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Research Article

PI3K Pathway Activation Mediates Resistance to MEK Inhibitors in KRAS Mutant Cancers

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Abstract

The RAS pathway is one of the most frequently deregulated pathways in cancer. RAS signals through multiple effector pathways, including the RAF/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK MAPK and phosphatidylinositol 3-kinase (PI3K)-AKT signaling cascades. The oncogenic potential of these effector pathways is illustrated by the frequent occurrence of activating mutations in *BRAF* and *PIK3CA* as well as loss-of-function mutations in the tumor suppressor *PTEN*, a negative regulator of PI3K. Previous studies have found that whereas *BRAF* mutant cancers are highly sensitive to MEK inhibition, *RAS* mutant cancers exhibit a more variable response. The molecular mechanisms responsible for this heterogeneous response remain unclear. In this study, we show that PI3K pathway activation strongly influences the sensitivity of *RAS* mutant cells to MEK inhibitors. Activating mutations in *PIK3CA* reduce the sensitivity to MEK inhibition, whereas *PTEN* mutations seem to cause complete resistance. We further show that down-regulation of *PIK3CA* resensitizes cells with co-occurring *KRAS* and *PIK3CA* mutations to MEK inhibition. At the molecular level, the dual inhibition of both pathways seems to be required for complete inhibition of the downstream mammalian target of rapamycin effector pathway and results in the induction of cell death. Finally, we show that whereas inactivation of either the MEK or PI3K pathway leads to partial tumor growth inhibition, targeted inhibition of both pathways is required to achieve tumor stasis. Our study provides molecular insights that help explain the heterogeneous response of *KRAS* mutant cancers to MEK pathway inhibition and presents a strong rationale for the clinical testing of combination MEK and PI3K targeted therapies. [Cancer Res 2009;69(10):4286–93]

Introduction

RAS is one of the most frequently mutated oncogenes in human cancer (1). Oncogenic mutations in RAS lead to deregulation of several effector pathways that control cell proliferation, survival, and migration and thus promote malignant transformation. The best-characterized RAS effector pathway is the RAF-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) MAPK cascade. RAF becomes activated, in part, through RAS-mediated recruitment to the plasma membrane, where RAF activates MEK1 and MEK2, which in turn activate ERK1 and ERK2. ERK1 and ERK2 phosphorylate several cytosolic and nuclear proteins, including transcription factors that control the G_1-S cell cycle transition (1). Approximately 30% of all human tumors exhibit signs of MAPK pathway activation based on high phosphorylation levels of ERK1/2 (2). Whereas many growth signaling pathways regulate MAPK activation, deregulation of this pathway in cancers occurs most frequently through activating mutations in *KRAS* or *BRAF*. Given the strong genetic evidence implicating the RAS pathway in the etiology of human cancer, much effort has focused on the development of therapeutic agents that inhibit critical downstream pathway components, such as RAF and MEK kinases (3, 4).

KRAS also stimulates signaling through the phosphatidylinositol 3-kinase (PI3K) pathway (5). The PI3K family encompasses lipid kinases that convert phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate, which in turn initiates a signaling cascade that promotes cell growth and survival (6). In addition to activation by oncogenic receptor tyrosine kinases, deregulation of the PI3K signaling pathway in cancers can also occur through activating mutations in *PIK3CA*, which encodes the catalytic p110α kinase subunit, or through loss-of-function mutations in the lipid phosphatase *PTEN* (7, 8). Extensive cross-talk has been observed between the PI3K and RAS-RAF-MEK signaling pathways. In addition to RAS activating both RAF-MEK and PI3K signaling, both pathways can modulate the activity of the mammalian target of rapamycin (mTOR) kinase through the negative regulation of the TSC1/2 complex (9–13).

Most cancers exhibit a myriad of genetic and epigenetic changes, and "driver" oncogenic lesions are selected during the clonal expansion of cancers if they provide a growth or survival benefit. Hence, mutations in components of the same signaling pathway often exhibit a pattern of mutual exclusivity when there is no or little selective advantage gained from the acquisition of multiple pathway lesions. For example, activating mutations in both RAS and BRAF are rarely found to coexist in the same tumor (14–16). Similarly, mutations in PI3K and PTEN are rarely concurrent (17). In the case of RAS and PI3K pathway mutations, however, the situation seems to be more complex. Whereas RAS and PI3K pathway mutations are found to be mutually exclusive in breast cancers, a significant fraction (22%) of colorectal cancers have genetic lesions in both pathways (18, 19). The molecular significance and therapeutic implications, however, of co-occurring mutations in the PI3K and RAS pathway are presently unclear.

A recent study found that the proliferation of *BRAF* mutant cancers is strongly affected by pharmacologic MEK pathway inhibition, whereas *RAS* mutant cell lines exhibited a more varied

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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response and were generally found to be less sensitive to a MEK1/2 inhibitor (20). The molecular mechanism responsible for this variable response of *RAS* mutant cancers is currently not known. In this study, we found that activation of the PI3K pathway strongly influences the response of *KRAS* mutant cancers to MEK inhibitors. Activating mutations in *PIK3CA* significantly decrease the response to MEK pathway inhibition, reflected by a more than 10-fold shift in IC_{50} required to inhibit cancer cell proliferation. Interestingly, loss of *PTEN* function leads to even more pronounced resistance to MEK pathway inhibition. We further show that p110α down-regulation is able to resensitize tumors with coexisting *KRAS* and *PIK3CA* mutations to MEK pathway inhibition both *in vitro* and *in vivo*. Together, these findings provide a strong rationale for combination therapies of PI3K and MEK inhibitors for cancers that harbor concurrent mutations in *KRAS* and *PIK3CA*.

Materials and Methods

Cell Culture and Reagents

LS513 and SW620 were generous gifts from Neal Rosen (Memorial Sloan-Kettering Cancer Center, New York, NY). All other cell lines were obtained from the American Type Culture Collection. All untransduced cells were cultured in DMEM (Invitrogen) and supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen). All short hairpin RNA (shRNA)–transduced cell lines were grown in DMEM supplemented with 10% Tet-approved FBS (Clontech) in the presence of appropriate antibiotic selection. Expression of shRNA was induced by growing cells in the presence of 10 to 100 ng/mL of doxycycline (Sigma).

