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Combination PI3K/MEK inhibition promotes tumor apoptosis and regression in *PIK3CA* wild-type, *KRAS* mutant colorectal cancer

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Abstract

PI3K inhibition in combination with other agents has not been studied in the context of *PIK3CA* wild-type, *KRAS* mutant cancer. In a screen of phospho-kinases, PI3K inhibition of *KRAS* mutant colorectal cancer cells activated the MAPK pathway. Combination PI3K/MEK inhibition with NVP-BKM120 and PD-0325901 induced tumor regression in a mouse model of *PIK3CA* wild-type, *KRAS* mutant colorectal cancer, which was mediated by inhibition of mTORC1, inhibition of MCL-1, and activation of BIM. These findings implicate mitochondrial-dependent apoptotic mechanisms as determinants for the efficacy of PI3K/MEK inhibition in the treatment of *PIK3CA* wild-type, *KRAS* mutant cancer.

Keywords

PI3K; MEK; KRAS; colorectal cancer; mouse model of cancer

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7. Conflict of Interest Statement
All authors report no conflicts of interest.

1. Introduction

Members of the RAS family of GTPases (HRAS, NRAS, and KRAS) are mutated in approximately 25% of human cancers, the majority of which are mutations in *KRAS*, most commonly $KRAS^{G12D}$ [1]. *RAS* mutations lock RAS into a constitutively activated state, which promotes tumorigenesis by activating the MAPK signaling pathway even in the absence of stimulation by receptor tyrosine kinases [2]. Oncogenic mutations in *KRAS* are present in 43-51% of colorectal cancers (CRCs), 27-37% of lung adenocarcinomas, and 70-90% of exocrine pancreatic cancers [3–8]. *KRAS* mutant colorectal and lung adenocarcinomas are resistant to receptor tyrosine kinase inhibitors [9,10]. Therefore, novel therapeutic strategies for *KRAS* mutant cancer are urgently needed.

No inhibitors of KRAS are clinically available despite three decades of efforts. Therefore, strategies to inhibit *KRAS* mutant cancers have focused on signaling proteins downstream of RAS and on parallel signaling pathways such as the phosphoinositide 3-kinase (PI3K) pathway [11]. Clinical trials of PI3K inhibitors have been limited to patients whose tumors harbor mutations in *PIK3CA*, which encodes the p110α subunit of PI3K. However, *PIK3CA* mutations are found in only 20-32% of CRCs, 1-4% of lung adenocarcinomas and are not found in pancreatic cancer; only 8-11% of CRCs are mutant in both *PIK3CA* and *KRAS* [3–6,12–14]. Thus, effective therapies are needed for the approximately 30% of CRCs that are *PIK3CA* wild-type, *KRAS* mutant, as well as for the vast majority of lung and pancreatic cancers.

We recently reported that inhibition of PI3K and the downstream mammalian target of rapamycin (mTOR) pathways are effective in a mouse model of *PIK3CA* wild-type, *KRAS* wild-type CRC. However, monotherapy of the PI3K pathway has demonstrated poor clinical efficacy for *KRAS* mutant cancer, likely due to adaptive resistance [15]. Here, we use a phospho-kinase array to rationally identify the MAPK pathway as a resistance mechanism to PI3K inhibition in *KRAS* mutant cancer. We then demonstrate that combination PI3K/MEK inhibition effectively treats a genetically engineered mouse model of *PIK3CA* wild-type, *KRAS* mutant CRC. Finally, we find that PI3K/MEK inhibition effectively blocks mTORC1, inhibits the BCL-2 anti-apoptotic family member MCL-1, and activates the BH3-only pro-apoptotic family member BIM. These findings support a role for combination PI3K/MEK inhibition in the treatment of *PIK3CA* wild-type, *KRAS* mutant cancer.

2. Materials and methods

2.1 In vitro treatment of human CRC cell lines

The human colorectal cancer cell lines DLD-1 ($KRAS^{G13D}$, $PIK3CA^{E545K}$ mutant) HCT116 ($KRAS^{G13D}$, $PIK3CA^{H1047R}$ mutant), and SW480 ($KRAS^{G13V}$, *PIK3CA* wild-type) human CRC cell lines were obtained from American Type Culture Collection (ATCC). Isogenic DLD-1 and HCT116 cells have been derived in which either the mutant or wild-type *KRAS* allele has been disrupted by targeted homologous recombination [16]. SW480 cells with shRNA-mediated knockdown of *KRAS* were obtained as kind gift from D. Chung. Cells were maintained in DMEM (Invitrogen) with 10% FBS and Penicillin/Streptomycin (Invitrogen). Cells were plated at different initial densities (HCT116: 3,000 cells/well,

DLD-1: 5,500 cells/well, and SW480: 4,500 cells/well) to account for differential growth kinetics. After 16 hours, media was exchanged for DMEM media containing 0.5% FBS and cells were incubated with increasing concentrations of NVP-BKM120 (Novartis), PD-0325901 (LC Pharmaceuticals), or a combination [17,18]. Cell viability was assessed 16 hours after the initial plating and 72 hours after initiation of drug treatment using the colorimetric MTS assay CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega), as per the manufacturer's instructions. Cell viability after drug treatment was normalized to that of cells treated with diluent (DMSO) also grown for 72 hours. For western blot analysis, cells were plated with various concentrations of NVP-BKM120, PD-0325901, or combination.

