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## Resistance to BRAF inhibition in BRAF-mutant colon cancer can be overcome with PI3K inhibition or demethylating agents

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### Abstract

**Purpose**—Vemurafenib, a selective inhibitor of *BRAF*<sup>V600</sup> has shown significant activity in *BRAF*<sup>V600</sup> melanoma, but not in the <10% of metastatic *BRAF*<sup>V600</sup> colorectal cancers (CRC), suggesting that studies of the unique hypermethylated phenotype and concurrent oncogenic activation of *BRAF*<sup>mut</sup> CRC may provide combinatorial strategies

**Experimental Design**—We performed comparative proteomic analysis of *BRAF*<sup>V600E</sup> melanoma and CRC cell lines, followed by correlation of PI3K pathway activation and sensitivity to the vemurafenib-analog PLX-4720. Pharmacologic inhibitors and siRNA were used in combination with PLX4720 to inhibit PI3K and methyltransferase in cell lines and murine models.

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**Results**—Compared to melanoma, CRC lines demonstrate higher levels of PI3K/AKT pathway activation. CRC cell lines with mutations in *PTEN* or *PIK3CA* were less sensitive to growth inhibition by PLX4720 ( $P=0.03$ ), and knockdown of *PTEN* expression in sensitive CRC cells reduced growth inhibition by the drug. Combined treatment of PLX4720 with PI3K inhibitors caused synergistic growth inhibition in BRAF-mutant CRC cells with both primary and secondary resistance. In addition, methyltransferase inhibition was synergistic with PLX4720 and decreased AKT activation. In vivo, PLX4720 combined with either inhibitors of AKT or methyltransferase demonstrated greater tumor growth inhibition than PLX4720 alone. Clones with acquired resistance to PLX4720 *in vitro* demonstrated PI3K/AKT activation with EGFR or KRAS amplification.

**Conclusions**—We demonstrate that activation of the PI3K/AKT pathway is a mechanism of both innate and acquired resistance to BRAF inhibitors in *BRAF*<sup>V600E</sup> CRC, and suggest combinatorial approaches to improve outcomes in this poor prognosis subset of patients.

### Keywords

BRAF; colon cancer; resistance; PI3K; vemurafenib; PLX4720

### Introduction

Outcomes in patients with colorectal cancer (CRC) have improved over the last decade, but this benefit has not extended to all subtypes of this disease (1). Less than 10% of metastatic CRC patients have tumors with a point mutation in *BRAF*, a component of the RAF/MEK/ERK signaling pathway (2, 3). Similar to other cancers, more than 95% of the *BRAF* mutations in CRC affect the V600 position of the protein, resulting in constitutive RAF/MEK/ERK pathway activation (4, 5). *BRAF*-mutant CRC is associated with hypermethylation of CpG islands and minimal chromosomal instability, which is molecularly distinct from the traditional model of adenoma-carcinoma progression associated with loss of APC, KRAS, p53, and chromosomal instability (6–8). Metastatic CRC patients with a *BRAF* mutation have a very poor prognosis, with median survival of only 10 months, as compared to 35 months for those with a wild type *BRAF* (3, 9). Thus, in these patients there is a critical need for more effective therapies.

Vemurafenib (PLX4032, Plexikkon/Roche) is a potent and selective inhibitor of the V600 mutant form of the BRAF protein. Vemurafenib, and its structural analogue PLX4720, has an IC<sub>50</sub> of 31nM for the kinase activity of the BRAF protein with the V600E mutation, which is more than 10-fold lower than the IC<sub>50</sub> for the wild-type BRAF protein (10). Vemurafenib achieved a response rates of 48–67% in in *BRAF*<sup>V600</sup> melanoma (11, 12). However, vemurafenib achieved a clinical response in only 1 of 21 patients with *BRAF*<sup>V600</sup> metastatic CRC, suggesting important differences in the biology of BRAF<sup>mut</sup> tumors in different cancer types (13).

