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Inhibition of PI3K/mTOR Leads to Adaptive Resistance in Matrix-Attached Cancer Cells

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SUMMARY

The PI3K/mTOR-pathway is the most commonly dysregulated pathway in epithelial cancers and represents an important target for cancer therapeutics. Here, we show that dual inhibition of PI3K/mTOR in ovarian cancer-spheroids leads to death of inner matrix-deprived cells, whereas matrix-attached cells are resistant. This matrix-associated resistance is mediated by drug-induced upregulation of cellular survival programs that involve both FOXO-regulated transcription and cap-independent translation. Inhibition of any one of several upregulated proteins, including Bcl-2, EGFR, or IGF1R, abrogates resistance to PI3K/mTOR inhibition. These results demonstrate that acute adaptive responses to PI3K/mTOR inhibition in matrix-attached cells resemble well-conserved stress responses to nutrient and growth factor deprivation. Bypass of this resistance mechanism through rational design of drug combinations could significantly enhance PI3K-targeted drug efficacy.

INTRODUCTION

The most prevalent forms of cancer are of epithelial origin. Normal epithelial cells form well-organized polarized cell layers under the influence of extracellular matrix (ECM), and attachment to ECM is required for the control of normal epithelial cell proliferation, differentiation, and survival (Debnath and Brugge, 2005). However, during cancer progression, the normal epithelial organization is disrupted and malignant cells proliferate and survive outside their normal niches (Chiarugi and Giannoni, 2008). This process is not well recapitulated in two-dimensional (2D) cell cultures and may explain, in part, the failure of many therapeutic approaches in clinical trials. Thus, there is a need and opportunity to explore mechanisms of drug response and resistance under culture conditions that more closely mimic the in vivo environment.

Three-dimensional (3D) cell culture models have been widely utilized in epithelial cancer research to probe mechanisms involved in tumor initiation and progression (Debnath and Brugge, 2005; Weigelt and Bissell, 2008; Yamada and Cukierman, 2007). Nontransformed epithelial cells cultured in reconstituted basement membrane form hollow, growth-arrested, polarized 3D structures that recapitulate many features of epithelial cells in vivo. Survival of 3D spheroid structures is dependent on attachment to ECM; inner cells, lacking ECM attachment, undergo apoptosis, generating a hollow lumen (Chiarugi and Giannoni, 2008; Debnath and Brugge, 2005). However, cells that harbor alterations that inhibit apoptosis are still compromised in their survival because of metabolic impairment caused by ECM deprivation leading to decreased growth factor signaling, mostly due to loss of nutrient uptake (Schafer et al., 2009). These findings suggest that survival of tumor cells

Significance

To fulfill the promise of PI3K/mTOR-pathway inhibition in cancer, it is critical to identify mechanisms of resistance and develop therapies to overcome it. Here, we show that resistance of matrix-attached cells to PI3K/mTOR-inhibitors is associated with upregulation of an evolutionarily well-conserved program that resembles nutrient deprivation, leading to translation of IRES-containing mRNAs previously associated with cellular stress responses. This adaptive response leads to upregulation of prosurvival proteins, and inhibition of this program abrogates resistance to PI3K/mTOR-inhibition in breast and ovarian cancer cells and ovarian cancer xenografts. These studies provide a platform for rational development of effective drug combinations through the use of three-dimensional (3D) model systems together with high-throughput proteomics approaches and identify drug combinations for further analysis as cancer therapeutics.

outside their natural matrix niches may require alterations that allow them to escape both apoptosis and metabolic impairment. Introduction of oncogenes into nontumorigenic cells in 3D can result in the formation of solid, filled structures that resemble tumor cells grown in 3D as well as the filled alveoli characteristic of ductal carcinoma in situ, a noninvasive form of breast cancer (Debnath and Brugge, 2005). Oncogenic alterations in the phosphatidylinositol 3-kinase (PI3K) pathway have been shown to rescue matrix-deprived cells from apoptosis and metabolic impairment and to cause filling of the luminal space in 3D cultures (Debnath et al., 2003b; Isakoff et al., 2005).

In epithelial cancers, the signaling pathway most frequently activated by genetic alterations is the PI3K pathway (Engelman, 2009; Samuels and Velculescu, 2004; Yuan and Cantley, 2008) and can be activated by multiple mechanisms, including altered DNA copy number and mutations of several of the components of this pathway (Carpten et al., 2007; Gewinner et al., 2009; Li et al., 1997; Samuels et al., 2004). Thus, this pathway presents an attractive candidate for targeted therapeutics. In recent years, many small molecule inhibitors targeting PI3K, AKT, and the downstream effector mTOR, have been developed; several are in clinical trials, and some (specifically, rapamycin analogs targeting mTOR) have been approved for therapy (Baselga, 2011; Courtney et al., 2010). Most initial in vitro studies of these inhibitors were performed in 2D monolayer cell cultures where it is not feasible to examine the effects of the targeted therapeutics on tumor-specific phenotypes such as loss of polarity and survival without ECM attachment. However, in studies using 3D cultures, the effects of PI3K inhibition on tumor growth in vivo correlated with effects on cell proliferation in 3D cultures more accurately than in 2D cultures, suggesting that 3D cultures better reflect drug sensitivity in vivo (Howes et al., 2007; Polo et al., 2010). In addition, other studies have shown that signaling pathway activation and drug responses in 3D cultures differ substantially from 2D cultures (Kenny et al., 2007; Liu et al., 2004; Weigelt et al., 2010), emphasizing the potential use of 3D culture to ascertain underlying mechanisms of sensitivity and resistance to targeted therapeutics as well as to predict effective drug combinations.

Inhibition of PI3K/AKT can lead to upregulation and activation of receptor tyrosine kinases (RTKs) through engagement of several homeostatic feedback loops aimed at maintaining the PI3K pathway in equilibrium. This includes a FOXO-dependent pathway (Chandarlapaty et al., 2011; Garrett et al., 2011), as well as compensatory activation of the MAPK pathway (Carracedo et al., 2008; Engelman et al., 2008; Serra et al., 2011). Inhibition of upstream components of the PI3K pathway has been shown to lead to compensatory activation of HER3 (Sergina et al., 2007). In addition, inhibition of mTORC1 by allosteric inhibitors (e.g., rapamycin family) relieves a negative feedback loop from S6K to IRS1, among others, leading to activation of IGF1R, PI3K, and AKT (Cloughesy et al., 2008; Harrington et al., 2004; O'Reilly et al., 2006). Activation of compensatory pathways in response to PI3K/mTOR inhibition may limit the efficacy of drugs targeting this pathway, leading to paradoxical responses and possibly contributing to emergence of drug-resistant clones.

In this report, we examined the response of ovarian and breast tumor cells in 3D spheroid cultures to PI3K and mTOR inhibitors in order to monitor drug-induced phenotypic changes that could not be assayed in standard 2D cultures.

RESULTS

Dichotomous Response to PI3K Pathway Inhibition in Tumor Spheroids

To examine how ovarian cancer cells cultured as 3D spheroids in reconstituted basement membrane (Matrigel) respond to inhibition of the PI3K/mTOR pathway, we treated several ovarian cancer cell lines with BEZ235, a dual-specificity PI3K/mTOR inhibitor (Maira et al., 2008). In 3D culture, OV2008, SKOV3, and OVCA432 cell lines form spheroid structures with a constitutive low level of proliferation (marked by Ki67 staining) localized predominantly in the outer matrix-attached cell layer (Figure 1A). A small number of apoptotic cells, marked by cleaved caspase-3 staining, were detected almost exclusively in the middle of the structures. Treatment of 6-day-old spheroids with BEZ235 induced a striking dichotomy in the induction of apoptosis between outer and inner cells, with apoptosis being exclusively localized in the inner region of the spheroids (Figure 1B; Figure S1A available online). MCAS cells, which form structures with hollow lumens, were largely resistant to apoptosis. Although apoptosis was restricted to inner cells, BEZ235 treatment suppressed proliferation throughout the structures (data not shown). OV2008 cells were treated cells with a wider selection of PI3K or mTOR inhibitors (Rapamycin, LY294002, GDC0941, and PIK-90) to determine the generality of this response. All inhibitors induced a similar dichotomy in apoptosis between the drug-resistant matrix-attached outer cells and the inner matrix-deprived cells (Figure S1B).