2.2 In vitro treatment of murine CRC cell lines

Genetically engineered colorectal tumors were induced in $Apc^{flox/flox}Kras^{G12D/+}p53^{flox/flox}$ and $Apc^{flox/flox}Kras^{+/+}p53^{flox/flox}$ mice [19]. *Kras* mutant and wild-type immortalized murine colorectal cancer cell lines were then derived from these tumors, as previously described [19]. Cell viability was assessed following treatment with NVP-BKM120, PD-0325901, or combination, as described above.

2.3 Sequencing of colonic tumors from a GEM model of CRC

C57BL/6J $Apc^{flox/flox}Kras^{G12D}$ (Apc-Kras) mice were treated with adenovirus expressing cre recombinase (University of Iowa), as previously described [20]. Following necropsy, 10 tumor specimens were sequenced for *PIK3CA* exons nine (helical domain) and 20 (kinase domain) mutations, as previously described [21].

2.4 In vivo treatment of GEM model of CRC

$Apc^{flox/flox}$ (Apc) and Apc-Kras mice were treated with adenovirus expressing cre recombinase and followed by optical colonoscopy, as previously described [20]. As a colonoscopic metric for tumor size, the Tumor Size Index (TSI) was calculated as (tumor area / colonic lumen area) x 100 (%) [21]. Experimental drugs were diluted in 10% 1-methyl-2-pyrrolidone/90% PEG 300. Tumor-bearing Apc mice were randomly assigned to treatment with control vehicle alone (n=8) or 40 mg/kg body weight NVP-BKM120 (n=8) by daily oral gavage for 7 days. Tumor-bearing Apc-Kras mice were randomly assigned to treatment with either control vehicle alone, 40 mg/kg body weight NVP-BKM120, 25 mg/kg PD-0325901, or combination treatment (N=6 per group). No toxicity was observed during the treatment period. Tumor biopsies were taken before the first treatment and one hour after the final treatment using biopsy forceps passed through the working channel of the endoscope sheath (Karl Storz), then flash-frozen in liquid nitrogen for subsequent western blot analysis [22]. Mice were sacrificed immediately following final biopsy, one hour after final treatment dose. Tumors were harvested for western blot analysis and immunohistochemistry. All protocols were approved by the Tufts Institutional Animal Care and Use Committee.

2.5 Phospho-kinase analysis

DLD-1 cells treated with or without 500 nM NVP-BKM120 for 24 hours in DMEM media containing 10% FBS were tested in an array of 43 antibodies against selected phosphorylated kinases (Proteome Profiler Human Phospho-Kinase Array Kit, R&D systems), as per the manufacturer's instructions. Phosphorylation of each target was then quantified using denisitometry (ImageJ).

2.6 Western blot analysis

Cells were seeded into six-well plates, and media was exchanged the following morning for DMEM containing 0.5% FBS. Cells were harvested at 70% confluency after 72 hours treatment. Western blot analysis of whole cell and tumor lysates was performed, as previously described [21]. P-AKT^{Thr308} (1:1000 dilution), AKT (1:1000 dilution) P-S6$^{Ser240/244}$ (1:3000 dilution), S6 (1:1000 dilution), P-JNK $^{Thr183/Tyr185}$(1:1000 dilution), JNK (1:1000 dilution), cleaved PARP (1:1000 dilution), BIM (1:1000 dilution), and MCL-1 (1:1000 dilution), were obtained from obtained from Cell Signaling Technologies (Beverly, MA). β-actin (1:5000 dilution) was obtained from Santa Cruz Biotechnology. Secondary antibody (1:10,000 dilution) was obtained from Jackson ImmunoResearch (West Grove, PA).

2.7 Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded tissue sections, as previously described [21]. P-AKT^{Ser473} (1:50 dilution), P-S6$^{Ser240/244}$ (1:50 dilution) were obtained from Cell Signaling Technologies (Beverly, MA). Ki-67 (1:100 dilution) was obtained from US Biological (Swampscott, MA). TUNEL assay (Apoptag) was purchased from Millipore (Billerica, MA). The Ki-67 staining was quantified as the mean number of Ki-67 positive cells / total number of glandular cells per high power field (mean of 16 high power fields) × 100. TUNEL positivity was quantified as mean number of TUNEL positive cells / total number of glandular cells per high power field (mean of 16 high power fields) × 100. Measurements were performed by three blinded, independent observers in four control and four treated tumors.

2.8 Cell cycle analysis

Cells were seeded at roughly 30-40% confluency and treated overnight with the indicated treatments in DMEM supplemented with 0.5% FBS, or control media, in triplicate. Cells were collected, washed in PBS, and resuspended in propidium iodide (PI) staining buffer (PBS containing 1% Triton X-100, 50 mg/ml PI and 50 mg/ml RNase). Cells were incubated for 30 min (37°C) and DNA content was measured by flow cytometry using a BD FACSCanto cytometer (BD Biosciences).

2.9 Statistics

Pre- and post-treatment Tumor Size Index values were compared with the Wilcoxon signed-rank test. All other comparisons between groups were performed using the two-tailed Student's T test. $P<0.05$ was considered significant for all analyses. All analyses were calculated using SPSS 18.0 for Windows (IBM, Inc).