To improve outcomes in CRC patients with a *BRAF* mutation, there is a critical need to better understand the mechanisms of resistance to BRAF inhibitors. Several studies have investigated mechanisms of resistance to selective BRAF inhibitors in melanoma (14–16). BRAF inhibition resistance has been shown to be mediated in part by EGFR in two recent publications, demonstrating the importance of studies in colorectal cancer models (17, 18). We use comparative proteomic analysis of human melanoma and CRC cell lines, and functional testing of *BRAF*-mutant CRC lines with primary (innate) and secondary (acquired) resistance to BRAF inhibition *in vitro* and *in vivo* to extend this line of research in colon cancer models. We hypothesized that the inherent CpG island hypermethylator phenotype and concurrent oncogenic activation of alternate pathways may impact sensitivity

to BRAF inhibition. Our results reveal a role for the PI3K/AKT pathway in resistance to BRAF inhibition in CRC. In addition, we have identified novel combinatorial approaches to overcome resistance to BRAF inhibitors that are candidates for further testing.

## Materials and Methods

### Cell lines and inhibitors

A panel of BRAF<sup>mut</sup> CRC and melanoma cell lines was utilized for comparative studies (Supplementary Table 1 for lines and conditions). The PLX4720-resistant Colo205 clone was a generous gift from Plexxikon, and was grown in 5  $\mu$ M PLX4720. To establish the PLX4720-resistant HT29 clone, HT29 cells were continuously exposed to step-wise increasing concentrations of PLX4720: 0.1, 0.5, 1, 2 and 4  $\mu$ M until the surviving cells reached 90% confluency. One resistant clone was isolated from viable cells growing in medium with 4  $\mu$ M PLX4720 after 2 month selection and characterized by comparing the PLX4720 IC<sub>50</sub> values to that of parental cells.

PLX4720 (Plexxikon), LY294002 (EMD Chemicals), 5-Azacytidine (Sigma-Aldrich) and GDC0941 (Selleck Chemicals) were dissolved in DMSO as 10 mM, 40 mM, 10 mM and 10 mM stocks, respectively.

### Proliferation and colony formation assays

Cells were seeded in a 96-well plate at a density of 2,000, allowed to attach for 24 h at 37°C, and then treated with inhibitors for 72 h, with quantification using MTS solution (Promega). For colony formation assay, cells were seeded in a 24 well plate at a density of 300, allowed to attach for 24 h at 37°C, and then treated with PLX4720. The cells were maintained at 37 °C for 2 weeks. Colonies of cells were then fixed with cold methanol for 25 min and stained with 1% crystal violet with quantification by ImageJ (NIH). Cell cycle analysis was performed within 1 h after labeling with propidium iodide (EMD Chemical) on the FACS Canto machine (BD Biosciences) with BD FACSDiva software.

### Protein analysis

For reverse phase protein array analysis (RPPA), proteins were isolated as described previously (19–21). Samples were analyzed for the expression of protein markers using RPPA-validated antibodies after correction of phosphor-specific antibodies for expression levels of total protein, and correlation for global protein content (21). Phospho-AKT<sup>S473</sup>AKT, phospho-p42/44 ERK<sup>T202/Y204</sup>p42/44 ERK1/2, phospho-GSK3 $\beta$ <sup>S9</sup>phospho-p70S6K<sup>T389</sup>EGFR, PARP, PTEN antibodies were purchased for immunoblotting from Cell Signaling Technology. Anti-IGFBP-3 was from Santa Cruz Biotechnology. Anti-vinculin was obtained from Sigma-Aldrich.

### Gene silencing by siRNA

Cells were seeded in 6-well plates at a density of 10<sup>5</sup> cells/well. The following day, cells were transfected with 50 nM PTEN-targeting siRNA (L-003023-00-0005, Thermo Fisher Scientific) or nontargeting siRNA using DharmaFECT 1 transfection reagent, according to the manufacturer's instructions.