Immunoblotting

Cells were lysed in a modified radioimmunoprecipitation assay buffer containing 50 mmol/L Tris-HCl, 1% NP40, 120 mmol/L NaCl, 25 mmol/L NaF, 40 mmol/L β-glycerol phosphate, and 1× Halt protease inhibitor cocktail (Pierce). All primary antibodies were obtained from Cell Signaling Technology, unless otherwise noted: anti-p110α, phospho-AKT, total AKT, total PTEN, phospho-S6, phospho-ERK1/2, phospho–glycogen synthase kinase 3β (GSK3β), total ERK, poly(ADP-ribose) polymerase (PARP), and cleaved caspase-7. Anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Imgenex, anti-PRAS40 from Invitrogen, and anti-KRAS from Santa Cruz Biotechnology. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and blocked in Odyssey blocking buffer followed by immunodetection with the indicated primary antibodies. Secondary antibody IRDye800 anti-rabbit and IRDye680 anti-mouse were purchased from Rockland Immunochemicals.

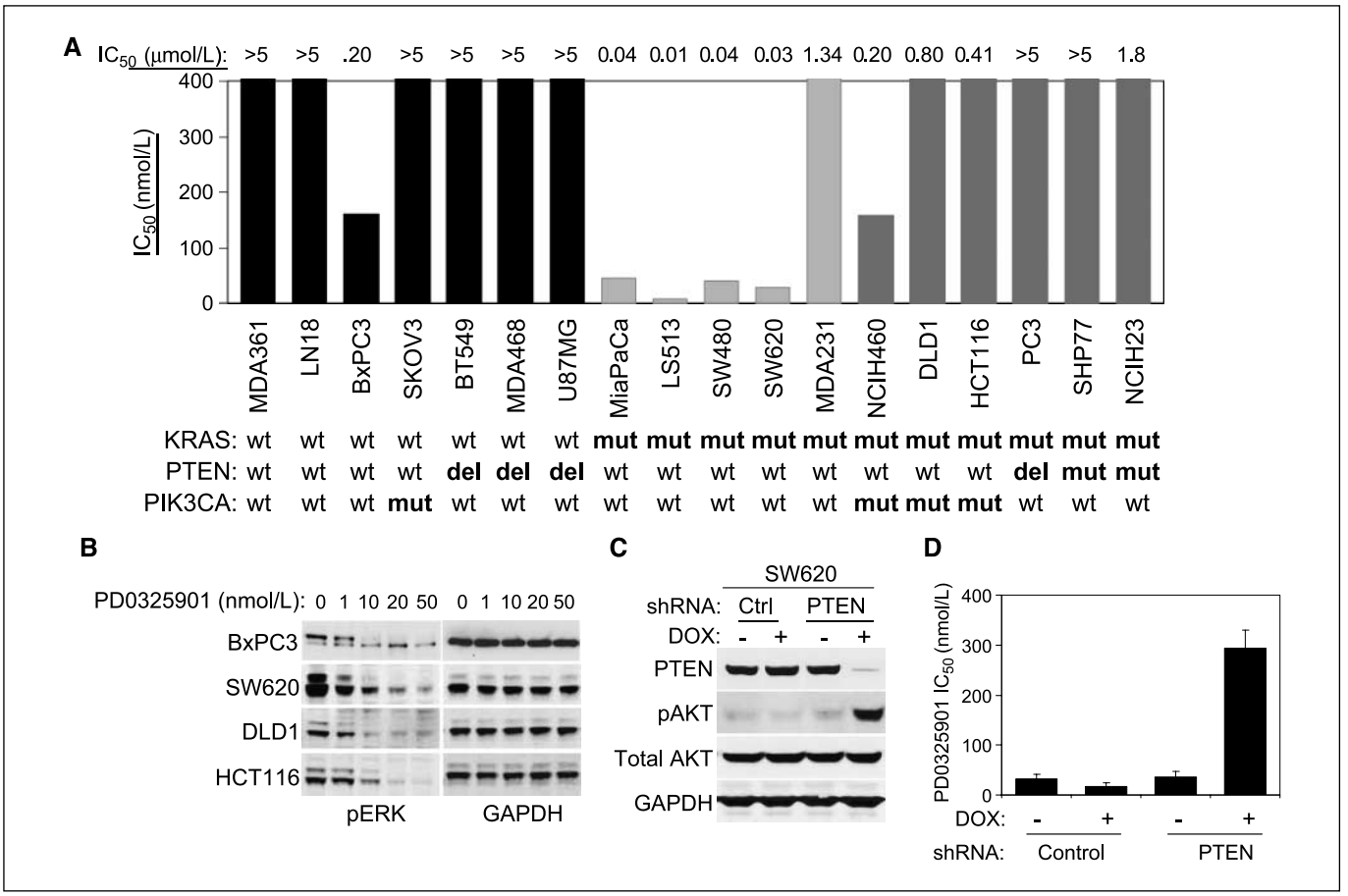

Figure 1. Mutations in the PI3K pathway correlate with resistance to MEK1/2 inhibitors in KRAS mutant cells. *A,* cell survival IC_{50} values for a panel of cancer cell lines treated with the MEK1/2 pharmacologic inhibitor PD0325901. The values are the average of three independent experiments. *Black columns,* KRAS wild-type (*wt*) cells; *light gray columns,* KRAS mutant (*mut*) cells; *dark gray columns,* cells harboring both KRAS and PI3K pathway mutations. The number of viable cells was determined 72 h after treatment as described in Materials and Methods. IC_{50} values are provided along the top of the graph. *B,* lysates harvested from cells treated for 2 h with the indicated concentration of PD0325901 were immunoblotted for phospho-ERK (*pERK*). GAPDH is shown as a loading control. *C,* KRAS mutant SW620 cells were stably transduced with either control or PTEN inducible shRNA. Lysates from cells grown in the presence or absence of doxycycline (*DOX*) for 96 h were harvested for immunoblot analysis. *D,* the IC_{50} value of SW620 cells containing either control or PTEN inducible shRNA in response to PD0325901 treatment is graphed. Effect on cell growth was determined 72 h after inhibitor treatment by CellTiter-Glo. PTEN depletion renders KRAS mutant cell lines insensitive to MEK inhibition.