3. Results

3.1 Human and murine KRAS mutant CRC cell lines are resistant to PI3K inhibition

To assess the efficacy of PI3K blockade in *KRAS* mutant CRC, we treated CRC cells with or without *KRAS* expression with 500 nM NVP-BKM120, a specific inhibitor of all four class I PI3K isoforms, and assessed cell viability after 72 hours [17]. The dose of 500 nM NVPBKM120 was selected based on data demonstrating that 500 nM is the IC50 required to inhibit PI3K activity *in vitro* [17]. NVP-BKM120 significantly inhibited proliferation of *KRAS* wild-type, but not mutant, cell lines (Figure 1A), and suppressed PI3K and downstream mTORC1 activity in both *KRAS* wild-type and mutant DLD-1 cells, as assessed by western blot for P-AKT and P-S6 (Figure 1B). Consistent with a prior report, both *PIK3CA* mutant and wild-type cells were sensitive to NVP-BKM120 [17]. We then confirmed these findings in low passage, immortalized colorectal cancer cell lines derived from *Pik3ca* wild-type / *Kras* wild-type and *Pik3ca* wild-type / *Kras* mutant genetically engineered murine tumors [19]. We found that NVPBKM120 effectively inhibited proliferation of *Pik3ca* wild-type / *Kras* wild-type, but not *Kras* mutant, murine cell lines (Figure 1C).

3.2 Genetically engineered Kras mutant murine tumors wild-type for Pik3ca do not respond to PI3K inhibition

We evaluated the efficacy of PI3K inhibition in genetically engineered mouse models of *PIK3CA* wild-type CRC. Consistent with our report that CRC tumors wild-type for *Kras* and *Pik3ca* are sensitive to NVP-BEZ235 (a dual PI3K/mTOR inhibitor), treatment of tumor-bearing *Apc* mice with NVP-BKM120 resulted in tumor regression and inhibition of P-AKT and P-S6 [21] (Figure 1D-F, Supplementary Figure 1). In contrast, treatment of tumor-bearing Apc-Kras mice had no effect on tumor size but effectively inhibited PI3K and mTOR activity (Figure 1G-I, Supplementary Figure 2). As expected, 10 of 10 Apc-Kras tumors tested negative for the most common *PIK3CA* mutations, H1047R and E545K (data not shown). These findings suggest that PI3K inhibition is an effective treatment for *PIK3CA* wild-type, *KRAS* wild-type CRC, but does not treat *PIK3CA* wild-type, *KRAS* mutant CRC.

3.3 A phospho-kinase array identifies MAPK activation as a resistance mechanism to PI3K inhibition

To identify possible resistant mechanisms to PI3K blockade in *KRAS* mutant CRC, we assessed the levels of 43 phosphorylated kinases in DLD-1 cells treated with 500 nM and 2500 nM NVPBKM120 relative to vehicle control using a phospho-kinase array (Proteome Profiler Human Phospho-Kinase Array Kit, R&D systems) (Figure 2A). 500 nM NVM-BKM120 treatment resulted in activation of P-JNK$^{Thr183/Tyr185,\ Thr221/Tyr223}$ (54%), P-mTOR^{Ser2448} (50%), P-ERK1/2$^{Thr202/Tyr204,\ Thr185/Tyr187}$ (32%), P-SRC^{Tyr419} (20%), and P-MEK1/2$^{Ser218/Ser222,\ Ser222/Ser226}$ (17%) (Supplementary Table 1). SRC and mTOR are well-established molecular pathways in *KRAS* wild-type CRC [23,24]. We therefore selected the JNK and MAPK pathway (which includes ERK and MEK) as candidate resistance mechanisms to PI3K inhibition.

We first sought to validate the findings from the phospho-kinase array with western blot to identify resistance mechanisms to PI3K inhibition that are specific to *KRAS* mutant cancer. Treatment of *KRAS* wild-type, but not *KRAS* mutant, isogenic DLD-1 cells with 1500 nM NVPBKM120 resulted in activation of P-JNK. This is consistent with evidence that JNK plays an important role in tumor progression in an *Apc* mutant, *Kras* wild-type mouse model of intestinal tumorigenesis [25]. In contrast, treatment of *KRAS* mutant DLD-1 cells and Apc-Kras tumors resulted in greater P-ERK1/2 and P-MEK activation compared to treatment of their *KRAS* wild-type counterparts (Figures 2B-2E, Figure 2C, Supplementary Figures 1 and 2). We therefore identified the MAPK pathway as a likely resistance mechanism to PI3K blockade in *KRAS*-mutant CRC.

3.4 Dual PI3K/MEK inhibition overcomes PI3K resistance in KRAS mutant human and murine CRC cells

To assess the role of dual PI3K/MEK blockade in treatment of *KRAS* mutant CRC, we treated *KRAS* wild-type and mutant CRC cells with 500 nM NVP-BKM120, 100 nM of the specific MEK inhibitor PD-0325901, or combination, and assessed cell viability [18]. For all three sets of cell lines, combination therapy was significantly more effective than monotherapy in inhibiting cellular proliferation in *KRAS* mutant cells (Figures 2D-2G). Corresponding western blot analysis for DLD-1 isogenic lines demonstrated elevated baseline P-ERK1/2 levels in *KRAS*-mutant lines, activation of P-ERK with NVP-BKM120 treatment, and maximal inhibition of PAKT and P-S6 with combination PI3K/MAPK inhibition (Figure 2E). Analogous experiments in *Kras* wild-type and mutant murine CRC cell lines demonstrated greater efficacy with combination therapy compared to NVP-BKM120 or PD-0325901 monotherapy (Figure 2H).