Multiple Signaling Pathways Are Upregulated by PI3K/mTOR Inhibition

Outer 3D tumor spheroid cells could be intrinsically drug resistant, or drug treatment could induce resistance. To examine these possibilities, we performed reverse phase protein array (RPPA) analysis of ~120 proteins and phosphoproteins representing many major signaling pathways (Figure 2A). As expected, BEZ235 treatment decreased phosphorylation of proteins downstream of PI3K and mTOR (p-FOXO3a, p-4E-BP1) and reduced cell proliferation markers (PCNA, cyclinB1, cyclinE1) (Figure 2A). However, BEZ235 also induced upregulation and/or activation of multiple prosurvival proteins (Figure 2A), including several RTKs (EGFR, HER2, c-Kit, and IGF1R), cytoplasmic kinases (p-p90RSK, p-SrcY), antiapoptotic proteins (Bcl-2, XIAP1), and transcription factors (p-STAT3, p-STAT6, p-c-Jun, p-SMAD3). This response was not unique to 3D cell cultures, as 2D cultures treated with BEZ235 displayed similar responses, with multiple survival pathways being activated by BEZ235 (Figure 2A). A comparable response was also observed in BEZ235-treated MCAS spheroids and monolayer cultures (Figures S2A and S2B), with upregulation of Bcl-2, IGF1Rβ, p-STAT3, p-STAT6, p-c-Jun, p-SMAD3, p-p90RSK, EGFR, and p-HER2; however, Bcl-xL and p-Erk were also upregulated in MCAS cells.

RPPA results were validated by western blot for Bcl-2, p-IGF1Rβ, c-Jun, p-p90RSK, p-EGFR, p-S6, and p-4E-BP1 (Figures 2B; Figure S2B). While detectable changes in total

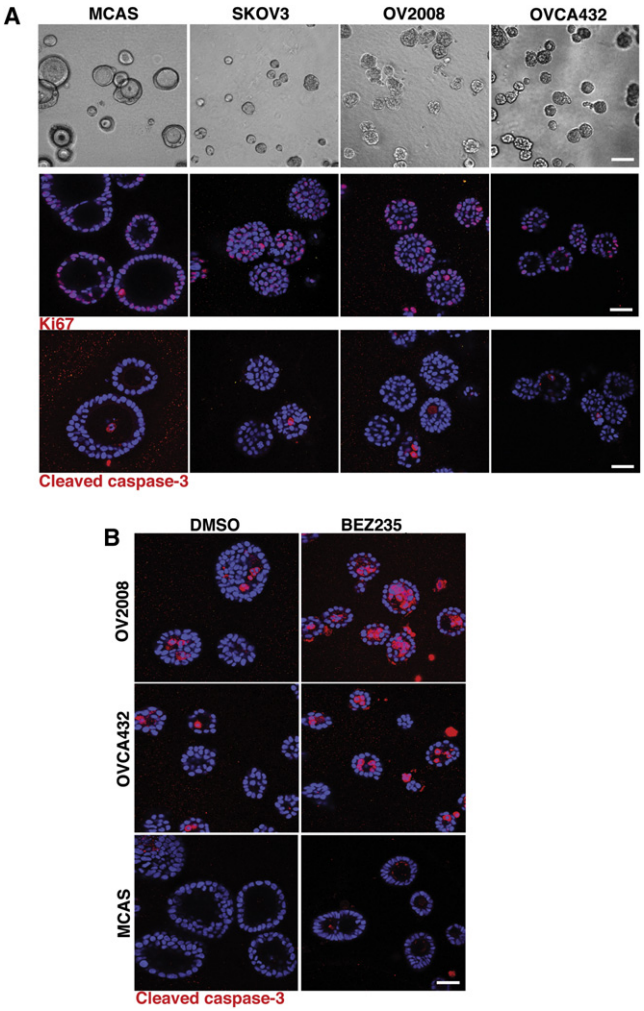

Figure 1. Ovarian Cancer Cell Lines Form Acinilike Structures in Reconstituted Basement Membrane and Matrix-Attached Cells Show Resistance to BEZ235-Induced Apoptosis

(A) Ovarian cancer cell lines (*PIK3CA* mutations indicated in parentheses) MCAS (H1047R), SKOV3 (H1047R), OVCA432 (unknown) and OV2008 (E545K) were cultured in Matrigel for 6 days and stained for Ki67 (red) or cleaved caspase-3 (red) and counterstained with DAPI (blue). (Mutation information was provided by S. Jones and V. Velculescu, personal communication.) One representative phase or confocal section is shown.

(B) OV2008, MCAS, and OVCA432 cells were cultured in Matrigel for 4 days and 1 μM BEZ235 was added for 48 hr before the cells were fixed, stained for cleaved caspase-3 (red) and DAPI (blue) and imaged by confocal microscopy. Confocal scale bar, 50 μm; phase contrast scale bar, 200 μm. See also Figure S1.

IGF1Rβ and p-AKT^{S473} were modest by RPPA, we reproducibly detected up- or downregulation, respectively, upon BEZ235 treatment by western blotting. Total protein levels of 4E-BP1 inversely correlated with those of p-4E-BP1 as previously reported (Yamaguchi et al., 2008). Furthermore, we confirmed that p-Erk was unaffected by BEZ235 treatment (Figure 2B).

To examine whether the proteins induced by BEZ235 were specifically induced in matrix-attached outer cells, we performed immunofluorescence analysis of Bcl-2 and Bcl-xL in BEZ235-treated OV2008 and MCAS cells. Indeed, upregulation of Bcl-2 and Bcl-xL was strongly enriched in outer, ECM-attached cells (Figure S2C). As ECM contact activates integrins, we addressed whether blocking integrin signaling could inhibit the adaptive response of the matrix-attached outer cells. Downregulation of β1-integrin, β4-integrin, FAK, or ILK alone did not abrogate Bcl-2 or IGF1R upregulation; however, inhibition of several integrin-pathway components in parallel, prevented upregulation of these proteins (Figure S2H). These results suggest that multiple integrins may contribute to the adaptive program; this possibility was supported by the evidence that additional integrins are induced at the transcriptional level by BEZ235 treatment (Figure S2I).

Inhibition of Bcl-2, IGF1R, or EGFR in Combination with BEZ235 Abrogates Matrix Protection

As BEZ235 treatment induced upregulation of several cell survival pathways, we assessed whether these pathways are critical for the resistance of matrix-attached cells. Given the critical role of Bcl-2 family members in regulation of cell survival (Walensky, 2006), we first examined the effects of inhibiting Bcl-2 family proteins in combination with BEZ235. Treatment with the Bcl-2 family inhibitor ABT-737, which targets Bcl-2, Bcl-xL, and Bcl-w (Oltersdorf et al., 2005), induced apoptosis of inner spheroid cells but did not affect survival of outer cells (Figure 2C). However, combined treatment with ABT-737 and BEZ235 induced massive disintegration of the spheroids. Immunostaining for cleaved caspase-3 confirmed extensive apoptosis throughout the entire spheroid structure (Figure 2C). Similar effects were observed using the structurally distinct Bcl-2 inhibitor, HA-14.1 (Figure S2D). Analysis of four additional ovarian cancer cell lines revealed similar synthetic lethality of ABT-737 and BEZ235 (Figure S2E).

We also examined whether the BEZ235-induced RTKs EGFR and IGF1R are essential for survival of BEZ235-treated cells. BEZ235 treatment together with EGFR inhibitors PD168393 or Gefitinib caused marked cell death (Figure S2F). In addition, downregulation of IGF1R with shRNAs caused death of ECM-attached cells treated with BEZ235 (Figure S2G). To address whether inhibition of other antiapoptotic signaling proteins that were not induced in the ECM-attached cells by BEZ235 would synergize with BEZ235, we incubated BEZ235-treated cells with inhibitors of MEK, PKC, PKA, Jak, or IKK. Inhibition of these proteins in combination with BEZ235 did not enhance death of outer, ECM-attached cells (Figure S2D; data not shown). These results imply that BEZ235-treatment selectively induces expression of several prosurvival proteins in ECM-attached cells and indicate that some of these induced proteins, such as Bcl-2, EGFR, and IGF-1R, are required for cancer cell survival. Our studies also validate RPPA as an efficient tool in identifying drug resistance proteins and pathways.