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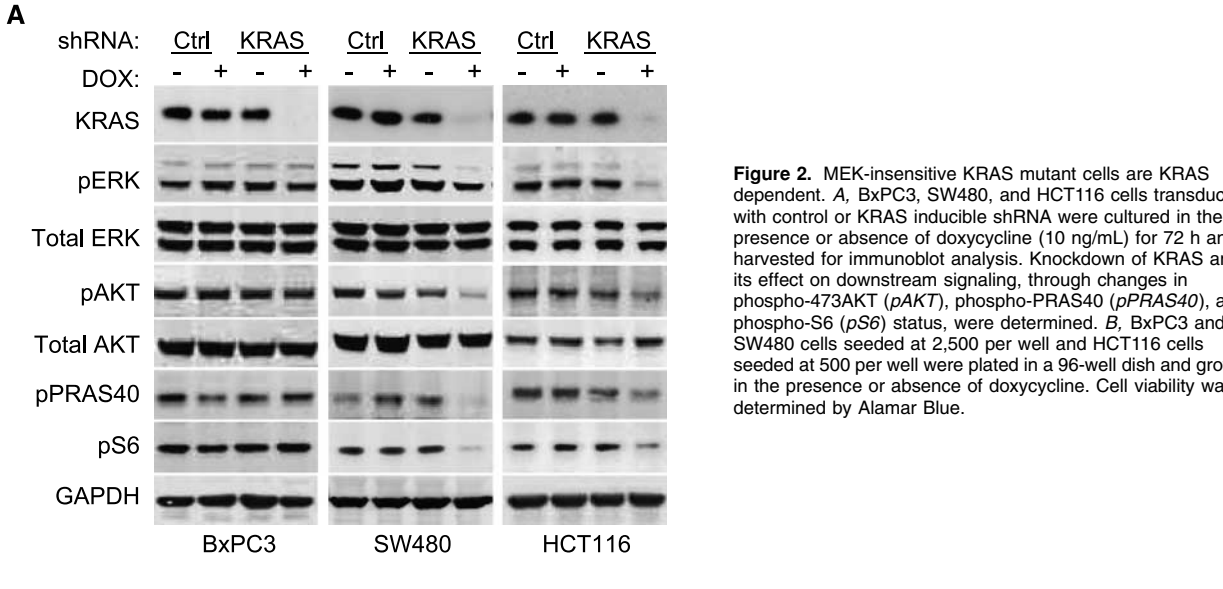

Figure 2. MEK-insensitive KRAS mutant cells are KRAS dependent. *A,* BxPC3, SW480, and HCT116 cells transduced with control or KRAS inducible shRNA were cultured in the presence or absence of doxycycline (10 ng/mL) for 72 h and harvested for immunoblot analysis. Knockdown of KRAS and its effect on downstream signaling, through changes in phospho-473AKT (*pAKT*), phospho-PRAS40 (*pPRAS40*), and phospho-S6 (*pS6*) status, were determined. *B,* BxPC3 and SW480 cells seeded at 2,500 per well and HCT116 cells seeded at 500 per well were plated in a 96-well dish and grown in the presence or absence of doxycycline. Cell viability was determined by Alamar Blue.

Apoptosis and Proliferation Analysis

Apoptosis. Cells were grown in 10 cm^2 dish for 48 h in the presence or absence of 10 ng/mL doxycycline followed by treatment with 50 nmol/L PD0325901 for 24 h. Cells were then trypsinized and resuspended in PBS containing 10% NP40, 37% formaldehyde, and 1 mg/mL Hoechst 33342. Apoptotic or fragmented nuclei were visualized and counted under a UV microscope.

Soft agar assay. Cells were plated at 2,000 to 4,000 per well in a 96-well plate (Costar). An equal volume of 0.6% agarose (FMC Bioproducts) was added to each well to a final volume of 100 μL/well. After an hour at room temperature, the cells were kept in a 37°C and 5% CO_2 incubator for 2 wk. Doxycycline, at a final concentration of 100 ng/mL, was replenished every 72 h. Colonies were then stained with the fluorophore Alamar Blue (Biosource International) and read 5 h afterwards in a fluorescence spectrophotometer equipped with a 540 excitation filter and 590 emission filter. Proliferation and colony formation assays were carried out as described previously (6).

Compound Treatment

The MEK1/2 inhibitor PD0325901 and etoposide were dissolved in DMSO to a final concentration of 10 mmol/L and stored at −20°C. Cells were plated in triplicate 96-well plates at 2,000 to 5,000 per well. After overnight attachment, cells were treated with serial dilutions of the indicated compound. Cells were incubated in the presence of compound for 72 h and viable cells were measured by CellTiter-Glo (Promega). The IC_{50} was calculated using Excel XLFit program using the one-site dose-response model 205.

Lentiviral RNA Interference

The inducible shRNA vector used in this study has been previously described in refs. 6, 21. shRNA sequences used in this study are as follows: control, 5′-TGGACTCTTGAAAGTACTATC-3′ (sense); *PIK3CA#1*, 5′-CCAGTACCTCATGGATTAGAC-3′ (sense); *PIK3CA#2*, 5′-GACAACTGTTTCATATAGATC-3′ (sense); KRAS#1, 5′-CGATACAGCTAATTCAGAATC-3′ (sense); KRAS#2, 5′-GGAGCTGGTGACGTAGGCA-3′ (sense); and PTEN, 5′-ACTTGAAGGCGTATACAGGAC-3′ (sense).

Tumor Xenografts

Female athymic nude mice (Harlan) were acclimated in Novartis Institutes for BioMedical Research animal facility with *ad libitum* access to food and water for 3 d before manipulation. Animals were handled in accordance with Novartis Animal Care and Use Committee protocols and regulations. HCT116 cells engineered with Tet-inducible shRNA against *PIK3CA* were cultured in DMEM supplemented with 10% Tet-approved FBS. Mice (6–8 wk old, $n = 8$) were inoculated s.c. with 1×10^6 cells in the right dorsal axillary region. Tumor volume was measured by calipering in two dimensions and calculated as (length × $width^2$) / 2. Drug treatment started 11 d after implant when average tumor volume was 110 mm^3. Animals received vehicle (5% dextrose, 10 mL/kg, orally, four times daily), doxycycline hyclate (25 mg/kg, orally, four times daily), or PD0325901 (6 mg/kg, orally, four times daily) for the duration of the study (17 d). Doxycycline was dissolved in 5% dextrose, whereas PD0325901 was formulated in 10% ethanol, 10% propylene glycol, and 16% Captisol. A separate set of animals ($n = 3$) was dosed for 5 d with vehicle control,

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doxycycline, and/or PD0325901 for biomarker analyses only. At termination of the study, tumor tissue was excised and snap frozen in liquid nitrogen for immunoblot analyses of biomarkers.

