity. *Cell Signal* **13:**777–785.
Lin NU and Winer EP (2004) Small molecule tyrosine kinase inhibitors. *Breast Cancer Res* **6:**204–210.
Lu Y, Zi X, Zhao Y, Mascarenhas D, and Pollak M (2001) Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J Natl Cancer Inst* **93:**1852–1857.
Lüdde T, Kubicka S, Plumpe J, Liedtke C, Manns MP, and Trautwein C (2001) RAS adenoviruses modulate cyclin E protein expression and DNA synthesis after partial hepatectomy. *Oncogene* **20:**5264–5278.
Moriuchi A, Hirono S, Ido A, Ochiai T, Nakama T, Uto H, Hori T, Hayashi K, and Tsubouchi H (2001) Additive and inhibitory effects of simultaneous treatment with growth factors on DNA synthesis through MAPK pathway and G1 cyclins in rat hepatocytes. *Biochem Biophys Res Commun* **280:**368–373.
Nahta R, Yuan LX, Du Y, and Esteva FJ (2007) Lapatinib induces apoptosis in trastuzumab-resistant breast cancer cells: effects on insulin-like growth factor I signaling. *Mol Cancer Ther* **6:**667–674.
Nelson MH and Dolder CR (2006) Lapatinib: a novel dual tyrosine kinase inhibitor with activity in solid tumors. *Ann Pharmacother* **40:**261–269.
Olayioye MA, Neve RM, Lane HA, and Hynes NE (2000) The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J* **19:**3159–3167.
Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, et al. (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* **435:**677–681.
Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG, and Varmus H (2005) Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* **2:**e73.
Peschard P and Park M (2003) Escape from Cbl-mediated downregulation: a recurrent theme for oncogenic deregulation of receptor tyrosine kinases. *Cancer Cell* **3:**519–523.
Qin B, Ariyama H, Baba E, Tanaka R, Kusaba H, Harada M, and Nakano S (2006a) Activated Src and Ras induce gefitinib resistance by activation of signaling pathways downstream of epidermal growth factor receptor in human gallbladder adenocarcinoma cells. *Cancer Chemother Pharmacol* **58:**577–584.
Qin JZ, Xin H, Sitailo LA, Denning MF, and Nickoloff BJ (2006b) Enhanced killing of melanoma cells by simultaneously targeting Mcl-1 and NOXA. *Cancer Res* **66:**9636–9645.
Raffo AJ, Perlman H, Chen MW, Day ML, Streitman JS, and Buttyan R (1995) Overexpression of bcl-2 protects prostate cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo. *Cancer Res* **55:**4438–4445.
Reed JC, Miyashita T, Takayama S, Wang HG, Sato T, Krajewski S, Aimé-Sempé C, Bodrug S, Kitada S, and Hanada M (1996) BCL-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. *J Cell Biochem* **60:**23–32.
Ross PJ, George M, Cunningham D, DiStefano F, Andreyev HJ, Workman P, and Clarke PA (2001) Inhibition of Kirsten RAS expression in human colorectal cancer using rationally selected Kirsten-RAS antisense oligonucleotides. *Mol Cancer Ther* **1:**29–41.
Rusnak DW, Lackey K, Affleck K, Wood ER, Alligood KJ, Rhodes N, Keith BR, Murray DM, Knight WB, Mullin RJ, et al. (2001) The effects of the novel, reversible epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and tumor-derived cell lines in vitro and in vivo. *Mol Cancer Ther* **1:**85–94.
Salomon DS, Brandt R, Ciardiello F, and Normanno N (1995) Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* **19:**183–232.
Schmidt-Ullrich RK, Contessa JN, Lammering G, Amorino G, and Lin PS (2003) ERBB receptor tyrosine kinases and cellular radiation responses. *Oncogene* **22:**5855–5865.
Shirasawa S, Furuse M, Yokoyama N, and Sasazuki T (1993) Altered growth of human colon cancer cell lines disrupted at activated Ki-ras. *Science* **260:**85–88.