3.5 Combination PI3K/MEK inhibition induces tumor regression and inhibits proliferation in Pik3ca wild-type, Kras mutant murine colorectal tumors

We then sought to validate our *in vitro* findings in the genetically engineered mouse model of CRC. We treated Apc-Kras tumors with NVP-BKM120, PD-0325901, or combination, for seven days, and assessed tumor size and activation of the PI3K, mTOR, and MAPK pathways before and after treatment. Combination PI3K/MEK therapy was required for maximal tumor regression in Apc-Kras tumors and inhibition of the PI3K, mTORC1, and MAPK pathways (Figure 3A-C, Supplementary Figure 2). Immunohistochemistry for Ki-67 revealed that while NVP-BKM120 was sufficient for inhibition of tumor cell proliferation in Apc tumors, combination NVPBKM120 / PD-0325901 was required for inhibition of proliferation in Apc-Kras tumors (Figure 4A-D).

3.6 Dual PI3K/MEK inhibition induces G1 phase arrest in KRAS mutant CRC cell lines

To further explore the mechanisms by which PI3K/MEK inhibition treats *KRAS* mutant CRC, we assessed cell cycle progression in DLD-1 and HCT116 *KRAS* isogenic cell lines. For both lines, NVP-BKM120 or PD-0325901 monotherapy was sufficient to induce G1 phase arrest in *KRAS* wild-type cells, while combination NVP-BKM120 / PD-0325901 treatment was required to induce G1 phase arrest in *KRAS* mutant cells (Supplementary Figure 3).

3.7 Combination PI3K/MEK blockade activates apoptotic pathways and inhibits anti-apoptotic pathways in KRAS mutant CRC

Based on our finding that PI3K/MEK treatment blocks cell proliferation by inducing cell cycle arrest, we asked whether combination treatment promotes apoptosis. In *KRAS* wild-type isogenic cells, NVP-BKM120 therapy was sufficient to induce cleavage of PARP by Caspase 3, a marker of cellular apoptosis. However, in *KRAS* mutant isogenic cells, combination NVP-BKM120 / PD-0325901 treatment was required to induce PARP cleavage (Figure 5A-B). *In vivo*, while PI3K blockade induced apoptosis in Apc tumors, combination PI3K/MEK inhibition was required to induce apoptosis in Apc-Kras tumors (Figure 5C-F).

MCL-1 is one of the most highly amplified genes in human cancers, particularly colorectal cancer [26]. MCL-1 is a member of the BCL-2 family of anti-apoptotic proteins and acts on the mitochondrial membrane to directly bind and sequester BH3-only pro-apoptotic family members such as BIM [27]. We therefore asked whether the efficacy of combination PI3K/MEK inhibition in *KRAS* mutant CRC is in part due to inhibition of MCL-1 and/or activation of BIM. We found that NVP-BKM120 or PD-0325901 was sufficient to inhibit MCL1 and activate BIM in *KRAS* wild-type CRC cell lines and genetically engineered colorectal tumors, while combination therapy was required to achieve the same apoptotic effect in *KRAS* mutant CRC cell lines and tumors (Figure 6).

4. Discussion

Therapeutic options for cancers with activating mutations in *KRAS* are limited. A number of studies have identified unbiased strategies to treat *KRAS* mutant cancers through RNA interference screens, including inhibition of TAK1, STK33, TBK1, WT1, GATA2, and BCL-XL / MEK [28–33]. However, follow up studies have been difficult to reproduce, possibly due to the off-target effects of RNA interference [34]. Moreover, clinical translation of these findings is limited by the need for selective, efficacious, and nontoxic drugs that target these pathways. In this study, we used a phospho-kinase array to identify adaptive mechanisms of resistance to PI3K inhibition in *KRAS* mutant CRC cells among 43 well-characterized kinases, most of which are targeted by known selective agents. This analysis revealed many potential phosphorylated mediators of PI3K resistance, including JNK, mTOR, ERK, MEK, SRC, p38a, HCK, p27, and FYN. The MAPK pathway has gained the most extensive interest as a resistance mechanism to PI3K inhibition due to multiple nodes of cross-talk between these pathways and the broad availability of selective pre-clinical therapeutics [35].

The importance of MAPK signaling in adaptive resistance to selective pan-class I PI3K inhibitors is supported by several studies demonstrating the combined benefit of PI3K/MEK inhibition in the treatment of *PIK3CA* mutant, *KRAS* mutant colorectal, ovarian, and lung cancers [36–42]. However, these studies did not examine the role of PI3K/MEK inhibition in *PIK3CA* wild-type cancers, which comprise the majority of *KRAS*-mutant cancers [3]. Despite studies suggesting that PI3K inhibitors are somewhat more effective in cancers with mutations in *PIK3CA*, we previously found that *PIK3CA* isogenic CRC cell lines and *PIK3CA* wild-type murine colorectal tumors are sensitive to PI3K/mTOR inhibition due to constitutive AKT activity [17,21,43]. Here, we show that combination PI3K/MEK inhibition

is effective in inducing regression of *PIK3CA* wild-type, *KRAS* mutant CRC by inhibiting mTORC1, blocking the anti-apoptotic activity of MCL-1 and increasing the pro-apoptotic activity of BIM.