Quantitative Reverse Transcription-PCR

mRNA levels were measured using Taqman Gene Expression Assays on an ABI Prism 7900 HT Sequence Detection System supplied by Applied Biosystems. RNA isolation was performed using the Qiagen TurboCapture mRNA kit and followed by cDNA synthesis (iScript; Bio-Rad). The *PIK3CA* probe was purchased from Applied Biosystems. VIC-MGB B-Actin primers were used in each reaction to coamplify the actin transcript. All experiments were performed in triplicate and normalized to β-actin levels. Relative mRNA expression was calculated using the formula $2^{-(CT \text{ of sample} - CT \text{ of } \beta\text{-actin})}$, where C_T (cycle count) is the threshold cycle value.

Results

PI3K pathway activation in KRAS mutant cancer cells confers resistance to MEK inhibitors. To identify molecular modifiers that influence the sensitivity of KRAS mutant cancers to MEK pathway inhibition, we profiled a panel of cancer cell lines to determine their response to the MEK1/2 inhibitor PD0325901, which inhibits ERK phosphorylation with an IC_{50} of 10 to 20 nmol/L (Fig. 1*B*; ref. 22). Given the frequent co-occurrence of KRAS and PI3K pathway lesions, many of these cell lines harbored both KRAS and PI3K pathway mutations (Fig. 1*A*). As expected, cell lines that are wild-type for KRAS were generally insensitive to PD0325901 (Fig. 1*A, black columns*). However, the sensitivity of the 11 KRAS mutant cell lines to the MEK1/2 inhibitor varied drastically. We noted that the sensitivity to MEK inhibitors seemed to strongly correlate with the PI3K pathway mutation status. For example, four of five cell lines that harbor *KRAS* mutations but have a wild-type PI3K pathway are very sensitive to MEK inhibitor treatment (Fig. 1*A, light gray columns*), with IC_{50} values similar to those required for complete phospho-ERK suppression (Fig. 1*B*). In contrast, cell lines harboring activating mutations in *PIK3CA* were significantly less sensitive to MEK pathway inhibition. It is important to note that the antiproliferative effect in HCT116 cells (IC_{50} of 400 nmol/L), for example, occurred at concentration of PD0325901 that was much higher than that required for the complete suppression of phospho-ERK (20 nmol/L). Strikingly, cancer cells with loss of PTEN function were far more resistant to MEK1/2 inhibitors, as their proliferation was not affected even at micromolar concentrations. The MEK1/2 inhibitor blocked MEK-ERK signaling with similar efficiency in sensitive and resistant cells, excluding the possibility that incomplete target modulation accounts for the varying responses.

These findings provide strong correlative evidence that activation of the PI3K pathway renders *KRAS* mutant cancer cells less sensitive to MEK pathway inhibition. To test this hypothesis directly, we engineered a *KRAS* mutant cell line to inducibly activate the PI3K pathway. We introduced an inducible shRNA targeting *PTEN* into the SW620 cell line, which harbors a KRAS mutation but has an intact PI3K pathway. PTEN depletion strongly increased the phosphorylation of AKT, a downstream marker of PI3K pathway activation (Fig. 1*C*). In addition, PTEN depletion strongly reduced the sensitivity of SW620 cells to the MEK1/2 inhibitor (Fig. 1*D*), providing direct evidence that PI3K pathway activation in KRAS mutant cells is sufficient to confer resistance to MEK-ERK inhibition.