Many Induced Proteins Are Upregulated at the mRNA Level through FOXO-Dependent Transcription

To address whether BEZ235-induced protein upregulation reflects changes in mRNA expression, we performed mRNA microarray analyses on drug-treated OV2008 and MCAS cells cultured in 3D. Several genes, such as *IGF1R*, *EGFR*, *BCL2*, *IRS1*, and *SMAD3*, displayed increased mRNA expression,

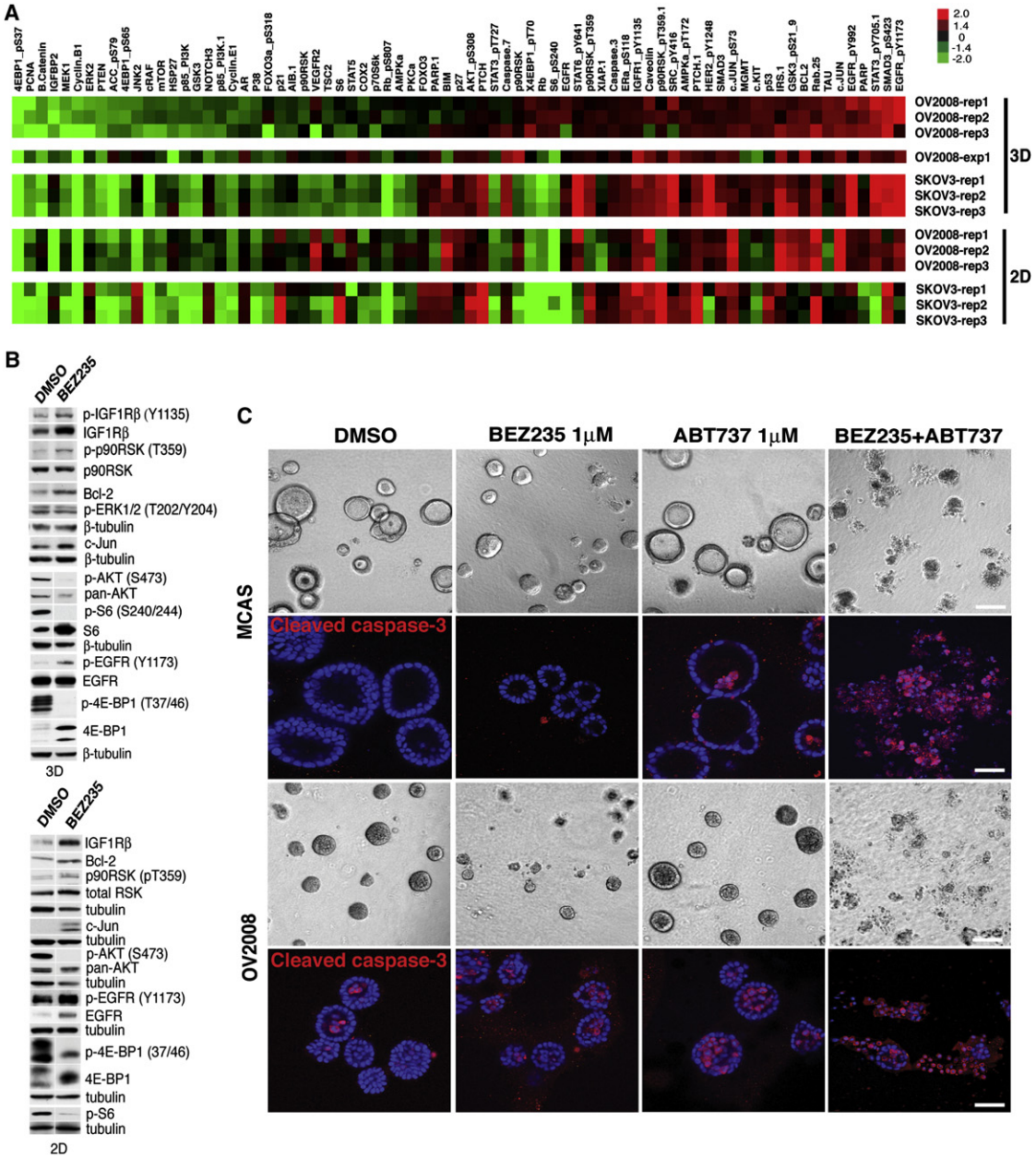

Figure 2. RPPA Reveals Multiple Proteins Upregulated in BEZ235-Treated Cells

(A) OV2008 and SKOV3 cells were cultured in Matrigel (3D) or monolayer (2D) and treated with BEZ235 or DMSO, and the protein lysates were analyzed by RPPA (red, increased signal; and green, decreased signal, upon BEZ235 treatment). Samples are normalized against DMSO-treated controls. Proteins with significant differences ($p < 0.05$, Student's t test) between BEZ235- and DMSO-treated OV2008 cells in an experiment performed in triplicate are shown for all experiments.

(B) Several of the up- and downregulated proteins from OV2008 RPPA were validated by western blot analysis.

(C) Bcl-2 inhibition abrogates outer cell resistance to PI3K/mTOR inhibition. MCAS and OV2008 cell lines were cultured in 3D and treated with DMSO, BEZ235, or ABT737 alone or in combination for 48 hr. Cells were imaged by phase contrast, fixed, stained for cleaved caspase-3 (red), and imaged by phase contrast or confocal microscopy. Confocal scale bar, 50 μm; phase contrast scale bar, 200 μm. See also Figure S2.

indicating that a subset of the RPPA results reflect, at least in part, changes in mRNA levels (Figure 3A).

Inhibition of PI3K prevents activation of AKT1/2/3, which phosphorylates FOXO family transcription factors, preventing their nuclear translocation (Brunet et al., 1999; Kops et al., 1999). Because FOXO family members were recently demonstrated to induce transcription of several RTKs upon AKT inhibition (Chandarlapaty et al., 2011), we analyzed the mRNA expression array for FOXO targets. A significant enrichment of known FOXO target genes were upregulated after BEZ235 treatment ($p = 0.00335$, hypergeometric probability distribution), including *CDKN1B* (p27Kip1), *TSC1*, *IRS2*, and *FOXO1/3* (Figure 3B).

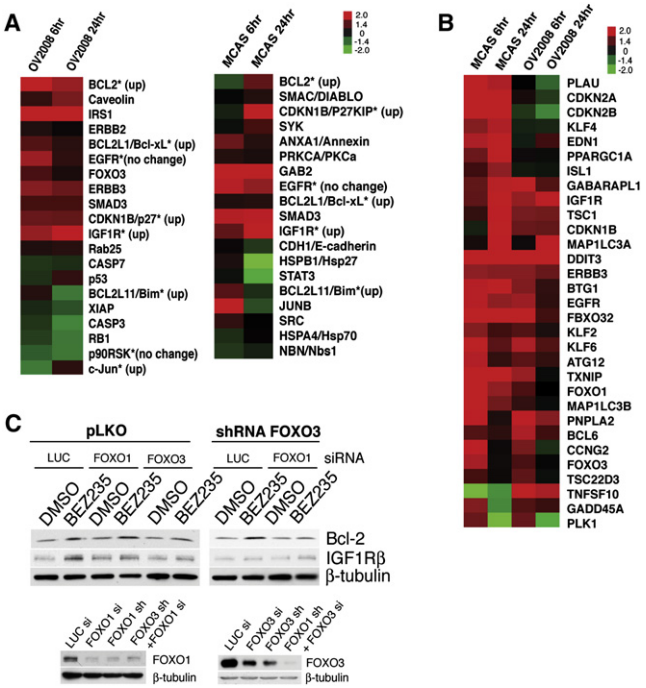

Figure 3. Many of the Induced Proteins Are Upregulated at the mRNA Level through FOXO-Dependent Transcription

(A) Heatmaps showing relative levels of mRNA of proteins that were upregulated in the RPPA in OV2008 and MCAS cells; additional RTKs not present in RPPA were also selected for analysis. mRNA values were normalized relative to DMSO-treated cells. Red, upregulation in response to BEZ235 treatment; and green, downregulation. Approximately 60% (12/20 for OV2008 and 11/19 for MCAS) of the proteins that are detectably upregulated in response to BEZ235 in RPPAs and detected in the mRNA expression array show elevated mRNA at either time point ($p = 0.000385$ for OV2008 and $p = 0.041$ for MCAS, hypergeometric probability distribution). Proteins validated by western blots are marked by an asterisk, and the direction of change is indicated in parentheses.