In vivo studies of PI3K/MEK inhibition have largely depended on 1) human cell lines and xenografts in immune-compromised mice that fail to recapitulate the complex host-stroma interaction in human tumors, or 2) genetic manipulations to over-activate PI3K in mouse models that are not commonly found in the cancer of study [37,38,41]. To avoid these limitations, we first tested our hypothesis in *PIK3CA* mutant (DLD-1 and HCT116) and wild-type (SW480) *KRAS* mutant human CRC cell lines. We then confirmed our findings in our *Pik3ca* wild-type murine CRC cell lines that are *Kras* mutant or *Kras* wild-type. These murine tumor-derived CRC cell lines are an excellent preclinical resource for drug discovery; they are genetically defined, recently derived (<5 passages), and recapitulate key genetic signatures of *KRAS* mutant and wild-type human cancers [19].

We validated our *in vitro* findings in genetically engineered mouse models of *PIK3CA* wild-type, *KRAS* wild-type (Apc) or *PIK3CA* wild-type, *KRAS* mutant (Apc-Kras) CRC. These models reproduce important features of human CRC: 1) tumors derive from somatic modification of driver genes such as *Apc*; 2) tumors recapitulate the adenoma-carcinoma-metastasis sequence; 3) only 1-3 tumors form; and 4) tumors are located only in the colon [20]. We have previously demonstrated the utility of endoscopic monitoring to assess tumor size before and after treatment [20,21,44]. Here, we show that tumors can be biopsied before and after drug therapy to assess mechanisms of response and resistance in individual mice. Taken together, our rigorous three-step approach (e.g., human cell line, murine cell line, and genetically engineered mouse model studies) represents a new model for preclinical therapeutic studies that maximizes the likelihood of translational relevance.

Our findings are relevant to the clinical care of patients with *KRAS*-mutant cancers for a number of reasons. First, although a number of Phase I clinical trials are currently examining the role of PI3K/MEK blockade in *KRAS*-mutant CRC and other cancers, patients harboring *PIK3CA* wild-type cancers are currently excluded [35]. Our findings suggest that the scope of these studies should be expanded to include *PIK3CA* wild-type patients. Second, our finding that tumor regression with combination PI3K/MEK therapy in *KRAS*-mutant CRC was associated with mTORC1 inhibition corroborates recent reports that mTORC1 suppression predicts sensitivity to MEK [45] or PI3K [46] inhibition. Together, these reports and our work suggest that efficacy of PI3K and MEK inhibitors as monotherapy or in combination depend on mTORC1 blockade. Finally, we report that modulation of the mitochondrial-dependent apoptotic program is an important mechanism for the effectiveness of PI3K/MEK inhibition. Previous studies have identified the anti-apoptotic BCL-2 family member MCL-1 and the pro-apoptotic BH3-only family member BIM as sentinel effectors in cancer cell apoptosis in response to targeted therapy [40,47–50]. Our results therefore support future studies on BCL-2 family inhibitors in *KRAS* mutant cancer.

Our findings raise the question of whether combination PI3K/MEK inhibition may be beneficial to patients harboring *KRAS* wild-type cancers. Prior reports have demonstrated

that *KRAS* wild-type cells are less sensitive to MEK inhibition than *KRAS*-mutant cells [37,42]. However, we found that DLD-1 *KRAS* wild-type cells, and one of two *Kras* wild-type CRC tumors, exhibited modest induction of P-ERK following treatment with PI3K inhibitor (Figures 2C and 2E). While *KRAS* wild-type cells were exquisitely sensitive to PI3K inhibition alone, combination treatment with a MEK inhibitor provided additional reduction in cell viability, induction of BIM, and inhibition of MCL-1 (Figures 2D, 2F-2H, and 6A) without increased cell cycle arrest or PARP cleavage (Supplementary Figure 1, Figures 5A-5B). Thus, our findings implicate a potential role for combination PI3K/MEK inhibition in *KRAS* wild-type cancer. Taken together, further studies are needed to explore this therapeutic paradigm.