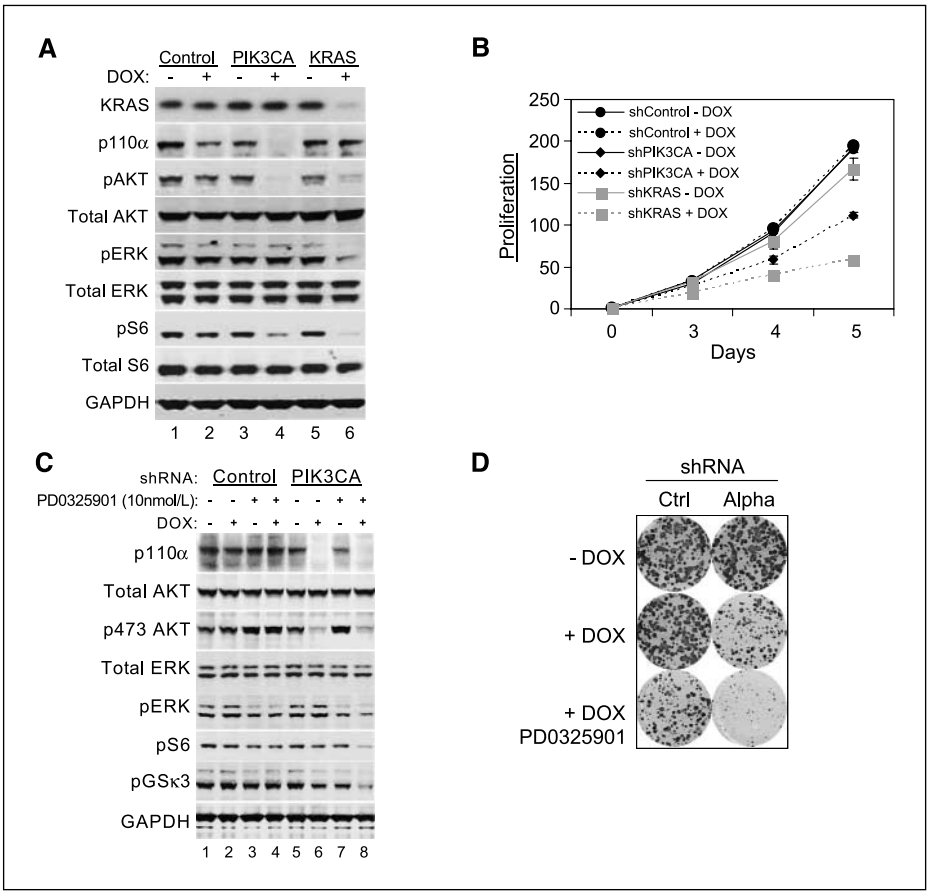

Figure 3. Inhibition of the PI3K pathway potentiates the antiproliferative effect of MEK1/2 pharmacologic inhibitors in HCT116 cells. HCT116 cells were transduced with control, *PIK3CA*, or *KRAS* inducible shRNAs. *A,* cells were grown in the presence or absence of doxycycline (10 ng/mL) for 72 h and harvested for immunoblot analysis with the indicated antibodies. *B,* cell proliferation in response to target gene knockdown was determined by Alamar Blue. *C,* cells grown in the presence or absence of doxycycline were treated with PD0325901 for 72 h. Harvested lysates were immunoblotted with the indicated antibodies. *D,* cells cultured in a six-well dish for 14 d in the presence (10 ng/mL) or absence of doxycycline were stained with crystal violet to visualize colony growth. A representative of triplicate experiment is shown.

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Cells with coexisting mutations in *KRAS* and *PIK3CA* still exhibit KRAS dependency. The observation that KRAS mutant cells with PI3K pathway activation were less sensitive to MEK inhibition raised the question as to whether these cells more generally lost dependence on KRAS signaling or only the MEK effector pathway. To specifically address this question, BxPC3, SW480, and HCT116 cell lines were engineered to inducibly express shRNAs targeting KRAS. The induction of KRAS shRNAs resulted in a significant reduction of KRAS mRNA levels (Supplementary Fig. S1*A–C*) and KRAS protein (Fig. 2*A*). KRAS depletion in the KRAS mutant colorectal cancer cell line SW480 strongly impaired proliferation (Fig. 2*B*) and efficiently suppressed downstream pathway signaling, as measured by the phosphorylation of ERK, AKT, and the AKT substrates S6 and PRAS40 (Fig. 2*A*). In contrast, the proliferation of BxPC3 pancreatic cancer cell line, which is wild-type for both KRAS and the PI3K pathway, was not affected by KRAS depletion. When we tested HCT116 cells, which harbor mutations in both *KRAS* and *PIK3CA*, we found that KRAS depletion strongly affected proliferation and downstream MAPK signaling. These results indicate that HCT116 cells remain dependent on KRAS signaling, consistent with previously published data (23). Interestingly, KRAS knockdown had a stronger effect on reducing phospho-ERK levels compared with phospho-AKT in HCT116 cells presumably because mutational activation of p110α at least partially bypasses the need for KRAS signaling for the activation of the AKT pathway. Taken together, our results show that cells harboring mutations in both the PI3K and KRAS pathways are still dependent on KRAS function despite their reduced sensitivity to MEK pathway inhibition.

Inhibition of p110α sensitizes HCT116 cells to MEK pathway inhibition. We and others have previously shown that cell lines with *PIK3CA* activating mutations, such as HCT116, are dependent on *PIK3CA* activity for proliferation and AKT pathway signaling (6, 7). However, blocking PI3K activity only reduced but did not completely prevent cell growth or *in vivo* tumor formation, indicating that additional pathways contribute to the transformed phenotype (Fig. 3*B*; Supplementary Fig. S2*A–D*). Based on our previous finding that KRAS depletion also caused a strong antiproliferative effect in HCT116 cells, we wanted to directly compare the effects of p110α and KRAS depletion. Whereas *PIK3CA* knockdown caused a marked reduction in PI3K pathway signaling (phospho-AKT and phospho-S6 in Fig. 3*A*), cell proliferation (Fig. 3*B*), and colony formation (Supplementary Fig. S2*A*), we found that KRAS depletion resulted in a significantly greater antiproliferative effect (Fig. 3*B*; Supplementary Fig. S2*A* and *C*). We reasoned that this more pronounced dependency may stem from the ability of KRAS to activate both PI3K and MEK-ERK pathways. Consistent with this hypothesis, we found that whereas *PIK3CA* depletion only affected AKT pathway activation, KRAS depletion strongly reduced both phospho-ERK and phospho-AKT downstream signaling (Fig. 3*A*).

These findings suggested that inhibition of both downstream effector pathways may be required to achieve maximal antiproliferative effects. We therefore tested the effects of combined inhibition of p110α and MEK using two independent inducible shRNAs targeting *PIK3CA* and the MEK1/2 inhibitor PD0325901. Indeed, blocking both pathways resulted in stronger antiproliferative effects compared with blocking either pathway alone (Fig. 3*C* and *D*; Supplementary Fig. S3*A* and *C*). Notably, whereas both MEK and PI3K pathway inhibition alone reduced phospho-S6 and phospho-GSK3 levels, only the combined inhibition of MEK and p110α resulted in complete suppression of phosphorylation of both proteins (Fig. 3*C*, *lane 8*). Together, these findings show that the combined inhibition of the PI3K and MEK-ERK pathways leads to more pronounced growth inhibition in HCT116 cells.