(B) Heatmap showing relative levels of mRNAs previously reported as FOXO targets; of 117 reported FOXO targets, 31 are upregulated in at least two conditions ($p < .01$), showing a significant enrichment ($p = 0.00335$, hypergeometric probability distribution).

(C) FOXO1 and FOXO3 were downregulated in OV2008 cells by siRNAs targeting FOXO1 or FOXO3 (left) or transduction of a lentiviral vector encoding shRNA for FOXO3, together with FOXO1 siRNAs (right). Lysates were probed with antibodies to Bcl-2 and IGF1Rβ to monitor upregulation upon 18-hr BEZ235 treatment. The efficacy of the knockdowns was verified by western blotting of FOXO1 and FOXO3.

Several FOXO-regulated RTKs (*ERBB3*, *EGFR*, and *IGF1R*) were upregulated in BEZ235-treated cells (Figure 3B), suggesting that FOXO could mediate transcription of some of the upregulated proteins. Knockdown of either FOXO1 or FOXO3 reduced IGF1Rβ upregulation by BEZ235, whereas FOXO3 knockdown reduced Bcl-2 as well; knockdown of both FOXO1 and FOXO3 inhibited upregulation of both Bcl-2 and IGF1Rβ (Figure 3C).

Inhibition of the mTORC1 Target 4E-BP1 Correlates with Upregulation of Bcl-2 and IGF1Rβ

BEZ235 is a dual inhibitor of both PI3K and mTORC1/2. To assess whether inhibition of either PI3K or TORC1/2 is necessary and/or sufficient to induce Bcl-2 and synergize with ABT-737, we examined several additional inhibitors of PI3K or mTOR: GDC0941 (PI3K inhibitor at 250nM, PI3K, and mTOR at 1 μM), rapamycin (allosteric mTOR inhibitor selective for mTORC1), and Torin1 (mTORC1/2 catalytic inhibitor). The most cell death was induced with ABT-737 in combination with either BEZ235, Torin1, or GDC0941 at a concentration that also inhibits mTOR (Figures 4A and 4B). Neither the PI3K-selective (250 nM GDC0941) nor the mTORC1-specific (rapamycin) inhibitors induced spheroid disintegration with ABT-737 (Figures 4A and 4B). Western blots demonstrated decreased p-4E-BP1 correlated with Bcl-2 upregulation (Figure 4C). 4E-BP1 phosphorylation is relatively insensitive to rapamycin, and long-term suppression of 4E-BP1 requires inhibition of mTOR catalytic activity (Choo et al., 2008; Feldman et al., 2009; Thoreen et al., 2009). We also noticed that inhibition of PI3K enhanced Bcl-2 upregulation, as 250 nM Torin1 significantly inhibited p-4E-BP1, but did not induce Bcl-2 as efficiently as 1 μM Torin or 1 μM GDC0941, which inhibit both 4E-BP1 and AKT phosphorylation (Figure 4C). These findings demonstrate that reduced 4E-BP1 phosphorylation correlates with upregulation of Bcl-2, but PI3K inhibition enhances this effect, likely through other mechanisms such as FOXO activation.

Increased Cap-Independent Translation in Response to mTOR Inhibition

mTORC1 activates cap-dependent translation by phosphorylating and inhibiting 4E-BP1. However, cell stress can suppress cap-dependent translation through inhibition of mTOR. Under these conditions, essential cell survival proteins (i.e., regulators of cell cycle, development, apoptosis, and stress response) are translated through a cap-independent mechanism (Holcik and Sonenberg, 2005; Silvera et al., 2010). A switch to cap-independent translation can also be achieved by direct inhibition of mTORC1 or perturbation of 4E-BP1 (Braunstein et al., 2007; Choo et al., 2008; Feldman et al., 2009; Hsieh et al., 2010; Moerke et al., 2007; She et al., 2010; Thoreen et al., 2009). mRNAs translated under these conditions have a highly structured 5′-untranslated region (5′-UTR), which often harbors an IRES sequence and several upstream AUGs. It is interesting that multiple proteins upregulated by BEZ235—including Bcl-2, Bcl-xL, IGF1R, XIAP, c-Jun, and p53—can all be translated in a cap-independent manner under cellular stress. To address whether cap-independent translation increases in response to PI3K/mTOR inhibition, we used a bicistronic luciferase reporter where Renilla luciferase is cap dependent and firefly luciferase is regulated by the cap-independent cricket paralysis virus IRES. Indeed, OV2008 and MCAS cells treated with BEZ235 or Torin1 (Figure 5A), but not rapamycin or 250 nM GDC0941 (Figures S3A and S3B), upregulated cap-independent translation, suggesting that an IRES translation is induced by mTOR inhibitors.

We also utilized a *BCL-2* IRES reporter that contains the *BCL-2* 5′-UTR upstream of firefly luciferase and is only translated by cap-independent translation (Suo et al., 2010). A short unstructured 5′-UTR reporter was used to monitor cap-dependent translation. BEZ235 treatment caused an ~38% increase in Bcl-2 IRES translation with a concomitant 60% decrease in cap-dependent reporter translation (Figure 5B). These results provide evidence