### Copy number and gene expression analysis

Parental and resistant cell lines were analyzed for copy number by SNP tiling (Affymetrix OncoScan) after DNA extraction as previously described (22–24). Total RNA was extracted with the Qiagen RNeasy Kit. Biotin-labeled cRNA samples for hybridization were prepared using Affymetrix followed by amplification, hybridization to Affymetrix HGU133plus2, and

imaging on GeneChip Scanner 3000 (Affymetrix), with data normalized by RMA prior to analysis. Data is deposited in NCBI's Gene Expression Omnibus with accession number GSE34299. Confirmatory FISH was performed for EGFR as previously described (25, 26).

### Animal studies

The HT29 or LS411N cells were injected subcutaneously (1 million per injection) in nude mice. In a variation of previous methyltransferase inhibitor murine studies, cells were pretreated *in vitro* for 4 weeks with 1  $\mu$ M 5-azacytidine (preprimed) or PBS prior to injection to accommodate the delayed epigenetics effects of methyltransferase inhibitors (27). When the tumor became visible, the mice were randomly grouped for treatment. MK2206 was dosed at 120mg/kg P.O. three times per week. The irradiated PLX4720 diet was purchased from Scientific Diets at a concentration of 417 mg/kg. 5-azacytidine was dosed at 0.8 mg/kg IP three times per week.

### Statistical analyses

Densitometry and colony counting was performed using ImageJ v1.45s (NIH). Comparison of the relative sensitivity of the cell lines to PLX4720 on the basis of genotype was performed using the Wilcoxon signed-rank test. Unpaired t-tests were utilized for comparisons of cytotoxicity between conditions or cell lines. IC<sub>50</sub> values, combination indices (using the synergy methodology of Chou and Talay) and IC<sub>90</sub> isobolograms were calculated using Calcsyn v2.0 (BioSoft, Cambridge, MA) (28).

## Results

### Comparison of levels of signaling proteins in colorectal cancer or melanoma cell lines

We first sought to determine if the clinical efficacy of vemurafenib in *BRAF*-mutant melanoma and CRC patients is due to baseline differences in signaling pathways. To test this hypothesis, we compared the expression of a set of proteins and phospho-proteins that are components of various cell signaling pathways in a panel of human melanoma and CRC cell lines (Supplemental Table 1) by RPPA analysis. This analysis demonstrated that *BRAF*<sup>mut</sup> CRC cell lines have higher activation of several proteins in the PI3K/AKT pathway, including phosphorylated (P-) AKT, P-P70S6K, and increased expression of eIF4E (Fig. 1A and 1B, Supplemental Table 3). This result is consistent with increased activation of the PI3K/AKT pathway in CRC cell lines as compared to melanoma cell lines. Conversely, CRC cell lines displayed lower levels of activation of the MEK pathway compared to *BRAF*<sup>mut</sup> melanoma lines, as indicated by lower levels of phosphorylated MEK, ERK and RSK, and lower levels of cyclin-D1 and Myc. Notably, HER2 and EGFR were expressed at higher levels in CRC than melanoma.

### CRC cell lines with concurrent PIK3CA mutation or PTEN loss are more resistant to BRAF inhibition

To evaluate the role of PI3K/AKT pathway activation on sensitivity to BRAF inhibition, an extended panel of *BRAF*<sup>mut</sup> CRC cell lines characterized for PIK3CA and PTEN mutation status were tested for PLX4720 sensitivity (Figure 2A). All of the *BRAF*<sup>mut</sup> CRC cell lines underwent varying degrees of growth inhibition in response to PLX4720 treatment compared to untreated controls, whereas only minimal effects were observed in *BRAF*<sup>wt</sup> CRC cell lines. The extent of growth inhibition varied considerably across the cell line panel. Colo201, Colo205 and VACO-5 displayed a reduction in the number of viable cells after treatment. CRC cell lines with PI3K/AKT pathway activating mutations in *PIK3CA* and/or *PTEN* loss were more resistant to growth inhibition by PLX4720 as compared to cell lines without these alterations (P=0.03 by Mann-Whitney U test). A similar analysis

examining levels of EGFR expression failed to show a correlation with PLX4720 sensitivity, but either loss of PTEN or higher EGFR expression is associated with PLX4720 resistance ( $P=0.048$ , Fisher's exact) (Supplemental Figure 1).