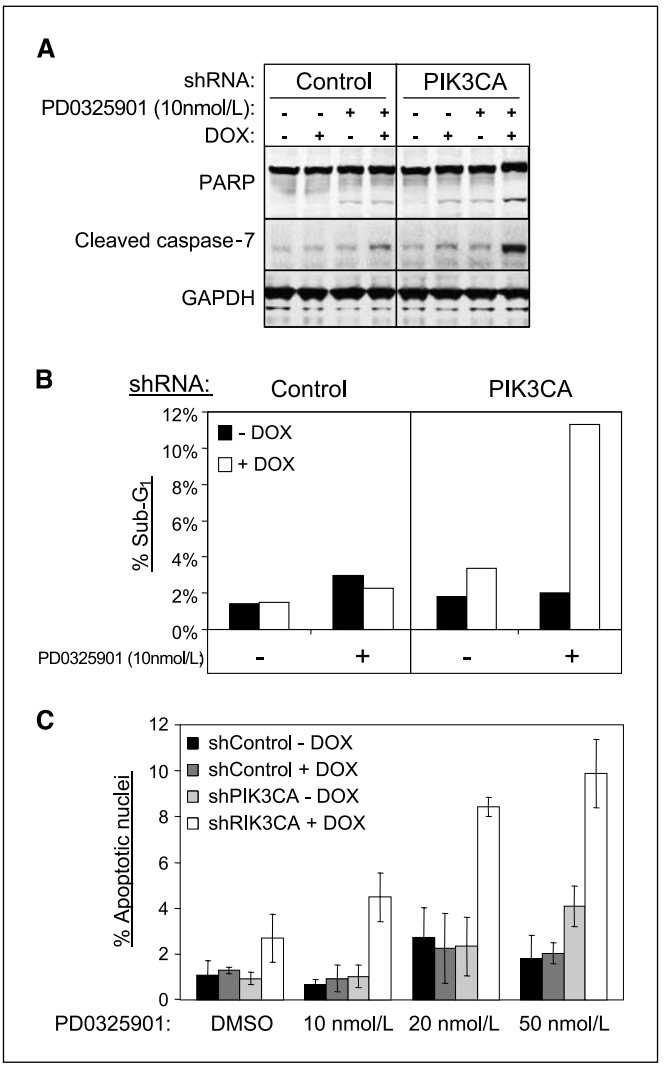

Figure 4. Inhibition of the PI3K and MEK-ERK pathway enhances cell death in HCT116 cells. HCT116 cells stably transduced with control or *PIK3CA* inducible shRNA were treated with PD0325901 in the presence or absence of doxycycline. *A,* lysate was harvested from cells treated with 10 nmol/L PD0325901 for 48 h. Effect on PARP and caspase-7 cleavage was determined by immunoblot analysis. GAPDH is shown as a loading control. *B,* percentage of cells in sub-G_1 in the presence or absence of *PIK3CA* knockdown was determined by propidium iodide staining and fluorescence-activated cell sorting analysis 48 h after treatment. For each sample, 10,000 cells were analyzed. *C,* HCT116 cells grown in the presence or absence of doxycycline were treated with PD0325901 for 48 h. Cells were subsequently fixed with formaldehyde and stained with Hoechst 33342 to visualize fragmented nuclei. For each treatment condition, 1,500 cells were counted under a fluorescence microscope.

Combined inhibition of the MEK and PI3K pathways induces cell death. We next wanted to explore the molecular mechanism responsible for the increased antiproliferative effect in response to combined inhibition of the MEK and PI3K pathways. We have previously shown that *PIK3CA* knockdown in HCT116 cells results in a prolonged G_1-phase arrest with little effect on cell survival (6). We therefore wanted to investigate whether the more potent growth-inhibitory effect elicited by the combination

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treatment may be a result of increased cell death. Depletion of either *PIK3CA* or MEK1/2 inhibitor treatment alone did not induce a significant apoptotic response (Fig. 4). However, combining the depletion of *PIK3CA* with MEK inhibitor treatment induced several markers of apoptosis, including cleavage of PARP and caspase-7 (Fig. 4*A*), sub-G_1 DNA content (Fig. 4*B*), and apoptotic nuclei (Fig. 4*C*). These findings indicate that the growth-inhibitory effects in response to simultaneous disruption of MEK-ERK and PI3K pathway signaling are at least in part mediated by an increase in apoptotic cell death.

Combined inhibition of p110α and MEK is most effective in cancer cells with coexisting mutations in *PIK3CA* and *KRAS*. Given the promising response of HCT116 cells to combined inhibition of p110α and MEK1/2, we wanted to further investigate which cancer cells are most responsive to this combination strategy. We therefore tested whether *PIK3CA* depletion increases the sensitivity to the MEK inhibitor PD0325901 across a panel of cancer cell lines that differ in their *KRAS, PIK3CA*, and *PTEN* status. Each cell line was stably transduced with an inducible shRNA targeting *PIK3CA*, and effective knockdown of *PIK3CA* in response to doxycycline treatment was verified by quantitative reverse transcription-PCR (RT-PCR; Fig. 5*A*). The IC_{50} for PD0325901 was measured in cells expressing *PIK3CA* (−DOX) and cells depleted for *PIK3CA* (+DOX). An IC_{50} ratio (−DOX/+DOX) of >1 indicates that the cell line is more sensitive to PD0325901 treatment when the PI3K pathway is inhibited (Fig. 5*B*). Strikingly, all cell lines with concurrent activating mutations in *KRAS* and *PIK3CA* (HCT116, NCI-H460, and DLD1) exhibited a markedly increased sensitivity to PD0325901 when *PIK3CA* expression was down-regulated. In contrast, the two cell lines with coexisting mutations in *KRAS* and *PTEN*, DU145 and PC3, were not sensitized by *PIK3CA* depletion. Whereas recent studies indicate that *PIK3CB* rather than *PIK3CA* is the major PI3K isoform driving the proliferation of PTEN mutant cancer cell lines (6, 24), we surprisingly found that knockdown of *PIK3CB* in PC3 cells did not increase its sensitivity to PD0325901 (Supplementary Fig. S4*A* and *B*). We confirmed that *PIK3CA* down-regulation did not cause a general sensitization to compound treatment, as *PIK3CA* depletion did not influence the sensitivity to the topoisomerase II inhibitor etoposide (Fig. 5*B*). Together, these findings indicate that inhibition of p110α sensitizes KRAS mutant cancer cells with *PIK3CA*, but not *PTEN* mutations, to MEK pathway inhibition.