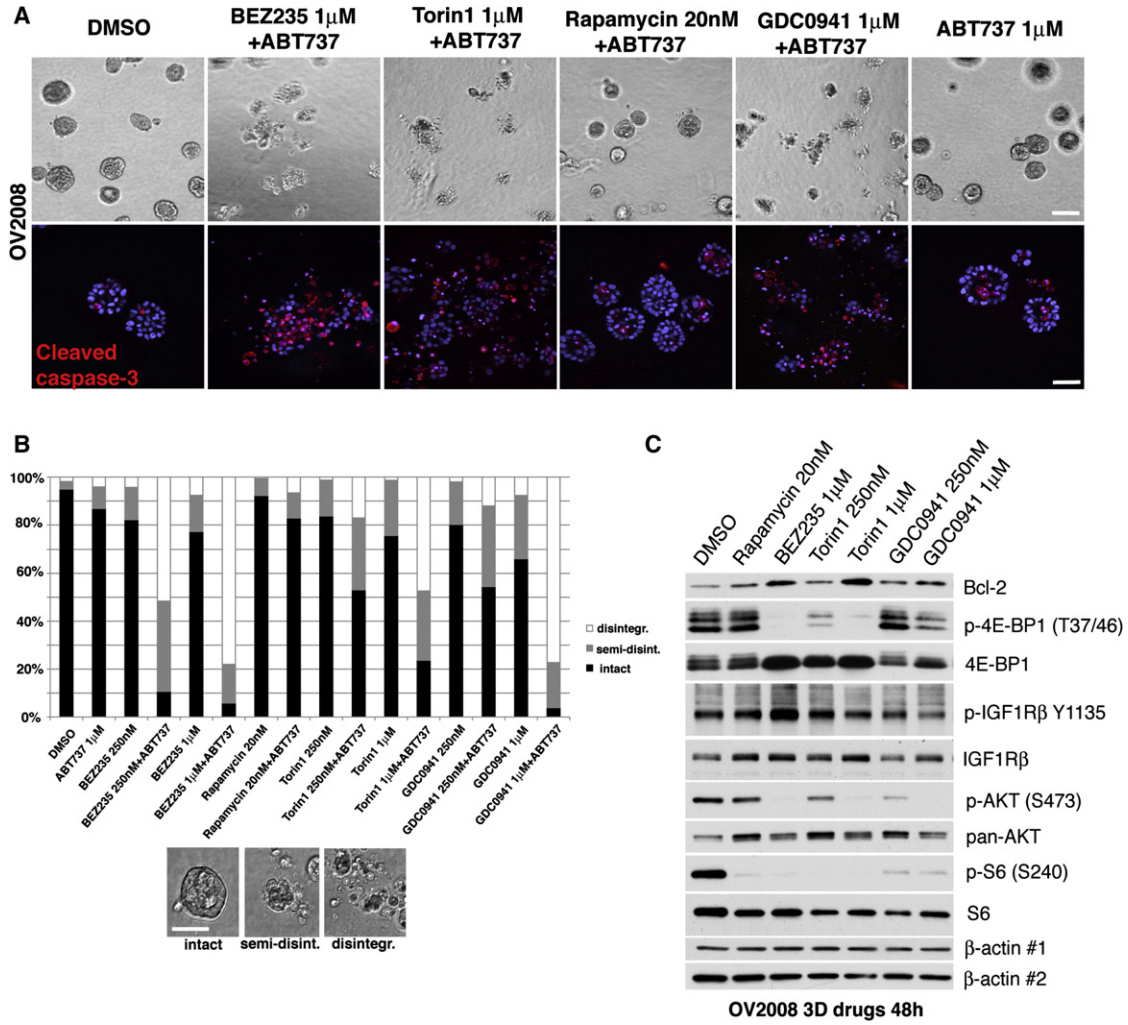

Figure 4. Inhibition of 4E-BP1 Phosphorylation Correlates with the Upregulation of Bcl-2 and IGF1R and Disintegration of 3D Spheroids

(A) OV2008 cells were grown in 3D cultures for 4 days and treated with indicated inhibitors targeting PI3K and/or mTOR in combination with ABT-737 for 48 hr. The cells were fixed, stained for cleaved caspase-3 (red) and DAPI (blue), and imaged with phase and confocal microscopy. Confocal scale bar, 50 μm; phase contrast scale bar, 200 μm.

(B) OV2008 cells were grown in 3D culture and treated with indicated drugs and quantitated for structural integrity after a 48-hr drug treatment as described in Experimental Procedures. Representative images of scored structures (intact, semidisintegrated, and disintegrated) are shown in the lower panel; scale bar, 100 μm.

(C) Lysates were harvested from structures cultured in 3D and analyzed for Bcl-2 and IGF1Rβ, p-4E-BP1$^{T37/46}$, p-S6^{S240}, and p-AKT^{S473} expression after the indicated 48-hr drug treatments.

that BEZ235 treatment increases cap-independent Bcl-2 translation while cap-dependent translation is inhibited.

Phosphorylation of the eukaryotic translation initiation factor 2 alpha (eIF2α), a component of the ternary translation initiation complex, selectively suppresses cap-dependent translation in response to cellular stress (Holcik and Sonenberg, 2005; Raven and Koromilas, 2008; Silvera et al., 2010; Wek et al., 2006). BEZ235 treatment increased eIF2α phosphorylation over time, reaching a plateau at 24 hr in OV2008 and 48 hr in MCAS cells (Figure 5C). Additionally, of 23 significantly upregulated proteins after BEZ235 treatment identified by RPPA, 10 have putative or validated IRES motifs. This represents a significant enrichment ($p = 0.0083$), as only 3%–5% of all mRNAs have potential IRES sequences (Johannes et al., 1999).

Breast Tumor Cell Lines Also Display the Adaptive Response to BEZ235 and Are Sensitive to Combined PI3K/mTOR and Bcl-2 Inhibition

To test whether the BEZ235 response is induced in other epithelial tumors, we performed RPPA on BEZ235-treated breast cancer cell lines harboring mutations in *PIK3CA* or deletions in *PTEN*. While there were variations in individual proteins that were affected by treatment, the adaptive response was strikingly similar, with PI3K/mTOR inhibition causing an induction of RTKs (HER3, IGF1Rβ, HER2, and EGFR), as well as Bcl-2 and Bcl-xL (Figure 6A). We also detected an adaptive response to BEZ235 in nontransformed MCF10A cells, although the most strongly upregulated proteins varied relative to the breast tumor cell lines. This likely reflects the dominance of the EGFR-ERK signaling

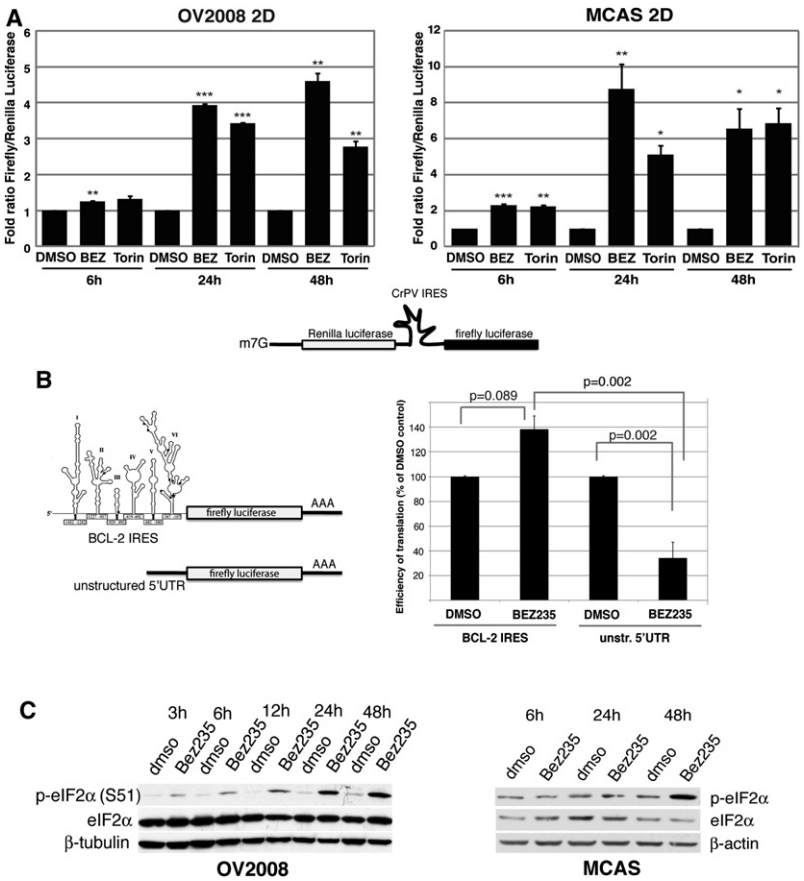

Figure 5. Cap-Independent Translation Increases in Response to BEZ235 Treatment

(A) A dual luciferase reporter (Renilla luciferase expression mediated by cap-dependent translation and firefly luciferase expression by CrPV IRES) was used to monitor cap-independent translation in OV2008 and MCAS cells in response to BEZ235 or Torin1 treatment. The fold increase in the ratio of the firefly/Renilla luciferase levels was calculated as described in Experimental Procedures. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

(B) *BCL-2* IRES translational activity was monitored by a reporter containing the *BCL2* IRES sequence upstream of firefly luciferase. Cap-dependent translation was monitored with a reporter containing a short unstructured 5′UTR fused to firefly luciferase. Luciferase expression from both reporters was normalized to luciferase mRNA expression and shown as fold change compared to DMSO control. Cartoon modified from Suo et al., 2010.

(C) Upregulation of eIF2α phosphorylation was monitored in response to BEZ235 treatment over time in OV2008 and MCAS cells. Error bars shown as ± SEM. See also Figure S3.

pathway in these cells (note upregulated p-EGFR, p-ERK, p-BAD^{S112}, and downregulated Bim) (Figures 6A and 6D).