In conclusion, we report the efficacy of combination PI3K/MEK inhibition of *PIK3CA* wild-type, *KRAS*-mutant human CRC cell lines, mouse CRC cell lines, and genetically engineered mice. Tumor regression with dual PI3K/MEK therapy was mediated by mTORC1 inhibition, MCL-1 inhibition, and BIM up-regulation. Our findings provide a rationale for testing PI3K/MEK inhibitors in patients with *PIK3CA* wild-type, *KRAS*-mutant cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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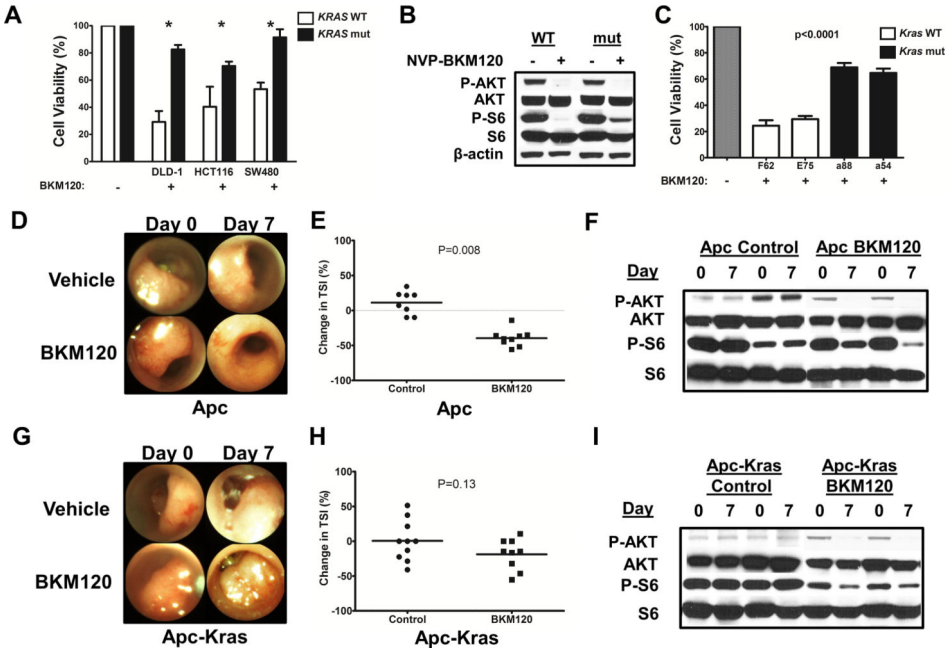

Figure 1. *KRAS* mutant CRC is resistant to PI3K inhibition

(A) *KRAS* mutant CRC cell lines DLD-1 and HCT116 and their wild-type isogenic counterparts and SW480 control or sh-*KRAS* cells were seeded in 0.5% FBS media, then treated with DMSO (-) or 500 nM NVPBKM120 (+) for 72 hours. Cell viability was assessed via MTS assay. (B) PI3K (P-AKT^{Thr308}) and mTORC1 (P-S6$^{Ser240/244}$) activity in DLD-1 *KRAS* wild-type and mutant isogenic cells was assessed via western blot. (C) *Kras*$^{+/+}$ (F62 and E75) and *Kras*$^{G12D/+}$ (a54 and a88) murine CRC cell lines were seeded in 0.5% FBS media, then treated with DMSO (-) or 500 nM NVPBKM120 (+) for 72 hours. Proliferation was assessed via MTS assay. Tumor-bearing *Apc*$^{flox/flox}$ (Apc) mice were randomized to vehicle or 40 mg/kg NVP-BKM120 for seven days. Colonoscopy images (D) were taken before and after treatment to calculate Tumor Size Index (tumor area / luminal area x 100) (E). Tumor biopsies were obtained before and after treatment and assayed for PI3K and mTORC1 signaling via western blot (F). Similar experiments were performed in *Apc*$^{flox/flox}$ / *Kras*$^{G12D/+}$ (Apc-Kras) mice (G-I). *P<0.01.

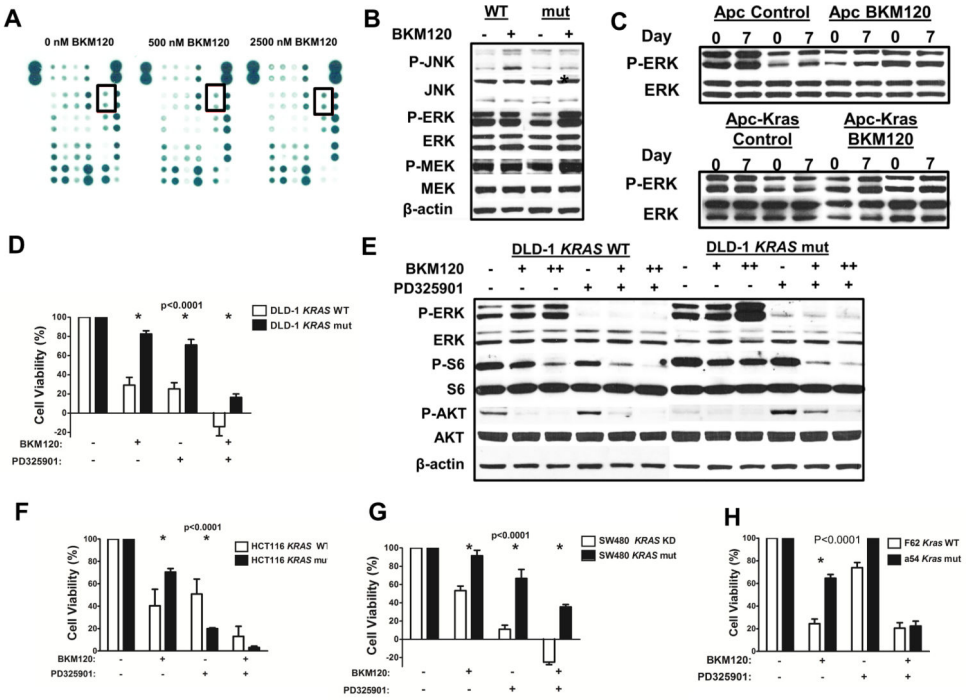

Figure 2. PI3K blockade induces MAPK activation in *KRAS* mutant CRC and is overcome with combination PI3K/MEK inhibition