To more directly assess causation, we next evaluated whether activation of PI3K/AKT signaling by PTEN knockdown altered sensitivity to PLX4720. As shown in Fig. 2B, PTEN knockdown reduced sensitivity to PLX4720 effects on colony formation and cell proliferation (Fig. 2C, Supplemental Figure 2), demonstrating that the PI3K/AKT pathway modulates sensitivity of CRC cells to BRAF inhibitors.

### Dual inhibition of mutant BRAF and PI3K synergistically inhibits growth of colon cancer cells

To further examine the impact of PI3K/AKT signaling on sensitivity to BRAF inhibitors, growth and proliferation of CRC cells were evaluated after treatment with PLX4720, and the PI3K inhibitors LY294002 or GDC0941. As shown in Fig. 3A, the combinations significantly reduced cell viability compared to single agent treatment. In formal synergy studies, the combination of PLX4720 and LY294002 showed synergistic effects in inhibiting cell growth ( $CI = 0.34$  and  $0.62$  at the  $IC_{50}$  for the combination in HT29 and RKO cells). A similar finding was observed with the combination of PLX4720 and GDC0941, which recapitulated the synergistic effects ( $CI = 0.42$  in HT29). An isobologram for these combinations is shown in Fig. 3B. When used as single agents, PLX4720 and LY294002 caused accumulation of cells in the G1 phase of cell cycle. Co-treatment resulted in more pronounced G1 arrest compared to either agent alone, indicating an additive effect of the combination on growth inhibition ( $p < 0.05$  for each cell line, Fig. 3C). Combination treatment with PLX4720 and LY294002 also induced apoptosis in Colo205 and RKO cells as denoted by an increase in the sub-G1 population (Fig. 3C, Supplemental Figure 3). Consistent with the flow cytometry data, cleaved PARP was detected in Colo205 and RKO cells, but not HT29 cells (Data not shown). To investigate if the combination of PLX4720 and AKT inhibitor MK2206 has synergistic effects *in vivo*, we utilized the LS411N cell line, which is resistant to PLX4720 alone. Greater growth inhibition was seen with dual administration of PLX4720 and the Akt inhibitor, MK2206 compared with the single administration of PLX4720 ( $P < 0.01$ ) or MK2206 ( $P < 0.05$ ) (Figure 3D).

### Effect of PLX4720 and LY294002 on MAPK and AKT signaling

We next investigated the effect of the combination therapy on signaling via the RAF/MEK and PI3K/AKT pathways in CRC cells. As expected, PLX4720 fully blocked ERK phosphorylation in HT29 and Colo205 cells, whereas LY294002 had minimal inhibitory effect (Fig. 4). After PLX4720 treatment, pAKT was increased in all three cell lines (range of 25% to 90% increase by densitometry), but decreased from control after the addition of the PI3K inhibitor. Intriguingly, RKO had only partial inhibition of pERK at  $1\mu\text{M}$  (60% by densitometry); however dual PI3K and BRAF inhibition reduced pERK further (90% inhibition). Similarly, more robust inhibition of pGSK3 $\beta$  was observed with dual inhibition than with single agent LY294002, further indication of the interaction of the two pathways.

### The DNA methyltransferase inhibitor 5-azacytidine sensitizes colon cancer cells to BRAF inhibition

BRAF<sup>mut</sup> colon cancer is strongly associated with the CpG island methylator phenotype (CIMP), which results in epigenetic silencing of multiple genes. To assess whether epigenetic effects were associated with innate resistance to PLX4720, RKO, Colo205, LS411N, and HT29 were treated with the DNA methyltransferase inhibitor 5-azacytidine in combination with PLX4720. As shown in Figure 5A, 5-azacytidine had minimal effect on colon cancer cell growth *in vitro* at the doses utilized. However, combination therapy

resulted in greater inhibition than PLX4720 alone in all evaluated cell lines. Formal synergy analysis confirmed that the combination had synergistic effects (CI range 0.3 to 0.8 across the cell lines; Fig. 5B). In an HT29 xenograft, enhanced tumor growth inhibition was seen with 5-azacytidine (preprimed *in vitro* with continued treatment *in vivo*) combined with PLX4720 administration *in vivo*, compared to either agent alone ( $P < 0.05$  for both comparisons).