We also examined whether the breast tumor cell lines would display enhanced cell death with combined inhibition of PI3K/mTOR and Bcl-2 in 3D cultures. In contrast to the well-organized ovarian cell lines, the breast cancer lines were less organized, precluding detection of a clear dichotomy between the outer and inner cell layers. However, as seen with ovarian lines, inhibition of PI3K/mTOR or Bcl-2 family members alone did not induce dramatic cell death, but combined treatment caused significant structural disintegration and cell death in MDA-MB-468 and HCC-1569 cells and, to a lesser extent, in T-47D cells (Figure 6B). In contrast, the overall integrity of non-transformed MCF-10A cell structures treated with both BEZ235 and ABT-737 was not significantly affected, as cells in contact with reconstituted matrix were resistant to apoptotic effects of the combination (Figure 6C). Nontransformed, nonimmortalized human mammary epithelial cells (HMECs) also showed no sensitivity to the dual treatment (Figure S4), indicating that cancer cells are more sensitive to this treatment than normal epithelial cells.

Mouse Xenografts and Primary Ovarian and Breast Cancer Patient Samples Show Sensitivity to Dual Inhibition of PI3K/mTOR and Bcl-2

To test the effectiveness of inhibiting PI3K/mTOR and/or the Bcl-2 family in vivo, we performed xenograft studies with subcutaneous injection of MCAS and OV2008 cells. Tumors were treated with the PI3K/mTOR inhibitor GNE493 (Sutherlin et al., 2010) and ABT-737 either alone or in combination every 24 hr for 7–9 days. Vehicle and ABT-737-treated MCAS tumors showed similar growth rates, whereas GNE493 significantly decreased tumor growth. Remarkably, combined treatment caused marked inhibition of tumor cell growth relative to GNE493 alone (Figure 7A). Similar results were obtained with OV2008 xenograft tumors (Figure 7A). Furthermore, hematoxylin and eosin (H&E) staining of tumor sections revealed that combination treatment induced increased cell death relative to single agents (Figure 7B). Combination treatment caused weight loss during the 7-day treatment (5% on average), and one mouse (of 23) showed more significant weight loss (~20%) and died on Day 7 of treatment, indicating that the combination may have some toxicity.

Lysates from GNE493-treated MCAS tumors showed the expected decrease in AKT, 4EBP1, and S6 phosphorylation, and it is important to note that Bcl-2, Bcl-xL, and IGF1Rβ were upregulated in response to treatment (Figure 7C). These results provide evidence that PI3K/mTOR inhibition in vivo induces a similar adaptive response as in 3D spheroids and monolayer culture in vitro and that Bcl-2 family inhibition can significantly enhance the efficacy PI3K/mTOR inhibition in vivo.

To address whether primary patient samples are sensitive to combination treatment, we isolated cancer cells from pleural and peritoneal fluid exudates from two breast cancer patients and two ovarian cancer patients. These samples were cultured in reconstituted basement membrane for 8 days followed by treatment with BEZ235, ABT-263 (BCL2 inhibitor similar to ABT-737; Ackler et al., 2008), PD168393, and combinations for 48 hr. Single-agent inhibition did not show significant efficacy in any of the treated patient samples (Figure 7D). The response to dual inhibition of PI3K/mTOR and EGFR was heterogeneous, only reaching significance in ovarian sample F44 (Figure 7D).

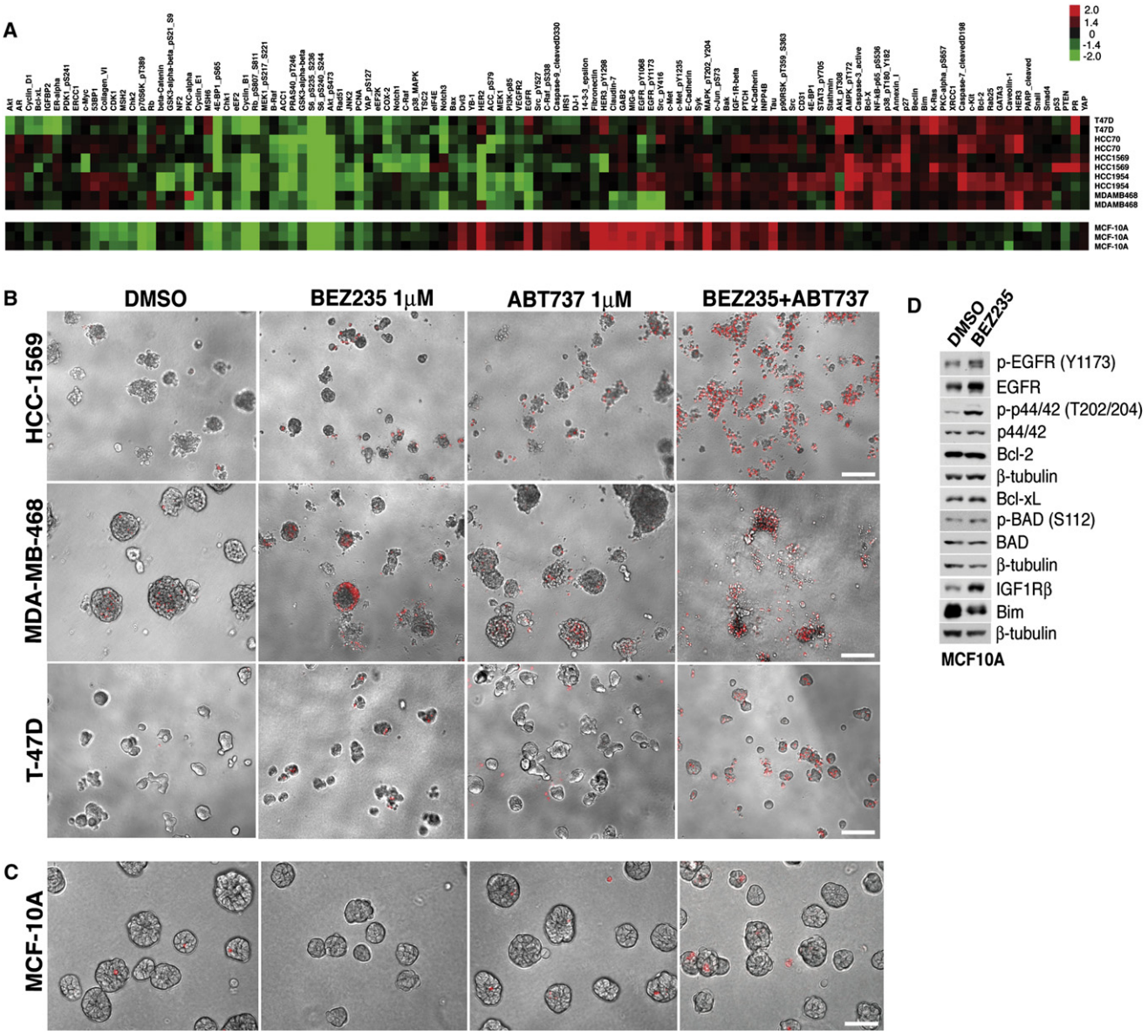

Figure 6. Analysis of BEZ235-Treated Normal Breast Epithelial Cells and Breast Tumor Cell Lines

(A) RPPA analysis of breast cancer cell line spheroids and MCF10A cell monolayers treated with BEZ235 for 48 hr (red, increased signal upon BEZ235 treatment; green, decreased signal upon BEZ235 treatment). Samples are normalized against DMSO-treated controls.

(B and C) T-47D, MDA-MB-468, and HCC-1569 breast cancer cells lines (in 6B) and nontransformed immortalized MCF10A breast epithelial cells (in 6C) were cultured in Matrigel and treated with DMSO, BEZ235, or ABT737 alone or in combination for 48 hr. Cells were imaged by phase contrast, fixed, stained with EtBr (red) to mark dead cells, and imaged by wide-field phase contrast microscopy. Scale bar, 200 μm.

(D) Immunoblots of MCF-10A cells treated with DMSO or BEZ235 for 48 hr. See also Figure S4.

However, all four samples showed significant response to dual treatment of BEZ235 and ABT-263 (Figure 7D). Together, these preliminary results suggest that further evaluation of this drug combination in more in vivo models and primary human tumor samples is warranted.