Combined inhibition of the MEK and PI3K pathways potently inhibits tumor growth of HCT116 *in vivo*. We next wanted to examine whether the synergistic antiproliferative effect of combined MEK and PI3K pathway inhibition can be recapitulated *in vivo*. To test this, HCT116 xenografts stably expressing inducible *PIK3CA* shRNA were implanted into nude mice. Doxycycline treatment (+DOX) strongly reduced the levels of p110α and decreased the phosphorylation of downstream pathway components, including AKT and PRAS40, whereas treatment with PD0325901 strongly inhibited the phosphorylation of ERK (Fig. 6*B*). In concordance with the results from our *in vitro* studies, we found that whereas either *PIK3CA* knockdown or PD0325901 alone

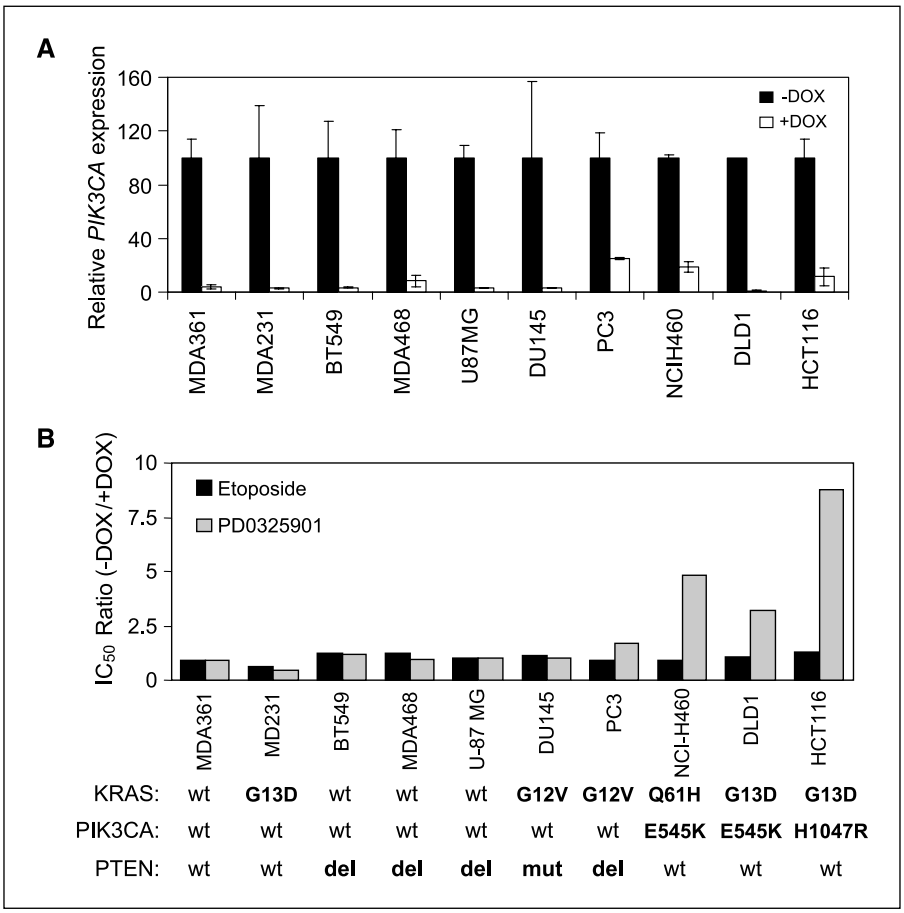

Figure 5. Modulation of the PI3K pathway alters the response to MEK pharmacologic inhibitors. *A*, cells transduced with control or *PIK3CA* shRNA were grown in the presence or absence of doxycycline (10 ng/mL) for 72 h. Knockdown of *PIK3CA* in all lines was confirmed by quantitative RT-PCR. *B*, cells were grown in 96-well plates and treated to a dilution series of PD0325901 in the presence or absence of doxycycline. The graph depicts the respective IC_{50} ratios resulting from the pharmacologic inhibition of MEK1/2 with PD0325901 in the presence of *PIK3CA* (*−DOX*) versus in the absence of *PIK3CA* (*+DOX*). Knockdown of *PIK3CA* in cell lines containing activating mutations in p110α sensitizes cells to PD0325901 treatment.

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reduced phospho-S6 levels, only the combined treatment led to complete ablation of S6 phosphorylation (Fig. 6*B*). In the tumor growth studies, we found that whereas inhibition of either pathway alone via *PIK3CA* depletion or PD0325901 treatment reduced tumor growth (70% and 28% growth compared with vehicle controls, respectively), tumor stasis was observed only with combination treatment (2% residual tumor growth compared with controls; Fig. 6*A*). In addition, significant tumor regression during the first 21 days was only observed in response to the combination treatment. These findings further support the notion that complete inhibition of downstream mTOR signaling may be required to achieve maximal antitumor effects. Collectively, our study shows that cancer cells harboring concurrent mutations in *KRAS* and *PIK3CA* rely on both signaling pathways for growth and survival and suggests a clear rationale for combination therapies for cancers harboring these genetic lesions.

Discussion

Due to its prevalent deregulation in cancer, the RAS/RAF/MEK/ERK pathway has been an attractive target for therapeutic intervention, resulting in the development of several potent MEK inhibitors (3). A previous study found that whereas BRAF mutant cancers are very sensitive to pharmacologic MEK inhibitors, RAS mutant cancers exhibit a more variable response to these compounds (20). The molecular underpinnings of this heterogeneous response, however, remain largely unexplored. In this study, we profiled a larger panel of *KRAS* mutant cancer cell lines for their response to the allosteric MEK inhibitor PD0325901. In agreement with the study of Solit and colleagues, we observed that the sensitivity of *KRAS* mutant cancer cells to MEK inhibitor varied extensively despite efficient MEK pathway inhibition in resistant and sensitive settings. We noted that the majority of MEK inhibitor–insensitive cell lines harbored activating mutations in the PI3K pathway as a result of either activating mutations in *PIK3CA* or loss-of-function mutations in *PTEN*. In contrast, four of five *KRAS* mutant cancers with an intact PI3K pathway exhibited exquisite sensitivity to the MEK inhibitor. In addition to this correlative evidence, we directly show that depletion of *PTEN* renders a previously MEK inhibitor–sensitive *KRAS* mutant cell line resistant to MEK pathway inhibition. Collectively, these findings show that PI3K pathway activation is a major resistance mechanism that impairs the efficacy of MEK inhibitors in *KRAS* mutated cancers. This observation parallels the clinical finding that PI3K pathway mutations confer resistance to trastuzumab therapy in HER2-overexpressing breast cancers (25).