Given the relationship between AKT activation and BRAF inhibitor sensitivity, we evaluated the impact of 5-azacytidine therapy on pAKT in HT29 and LS411N cell lines. In both cell lines, treatment with 1  $\mu$ M or 2  $\mu$ M of 5-azacytidine for 72 h resulted in decreased pAKT expression (Fig. 5C). As both PTEN and IGFBP3 have been reported to be under epigenetic regulation and are known modulators of AKT signaling, we evaluated their expression in these two cell lines (29, 30). In HT29, PTEN was re-expressed in a dose-dependent manner (Fig. 5C). LS411N has a known truncating mutation in PTEN; no PTEN was detected in these cells. Conversely, IGFBP3 was increased in LS411N with 5-azacytidine treatment, but was not detected in HT29. To assess if increase in PTEN expression may be an important mechanism of PLX4720 and 5-azacytidine synergy in HT29 cells, the combination therapy was repeated in HT29 cells first treated with siRNA to PTEN as previously described. Silencing PTEN was not sufficient to fully abolish the synergy seen with the combination, suggesting that additional epigenetically regulated proteins may also account for the observed synergy (Supplemental Fig. 4).

#### **Acquired resistance to PLX4720 is associated with increased expression of activated AKT in human CRC cell lines**

In the clinical trial of vemurafenib in CRC patients, minor responses and mixed responses were seen in almost half the patients, suggesting that resistance may not be solely due to inherent factors, but may also be due to rapidly acquired alterations. Therefore, analyses of the potential mechanisms of acquired resistance were undertaken by developing resistant clones of Colo205 and HT29 with  $IC_{50}$  values 5 to 10-fold above those of the parental lines (Fig. 6A). Unlike the complete inhibition of pERK with PLX4720 in the parental lines, retreatment of the resistant clones resulted in partial inhibition of pERK (Fig. 6B), consistent with previous findings with BRAF inhibitors in melanoma and MEK inhibitors in colon cancer (31). There were no acquired mutations in PIK3CA, NRAS, BRAF, or MEK1 or change in IGF1R, PDGFR, MET, or PTEN gene expression. Copy number analyses demonstrated a gain in copy number in Chr7 (cytoband p11.2) in the HT29 cells, and gain of a smaller region in Chr12 in Colo205 (p11.1-p12.1). Genes of potential relevance in these amplified regions include EGFR and PHKG1, a serine/threonine kinase, in 7p11.2, and in p11.1-p12.1, KRAS and RASSF8, a member of the Ras-association domain family of tumor suppressors (Figure 6C, Supplemental Figure 5). EGFR amplification in HT29RC was confirmed by FISH (Supplemental Figure 6). The increased copy number of EGFR and KRAS translated into increased gene expression in the respective cell lines (Figure 6C), but not for RASSF8 and PHKG1 or other genes previously shown to affect BRAF-inhibitor resistance, including the genes encoding for MEK1/2 (Supplemental Figure 7). Colo205 also demonstrated increased EGFR gene expression and protein levels independent from changes at the DNA level (Figure 6D). Elevations in both phosphorylated ERK and total ERK were seen compared to parental cells (1.5 to 2.5 fold increases), but the ratio of pERK to ERK by densitometry did not change. Notably, expression of activated AKT increased in these chemoresistant clones (2.1 and 2.6-fold for HT29 and Colo205, respectively). Knockdown of EGFR in the resistant clone resulted in sensitivity to PLX4720 similar to that seen in the parental cell line (Supplemental Figure 8).

## Discussion

The identification of BRAF mutations in melanoma and CRC, among other cancers, has led to rapid clinical testing of BRAF inhibitors, including the mutant BRAF isoform inhibitor vemurafenib. Unfortunately, the high response rates seen in melanoma have not extended to BRAF<sup>mut</sup> CRC. Here, we have studied mechanisms that might account for lack of response to BRAF inhibitors in CRC, with the goal of identifying rational clinical combinations with BRAF inhibitors.