DISCUSSION

Here, using 3D spheroid cell cultures, we show that matrix-attached tumor cells are specifically resistant to dual PI3K/mTOR inhibition by inducing an adaptive response involving up-regulation of multiple prosurvival proteins. Induction of the adaptive response involves increased cap-independent translation and FOXO mediated transcription. It is interesting that this response resembles the highly conserved stress response induced in organisms deprived of nutrients and growth factors (Gilbert et al., 2007; Jefferson and Kimball, 2003; Marr et al., 2007; Puig and Tjian, 2005; Villa-Cuesta et al., 2010). In addition, this work establishes the 3D-based model system, particularly in combination with a high throughput proteomics approach, as

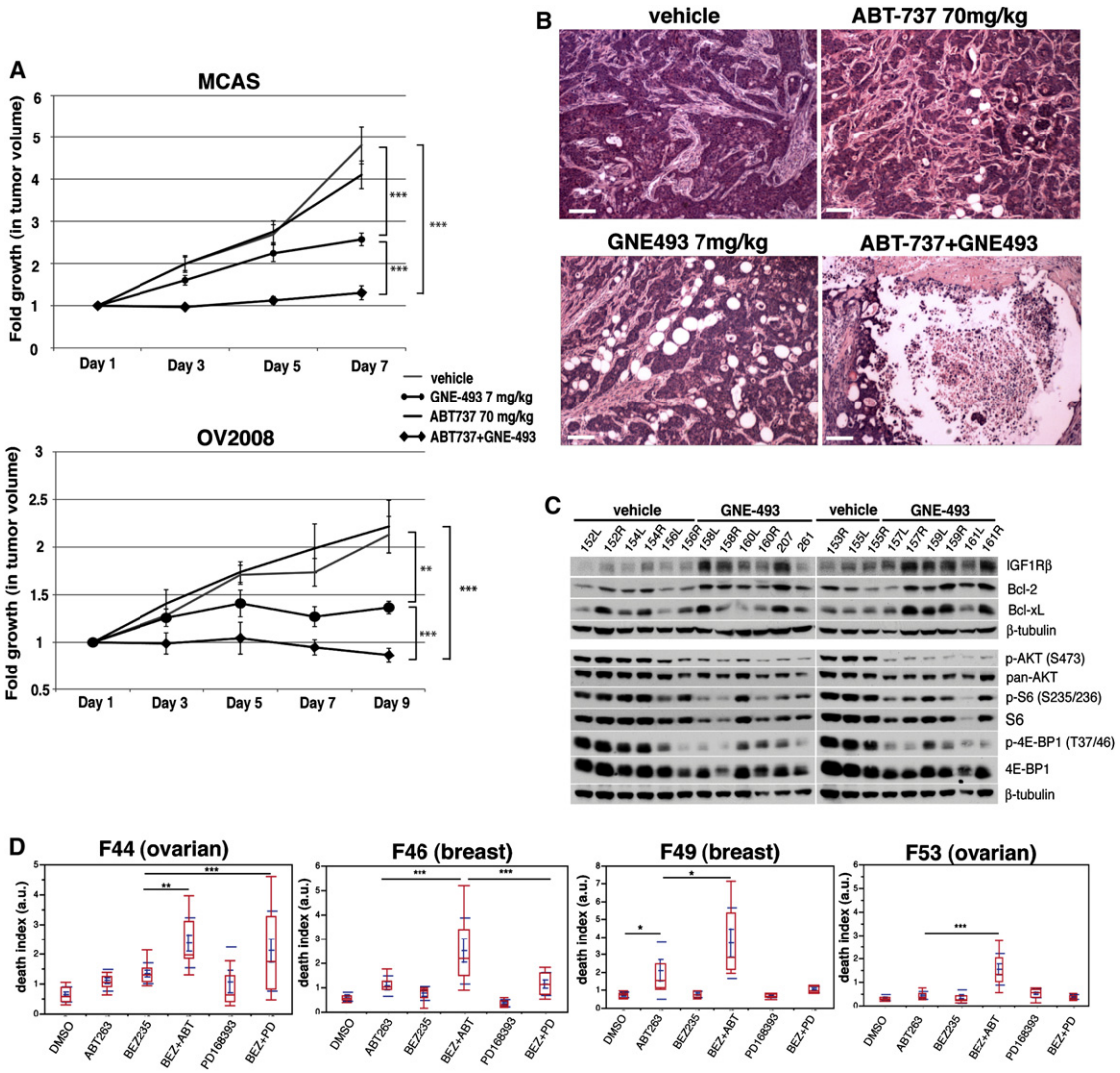

Figure 7. Dual Inhibition of Bcl-2 and PI3K/mTOR in an In Vivo Xenograft Model and in Primary Patient Samples Causes Decreased Tumor Growth and Enhanced Cell Death

(A) MCAS and OV2008 cells were injected subcutaneously into female nod/scid mice and, after tumors were palpable, mice were treated every 24 hr with vehicle, GNE493 (mTOR inhibitor, 7mg/kg), ABT-737 (70 mg/kg), or a combination of both. Tumors were measured on indicated days, and the data are reported as the fold change relative to size of the same tumor on Day 1. The data, represented as the average ± SEM, were derived from experiments in which 72 tumors were monitored in 43 mice for MCAS and 40 tumors were monitored in 20 mice for OV2008. ***$p < 0.001$; **$p < 0.005$.

(B) Tumor sections from MCAS xenografts were stained with H&E and imaged at 4×. Representative images of tumors of similar size (~300mm^3) for each treatment are shown. Scale bar, 100 μm.

(C) Western blot analysis of vehicle- and GNE493-treated MCAS tumors. Tumors were harvested 4 hr after the last dosing on Day 7 and probed with the indicated antibodies. Numbers above each lane indicate tumor numbers.

(D) Tumor cells isolated from peritoneal or pleural fluid exudates were cultured in Matrigel for 8 days and treated with the indicated inhibitors (ABT-263, an identical Bcl-2 inhibitor to ABT-737). Cell death was quantified by analysis of dead cells (as marked by EtBr) over total cell number (as analyzed by Hoechst stain). *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$. Red boxes indicate average data distribution, and highest and lowest value are indicated with connected red caps. Single blue caps indicate SD and connected blue caps indicate ± SEM.

a valuable approach for rational prediction of effective drug combinations.

Although treatment of cancer cells in monolayer cultures with PI3K/mTOR inhibitors suppresses cell proliferation, apoptosis is not commonly observed. In contrast, these inhibitors are potent inducers of apoptosis of inner cells within 3D spheroid cultures that are not attached to matrix, whereas cells in 2D cultures and the outer cells of tumor spheroids induce a strong survival program and provide an explanation for the lack of toxicity of PI3K/mTOR inhibitors in traditional 2D cultures. The enhanced sensitivity of the inner cells of 3D spheroids to apoptosis infers that PI3K activity is specifically required for survival of this population of tumor cells. The inner cells in 3D spheroids lack exposure to ECM components and likely rely on sustained PI3K activity for anchorage-independent survival. Indeed, oncogenic mutant variants of PI3K have been shown to rescue cells

from apoptosis caused by loss of matrix signaling (Isakoff et al., 2005; Martin et al., 2006; Schafer et al., 2009). These studies, together with our studies of spheroid cultures, highlight the critical role of the PI3K pathway in the survival of matrix-deprived cells and raise the question whether human epithelial tumor cells may display differential sensitivity to PI3K inhibitors depending on their differential association with ECM components.

The matrix-attached tumor cells initiate a transcriptional and translational program in response to PI3K/mTOR inhibition involving expression of signaling proteins that regulate survival. Whether the induction of this program by PI3K/mTOR inhibitors is exclusively due to ECM attachment or is dependent on other features of the outer cells (e.g., cell-cell adhesion or polarity) remains to be established. Weaver and coworkers have shown that α6/β4-integrin-dependent cell polarity controls responses to apoptotic stimuli via NFκB activation in normal epithelial cells (Friedland et al., 2007; Weaver et al., 2002); however, polarity of the outer cells was not required for protection in this study, and inhibition of NFκB signaling by expression of the NFκB super-repressor did not induce cell death in OV2008 BEZ235-treated cells. It has also been shown that β4-integrin signaling can enhance RTK signaling, invasion, and metastasis through c-Jun and STAT3 (Guo et al., 2006); however, expression of dominant-negative c-Jun or downregulation of STAT3 did not prevent the adaptive response to PI3K/mTOR inhibition in our system (T.M., unpublished results). While integrins are likely critical for the induction of the adaptive response, we were unable to identify a specific integrin or downstream component that plays a dominant role in the response, suggesting that this adaptive response is robust and likely includes several components of the integrin-signaling pathway.