(A) DLD-1 cells were treated with DMSO, 500 nM NVP-BKM120, or 2000 nM NVP-BKM120 for 24 hours. Cell lysates were probed with a phospho-kinase array (Proteome Profiler Human Phospho-Kinase Array Kit, R&D systems). (PERK1/2$^{Thr202/Tyr204}$, red boxes) (B) DLD-1 *KRAS* WT and mutant isogenic cells were treated with 2000 nM BKM120 for 72 hours, and probed for JNK and MAPK signaling with western blot. (C) Tumor biopsies from two Apc and Apc-Kras mice before and after 7 days treatment with vehicle or NVP-BKM120 were assessed for P-ERK1/2 $^{Thr202/Tyr204}$ signaling with western blot. DLD-1 (D), HCT116 (F), SW480 (G), and murine (H) *KRAS* WT and mutant cells were treated with DMSO, 500 nM NVP-BKM120, 100 nM PD325901, or combination for 72 hours. Cell viability was assessed via MTS assay. (E) *KRAS* WT and mutant isogenic DLD-1 cells were treated for 72 hours with DMSO, 500 nM NVP-BKM120 (+), or 2000 nM NVP-BKM120 (++), with or without 100 nM PD325901, then probed for MAPK (P-ERK1/2$^{Thr202/Tyr204}$), mTORC1 (P-S6$^{Ser240/244}$), and PI3K (P-AKT^{Thr308}) signaling. *P<0.001.

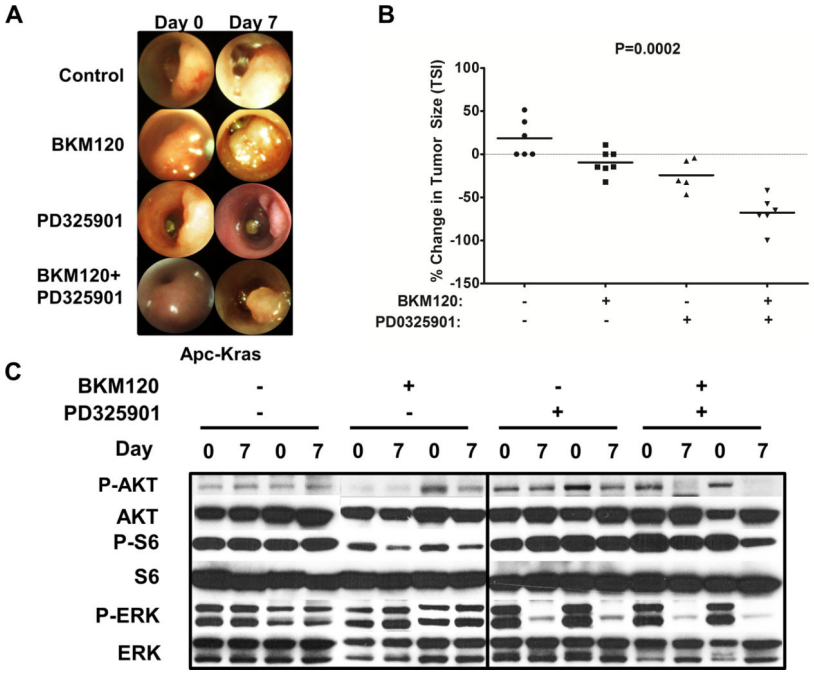

Figure 3. Combination PI3K/MEK inhibition induces tumor regression in a mouse model of *PIK3CA* wild-type, *KRAS*-mutant CRC

Tumor-bearing Apc-Kras mice were treated with vehicle, 40 mg/kg NVP-BKM120, 25 mg/kg PD-325901, or combination for seven days. (A) Representative colonoscopic tumor images are shown before and after treatment. (B) Tumor size index (tumor area / luminal area x 100) was assessed for each tumor before and after treatment to calculate percent change in tumor size. (C) Biopsy specimens obtained before and after treatment for two tumors in each treatment group were probed for PI3K, mTORC1, and MAPK signaling with western blot.

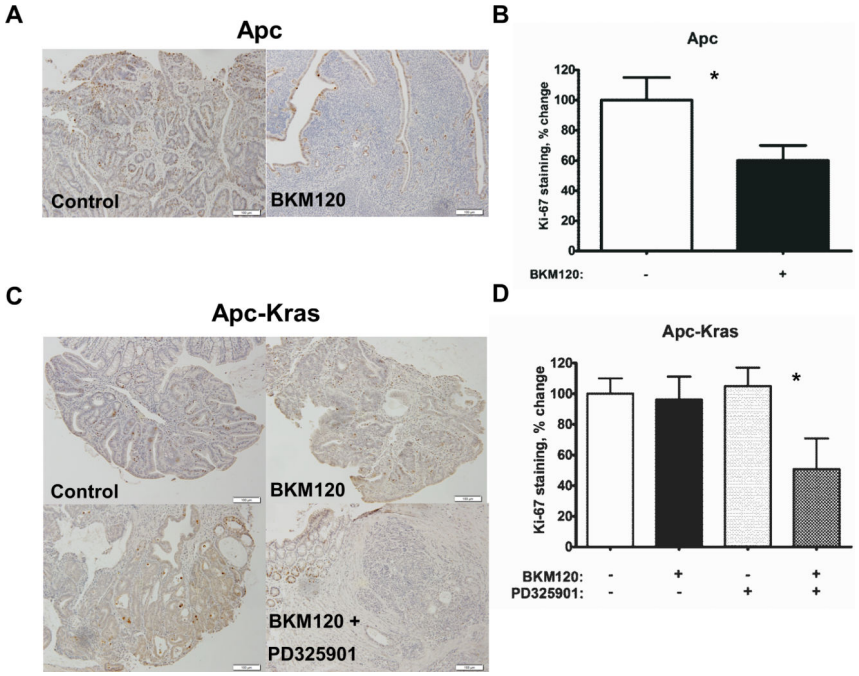

Figure 4. Dual PI3K/MEK inhibition inhibits proliferation of *Kras*-mutant CRC *in vivo*