Despite the fact that BRAF<sup>mut</sup> colon cancers and melanomas share the similar V600 mutation patterns, our study demonstrated important differences in pathway activation and protein expression, including higher EGFR expression and AKT signaling in colon cancer cell lines than in melanoma cell lines. While there was not a correlation between EGFR expression and BRAF inhibitor sensitivity in the limited cell line panel, this type of analysis may be limited by the small number of BRAF mutant cell lines available. However, cell lines with PIK3CA mutations and PTEN loss were more resistant to the BRAF inhibitors. Colo205, as a cell line lacking PIK3CA mutation or PTEN loss, appears to be as sensitive to BRAF inhibition as melanoma cell lines, and may be an appropriate model for studying the rare subset of vemurafenib-sensitive CRC. This result is consistent with previous work with MEK inhibitors, in which a correlation between AKT phosphorylation status and MEK inhibitor sensitivity has been seen in melanoma, further supported by knockdown of PTEN, leading to Akt activation and conferring resistance to MEK inhibitors (32, 33).

While the frequency of PTEN loss and/or PIK3CA mutations was high in the BRAF<sup>mut</sup> colon cancer cell lines, this phenotype is not seen as frequently in patient samples (34, 35). This likely represents a bias for tumors able to be established as cell lines, as seen in the over-representation of PIK3CA and KRAS dual mutations in colon cancer cell lines in contrast to primary tumors (36). A review of the COSMIC database for BRAF<sup>mut</sup> colon adenocarcinoma samples annotated for PIK3CA and PTEN demonstrates co-mutation rates of 13% (25/194) and 22% (8/37), respectively, consistent with other published series (35, 37). However, PIK3CA and PTEN mutations represent only a subset of mechanisms whereby AKT pathway can be activated. Epigenetic events, including silencing of PTEN by promoter hypermethylation and overexpression and/or high basal activity of receptor tyrosine kinases such as EGFR, also increase activation of the PI3K pathway (36).

Combination treatment with the BRAF inhibitor PLX4720 and PI3K/AKT inhibitors demonstrated synergistic growth inhibition in the BRAF-mutant CRC cell lines, including an in vivo study with a cell line resistant to single agent BRAF inhibition. Yang et al presented a murine study of the combination of vemurafenib and the AKT inhibitor MK2206 in the RKO cell line (38). Similarly, Oikonomou et al demonstrated pharmacological suppression of the PI3K pathway further enhances the synergistic effect between TRAIL and PLX4720 in the same cell line (39). Given the selectivity of vemurafenib for the mutant form of BRAF, the combination of vemurafenib with a PI3K/AKT pathway inhibitor is anticipated to be tolerable in patients and is worthy of further development. While the choice of EGFR, AKT, pan-PI3K or isoform selective PI3K inhibitors to combine with BRAF inhibition would ideally be based in part on the individual mechanisms of PI3K pathway activation in BRAF<sup>mut</sup> CRC (for example PI3K- $\beta$  inhibitors in the setting of PTEN loss), this will need to be balanced by the practical limitations of study designs for this small population. In our studies, single agent PLX4720 treatment failed to induce significant apoptosis in BRAF-mutant colorectal cell lines, in contrast to that seen in melanoma cell lines (10). One likely explanation for this disparity is the higher basal activation status of the pro-survival PI3K pathway in CRC cells. Consistent with this possibility, PTEN-null and AKT-activated melanoma cell lines undergo lower levels of

apoptosis induction in response to PLX4720 treatment, supporting the concept that constitutive activation of the PI3K signaling pathways confers resistance mechanism to mutant BRAF inhibition (33, 40). While we demonstrated that combination therapy with PI3K inhibitors was able to induce apoptosis in some cell lines, the level of apoptosis induction remains below that induced by single agent BRAF inhibition in melanoma (10, 21). This result may be due to alternate survival signaling independent of MAPK and AKT pathways, for example Src-family signaling (41, 42). It is therefore possible that clinical trials of PI3K and BRAF inhibitors in colon cancer should be designed to detect prolonged stabilization of disease rather than objective responses.