Inhibition of PI3K pathway has been shown to relieve negative feedback inhibition of upstream pathways. Recently, two reports (Chandarlapaty et al., 2011; Garrett et al., 2011) described induction of expression of RTKs by FOXO transcription factors after inhibition of HER2 or AKT. We also observed significant enrichment in FOXO transcriptional targets after BEZ235 treatment, suggesting a role for FOXO transcription factors in the induction of the adaptive response at the mRNA level. However, our studies involving a more comprehensive proteomic approach revealed a much broader BEZ235-induced program involving significant translational regulation. Several lines of evidence support a role for cap-independent translation in the adaptive response. First, decreased p-4E-BP1 correlated with upregulation of Bcl-2 and IGF1Rβ. Because 4E-BP1 is a potent inhibitor of cap-dependent translation (Choo et al., 2008; Hsieh et al., 2010; Thoreen et al., 2009), this evidence, together with the presence of predicted or validated IRES sequences in many of the upregulated proteins, raised the possibility that cap-independent translation contributes to this program. In addition, using two reporters, we found that BEZ235 (but not rapamycin, which does not inhibit 4E-BP1 significantly, or a selective PI3K inhibitor) caused an increase in IRES-mediated translation and a significant decrease in cap-dependent translation, supporting the utilization of cap-independent translation in the context of mTOR inhibition.

Cells downregulate global translation under stress and rely on the less efficient cap-independent translation for production of proteins critical for cell survival (Holcik and Sonenberg, 2005; Silvera et al., 2010). Our results suggest that cancer cells may use a similar program to mediate survival under the stress of PI3K/mTOR inhibition. PI3K pathway inhibition not only reduces growth factor signaling but also inhibits nutrient uptake, mimicking nutrient starvation. It is not surprising that the adaptive response to PI3K/mTOR inhibition resembles the highly conserved stress responses observed in lower eukaryots in response to nutrient deprivation as well as endoplasmic reticulum, irradiation, or oxidative stress (Fels and Koumenis, 2006; Sengupta et al., 2010; Wek et al., 2006). In *Drosophila melanogaster*, nutrient deprivation leads to repression of cap-dependent translation and dFoxO-mediated induction of insulin receptor expression. Both dFoxO and insulin receptor can be translated via IRES-mediated mechanisms and are required for survival (Marr et al., 2007; Villa-Cuesta et al., 2010). A similar response to dietary restriction is also observed in *Caenorhabditis elegans* (Syntichaki et al., 2007). The adaptive response to nutrient or amino acid starvation also requires eIF2α phosphorylation, which decreases translation initiation generally; however, some IRES-containing mRNAs are preferentially translated (Allam and Ali, 2010; Fernandez et al., 2002; Gerlitz et al., 2002; Terenin et al., 2008). It is interesting that eIF2α phosphorylation also contributes to cell survival upon glucose deficiency and is associated with increased XIAP and Bcl-xL translation, both of which have been shown to harbor IRES sequences (Muaddi et al., 2010). Given that PI3K pathway inhibition suppresses glucose and amino acid transport and that mTOR inhibition suppresses cap-dependent translation (Choo et al., 2008; Hsieh et al., 2010; Thoreen et al., 2009), the evidence that combined PI3K/mTOR inhibitors elicit responses similar to those induced by nutrient starvation is not surprising. However, it has not been appreciated that targeted therapies would elicit these types of responses and that these would be amenable to rational therapeutic targeting.

Among the PI3K/mTOR inhibitor upregulated proteins, RTKs and antiapoptotic Bcl-2 family members were the most attractive targets for combination therapies, given their known role in cell survival. It is of interest that tamoxifen- or HER2/EGFR-resistant tumors display upregulation of several of the same targets, such as IGF1R, EGFR, and HER2 (Creighton et al., 2008; Massarweh et al., 2008; Plati et al., 2011; Riggins et al., 2007), and chemoresistance in ovarian carcinomas is associated with upregulation of Bcl-2 and Bcl-xL (Giménez-Bonafé et al., 2009; Jain and Meyer-Hermann, 2011; Plati et al., 2011; Walensky, 2006). In addition, irradiation (Holcik et al., 1999; Holcik et al., 2000) and hypoxia can both induce the switch to cap-independent translation (Braunstein et al., 2007), suggesting that upregulation of the pathways that we observed in this study could represent a mechanistic explanation of resistance to multiple types of cancer therapy.

Although further studies are required, our initial in vivo xenograft studies and analysis of breast and ovarian cancer patient samples suggest that inhibition of both Bcl-2 and PI3K/mTOR may be more effective than either alone. Furthermore, the effects of inhibition of Bcl-2 family members and PI3K/mTOR may be more general than the combination of PI3K/mTOR and EGFR if different RTKs are activated in tumor samples. It is interesting that nontransformed epithelial cells were highly resistant to combined treatment with BEZ235 and ABT737. We speculate

that one contributing factor might be the proapoptotic protein Bim, which is upregulated in the BEZ235-treated tumor cell lines but not in MCF-10A cells.

The results presented here demonstrate that inhibition of PI3K/mTOR in matrix-attached cancer cells initiates an evolutionarily conserved cellular stress response, mediated by FOXO transcription factors and preferential translation of IRES-containing mRNAs. This response leads to upregulation of signaling pathways required for cancer cell survival but only in populations of tumor cells within a specific niche (i.e., matrix-attached cells). It is possible that this stress response could contribute to relapse after treatment and possibly to the development of resistant tumor cells within matrix-associated niches in human tumors exposed to PI3K/mTOR inhibitors.

EXPERIMENTAL PROCEDURES

Additional experimental procedures are described in detail in the Supplemental Experimental Procedures.

Antibodies and Reagents, Cell Culture, Cell Lines, and Virus Production

These are described in the Supplemental Experimental Procedures.

In Vivo Xenograft Experiments

One million cells (MCAS and OV2008) were injected per flank, subcutaneously, into 10- to 12-week-old female Nod/Scid mice in a 1:1 mix of PBS and Matrigel. Once tumors became palpable (~250 mm^3), generally Day 8 postinjection for MCAS and Day 28 for OV2008, drugs were administered daily intraperitoneally. GNE493 (70mg/kg) was dissolved in 0.5% methylcellulose/0.2% Tween 80, and ABT-737 was dissolved in 30% propylene glycol/5% Tween 80/65% D5W (5% dextrose in water). All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee, the Standing Committee on Animals at Harvard University.

Primary Patient Samples

Primary cells were obtained from patients at Brigham and Women's Hospital (BWH) who underwent paracentesis for malignant ascites. The protocol was approved by the BWH Institutional Review Board (IRB), the Harvard Medical School Office for Research Subject Protection, and the Partners Human Research Committee. Consent from patients was obtained as per IRB guidelines. The fluid exudate samples were briefly centrifuged, and the cell pellet was washed with PBS. Red blood cells were lysed with Hybri-Max red blood cell lysis buffer (Sigma No. 7757), and the tumor cells were washed in PBS and media and allowed to grow one passage in 2D culture before they were used for the 3D assays. Media for the primary samples was same as for the ovarian cell lines.

3D Acinar Morphogenesis Assay and Scoring of 3D Structures

Our previously reported MCF-10A 3D cell culture protocol (Debnath et al., 2003a) (https://brugge.med.harvard.edu/) was modified in the following way for 3D culture of ovarian cancer cell lines: Cells were grown in MCBD105:199 media (described in Supplemental Experimental Procedures) supplemented with 2% inactivated calf serum that was replaced every 4 days. For protein and mRNA, cells were grown on poly-HEMA-coated plates with 2% matrigel. The 3D structures were scored according to 3D structure integrity based on the resemblance to images shown in Figure 3B. Over 200 structures were scored for each condition.

RPPA Assay

RPPA experiments were performed as previously described (Hennessy et al., 2010).

ACCESSION NUMBERS

The data from the microarrays have been deposited in the GEO database with accession number GSE28992.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2011.12.024.

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