Although our study clearly shows that PI3K pathway activation renders *KRAS* mutant cancers less sensitive to MEK inhibitors, we noted that the extent of resistance conferred by *PIK3CA* and *PTEN* mutations seems to differ significantly. Activating mutations in *PIK3CA* caused a 5- to 10-fold shift in IC_{50}, whereas loss of *PTEN* function apparently conferred complete resistance. It is important to note that the more modest increase in IC_{50} in the presence of *PIK3CA* mutations is likely to be clinically relevant, as increased dosing of MEK inhibitors may not be an option due to dose-limiting toxicities. Moreover, these findings further support the notion that mutational activation of *PIK3CA* is not functionally equivalent to loss of *PTEN*. It is currently not clear, however, whether this phenotypic difference is due to more potent activation of downstream PI3K pathway by *PTEN* loss or due to PI3K pathway–independent functions of PTEN (26–28).

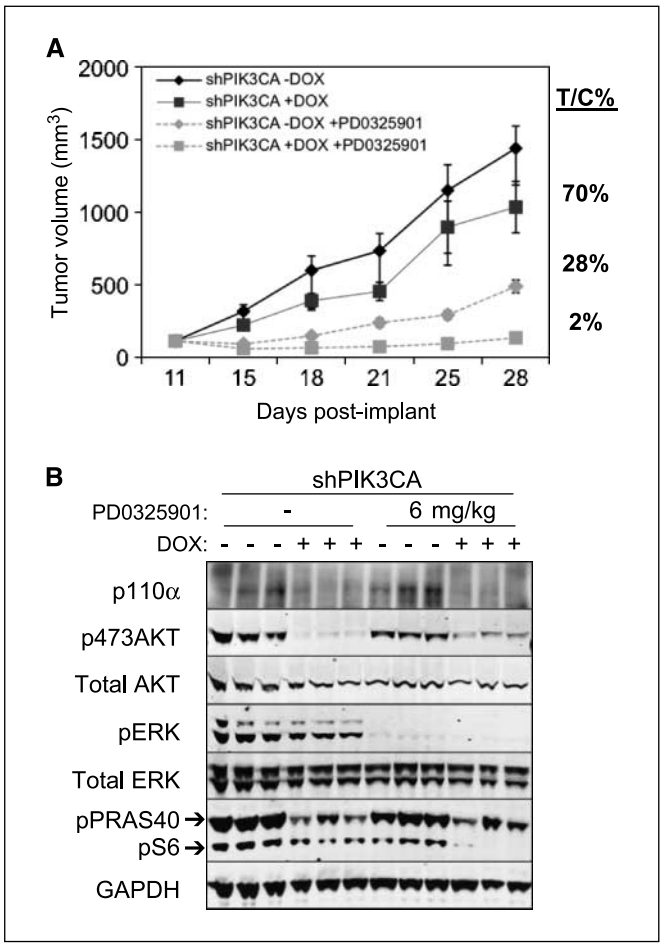

Figure 6. Combined inhibition of the PI3K and MEK-ERK pathways results in tumor stasis *in vivo*. *A,* stable HCT116 cells containing *PIK3CA* inducible shRNA were implanted into nude mice and administered vehicle control or doxycycline in combination with PD0325901 (6 mg/kg). Tumor size was measured using calipers. *Points,* mean; *bars,* SE. *B,* tumors harvested from vehicle-, doxycycline-, and/or PD0325901-treated mice for 5 d were analyzed by immunoblot with the respective antibodies.

The observation that co-occurring PI3K pathway mutations render *KRAS* mutant cancer cells less sensitive to MEK inhibitors prompted us to investigate whether we could use these insights to design rational combination strategies. We focused our studies on down-regulation of *PIK3CA*, as this is the most frequently deregulated PI3K isoform in human cancers. Indeed, we found that *PIK3CA* depletion in cancer cell lines with concurrent *PIK3CA* and *KRAS* mutations sensitized those cells to MEK inhibitors. However, in cancer cell lines with coexisting *PTEN* and *KRAS* mutations, neither *PIK3CA* nor *PIK3CB* depletion was sufficient to sensitize these cells to MEK inhibitors. This finding suggests that in the background of a *KRAS* mutation, PTEN mutant cells may rely on multiple p110 isoforms for growth and survival signaling. Thus, it will be important to test in future studies if pan-PI3K inhibitors will synergize with MEK inhibitors in cancers with coexisting *PTEN* and *KRAS* lesions.

We previously found that inhibition of p110α in HCT116 cells, which harbor *PIK3CA* and *KRAS* mutations, causes cells to arrest in G_1 but not cell death (6). In this study, we show that combined inhibition of the MEK and PI3K pathways is required to induce an apoptotic response. This enhanced apoptotic response translates

into augmented antitumor effects *in vitro* and *in vivo*. At the molecular level, we noted that the phosphorylation status of the ribosomal protein S6 correlates much better with antiproliferative response compared with more upstream pathway markers such as phospho-AKT and phospho-ERK. Whereas the inhibition of MEK and p110α reduced phospho-S6 levels, only the combined pathway inhibition led to a complete suppression of S6 phosphorylation in both *in vitro* and *in vivo* studies. Given that the MEK-ERK and PI3K-AKT pathways converge on the regulation of mTOR through their regulation of TSC1/2, pharmacodynamic response markers downstream of mTOR may serve as an integrating signal of both upstream pathways.

In summary, our study provides a molecular explanation for the heterogeneous response of KRAS mutant cancer cells to MEK inhibitors and further underscores the need to tailor cancer therapies to match the genetic context of the cancers to achieve optimal treatment response. Our findings and other recent studies (17, 29, 30) provide a strong rationale for the combination of PI3K and MEK inhibitors in cancers with coexisting *PIK3CA* and *KRAS* mutations. In addition, based on our observations, we predict that PI3K pathway lesions are likely to present a major mechanism of acquired resistance in clinical settings with MEK inhibitors. In this light, it will be important in future studies to explore whether combination therapy with PI3K and MEK inhibitors will be able to prevent the emergence of such resistance.

Disclosure of Potential Conflicts of Interest

S. Wee is an employee of Bristol-Myers Squibb. C. Lengauer is an employee of Sanofi-Aventis. The other authors disclosed no potential conflicts of interest.

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PI3K Pathway Activation Mediates Resistance to MEK Inhibitors in KRAS Mutant Cancers

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