Sizeland AM and Burgess AW (1992) Anti-sense transforming growth factor α oligonucleotides inhibit autocrine stimulated proliferation of a colon carcinoma cell line. *Mol Biol Cell* **3:**1235–1243.
Sizemore N, Cox AD, Barnard JA, Oldham SM, Reynolds ER, Der CJ, and Coffey RJ (1999) Pharmacological inhibition of Ras-transformed epithelial cell growth is linked to down-regulation of epidermal growth factor-related peptides. *Gastroenterology* **117:**567–576.
Sklar MD (1988) The ras oncogenes increase the intrinsic resistance of NIH 3T3 cells to ionizing radiation. *Science* **239:**645–647.
Sok JC, Coppelli FM, Thomas SM, Lango MN, Xi S, Hunt JL, Freilino ML, Graner MW, Wikstrand CJ, Bigner DD, et al. (2006) Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. *Clin Cancer Res* **12:**5064–5073.
Sumitomo M, Tachibana M, Nakashima J, Murai M, Miyajima A, Kimura F, Hayakawa M, and Nakamura H (1999) An essential role for nuclear factor kappa B in preventing TNF-alpha-induced cell death in prostate cancer cells. *J Urol* **161:**674–679.
Sunpaweravong P, Sunpaweravong S, Puttawibul P, Mitarnun W, Zeng C, Barón AE, Franklin W, Said S, and Varella-Garcia M (2005) Epidermal growth factor receptor and cyclin D1 are independently amplified and overexpressed in esophageal squamous cell carcinoma. *J Cancer Res Clin Oncol* **131:**111–119.
Szakács G, Paterson JK, Ludwig JA, Booth-Genthe C, and Gottesman MM (2006) Targeting multidrug resistance in cancer. *Nat Rev Drug Discov* **5:**219–234.
Trudel S, Li ZH, Rauw J, Tiedemann RE, Wen XY, and Stewart AK (2007) Preclinical studies of the pan-Bcl inhibitor obatoclax (GX015–070) in multiple myeloma. *Blood* **109:**5430–5438.
Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, Adams JM, and Huang DC (2005) Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* **19:**1294–1305.
Witters LM, Witkoski A, Planas-Silva MD, Berger M, Viallet J, and Lipton A (2007) Synergistic inhibition of breast cancer cell lines with a dual inhibitor of EGFR-HER-2/neu and a Bcl-2 inhibitor. *Oncol Rep* **17:**465–469.
Wood ER, Truesdale AT, McDonald OB, Yuan D, Hassell A, Dickerson SH, Ellis B, Pennisi C, Horne E, Lackey K, et al. (2004) A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. *Cancer Res* **64:**6652–6659.
Xia W, Bacus S, Hegde P, Husain I, Strum J, Liu L, Paulazzo G, Lyass L, Trusk P, Hill J, et al. (2006) A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. *Proc Natl Acad Sci U S A* **103:**7795–7800.
Xia W, Husain I, Liu L, Bacus S, Saini S, Spohn J, Pry K, Westlund R, Stein SH, and Spector NL (2007) Lapatinib antitumor activity is not dependent upon phosphatase and tensin homologue deleted on chromosome 10 in ErbB2-overexpressing breast cancers. *Cancer Res* **67:**1170–1175.
Yan J, Roy S, Apolloni A, Lane A, and Hancock JF (1998) RAS isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase. *J Biol Chem* **273:**24052–24056.
Yarden Y and Sliwkowski MX (2001) Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* **2:**127–137.
Zhou Y, Li S, Hu YP, Wang J, Hauser J, Conway AN, Vinci MA, Humphrey L, Zborowska E, Willson JK, and Brattain MG (2006) Blockade of EGFR and ErbB2 by the novel dual EGFR and ErbB2 tyrosine kinase inhibitor GW572016 sensitizes human colon carcinoma GEO cells to apoptosis. *Cancer Res* **66:**404–411.

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