Tumor-bearing Apc mice were treated with control or NVP-BKM120 for seven days. After sacrifice, Ki-67 staining of tumors was assessed by immunohistochemistry (20X) (A-B). Tumor-bearing Apc-Kras mice were treated with control, NVP-BKM120, PD-325901, or combination for seven days. Ki-67 staining of tumors was assessed by immunohistochemistry (20X) (C-D). *P<0.01.

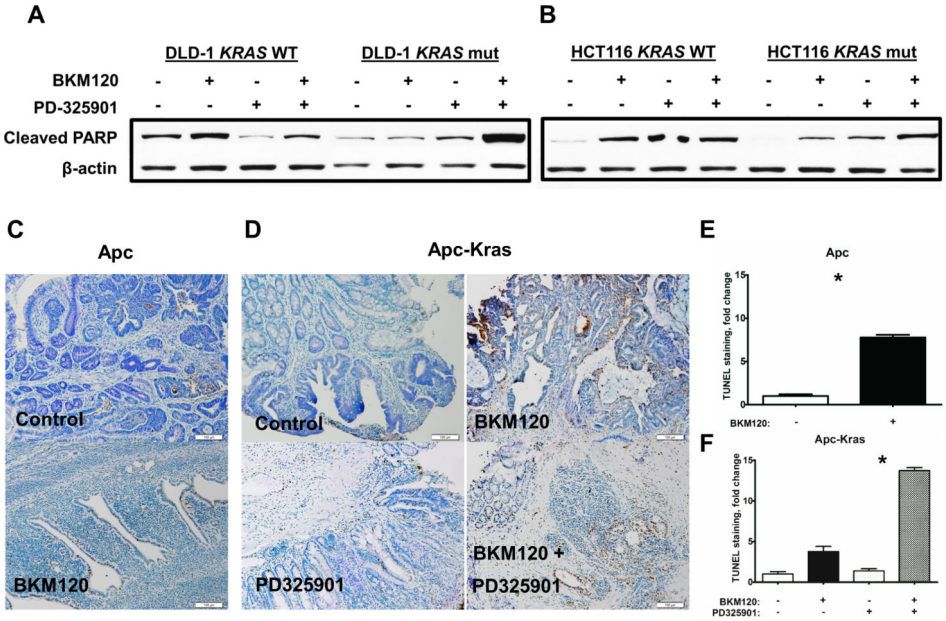

Figure 5. Combination PI3K/MEK inhibition is required for induction of apoptosis in *KRAS* mutant CRC

Isogenic *KRAS* mutant and wild-type DLD-1 (A) and HCT116 (B) CRC cells were treated with DMSO, 500 nM NVP-BKM120, 100 nM PD325901, or combination for 72 hours. Cell lysates were probed for Cleaved PARP. (C-F) Control and treated Apc and Apc-Kras tumor specimens were assessed for TUNEL staining (20X). *P<0.01.

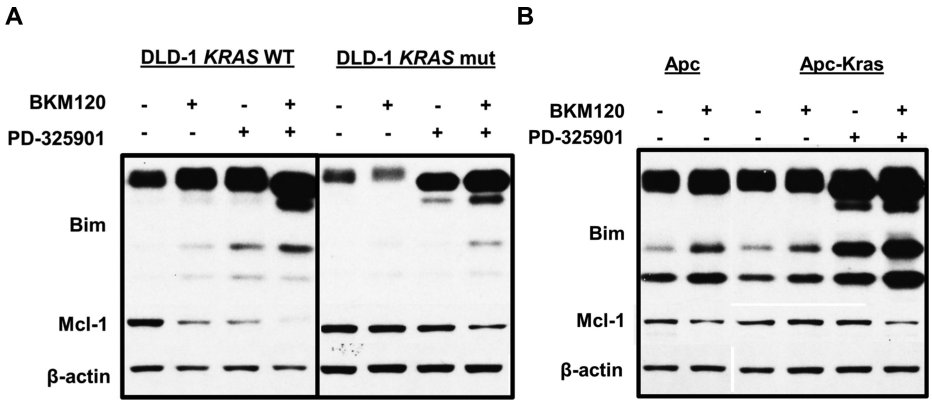

Figure 6. Dual PI3K/MEK blockade promotes apoptosis by inhibiting MCL-1 and activating BIM in *KRAS* mutant CRC

(A) Isogenic DLD-1 KRAS wild-type and mutant cells were treated with DMSO, 500 nM NVP-BKM120, 100 nM PD-325901, or combination for 72 hours. Cell lysates were assessed for BIM and MCL-1 expression. (B) Tumor biopsies from Apc and Apc-Kras mice before and after treatment with vehicle, NVP-BKM120, or PD-325901 were assessed for BIM and MCL-1 expression.