CpG island promoter hypermethylation is present in nearly all BRAF<sup>mut</sup> CRC, and the presence of BRAF mutation is used to confirm promoter hypermethylation of hMLH1 as a causative factor for non-familial microsatellite instability (43). The use of 5-azacytidine, a methyltransferase inhibitor currently under evaluation in colorectal cancer, was shown to inhibit phosphorylation of AKT in a dose and time-dependent manner and synergize with BRAF inhibition. Several genes in the PI3K/AKT pathway are commonly cited as being under epigenetic control, including IGF binding proteins, PIK3CG, and PTEN (29, 30, 44, 45). We show that potential mediators of the AKT inhibition include PTEN and IGFBP3, although additional effectors are likely given the pleiotropic effects of methyltransferase inhibition. PTEN loss in melanoma is one of the potential cooperative events in BRAF mutant associated transformation in melanoma, implying that a similar mechanism may be relevant in CRC and driven by hypermethylation (46). These findings provide a possible colon cancer-specific approach for combination therapy. However, several months of demethylator therapy is usually required to fully inhibit aberrant methylation, and such duration of therapy may be difficult to achieve in this rapidly progressive subset of metastatic CRC. In our *in vivo* study, the pretreatment with 5-azacytidine *in vitro* significantly increased the efficacy with subsequent BRAF inhibition. Further efforts to optimize the epigenetic component of the therapy will be necessary prior to translation to the clinic (47).

In the clinical trial of vemurafenib in CRC patients, many patients appeared to derive transient and mixed benefit, manifesting as tumors with both regressing and progressing tumors. These findings suggest that resistance may not be solely due to inherent sensitivity, but may also be due to rapidly acquired resistance factors. Hence, the acquired resistance cell lines may still be relevant to the tumor biology seen in colon cancer patients. As described previously in melanoma, acquired resistance to BRAF inhibition is commonly associated with restored ERK activation through multiple mechanisms, including overexpression or utilization of alternate MAPK family members or RAF isoforms (14–16). Consistent with these previous findings, we observed partial restoration of pERK in PLX4720 resistant colon cancer cell lines, which has been attributed in part to overexpression of EGFR in recently publications (17, 18). Consistent with this, EGFR expression was increased in both resistant cell lines, through both copy number changes and transcriptional/translational mechanisms. Intriguingly, our model shows an apparent predilection to amplification events, instead of acquired mutations, suggesting that mechanisms of resistance may vary by tumor type, even in the setting of similar oncogenes and selective pressure. KRAS amplification has also been seen in cell lines after selective pressure from EGFR inhibition (48). A recent publication similarly demonstrated the role of receptor-tyrosine kinases in activating PI3K and inhibiting apoptosis despite MEK inhibition in KRAS<sup>mut</sup> CRC (49). Given these findings and the relevance of EGFR signaling in CRC, dual EGFR and BRAF inhibition may represent an additional therapeutic possibility and an opportunity for further preclinical study, as suggested by others (17, 18).



In summary, this work highlights important differences between CRC and melanoma and implicates the PI3K/AKT pathway in innate and acquired resistance to BRAF inhibition. Further, we demonstrated that a demethylating agent inhibits the PI3K pathway and also synergizes with PLX4070 in BRAF-mutant CRC cell lines. Targeting EGFR and PI3K are viable combination strategies worthy of immediate clinical implementation in doublet or triplet regimens, while combinations with DNA methylation inhibition will require additional optimization of schedule to maximize likelihood of success in the clinic. In addition, the differential, clinical and biological response of BRAF mutant CRC and melanoma to BRAF inhibitors has provided an important warning about treatment selection done solely on the basis of gene mutations without regard to the broader molecular context of the tumor.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

1. Kopetz S, Chang GJ, Overman MJ, Eng C, Sargent DJ, Larson DW, et al. Improved survival in metastatic colorectal cancer is associated with adoption of hepatic resection and improved chemotherapy. *J Clin Oncol.* 2009; 27:3677–3683. [PubMed: 19470929]
2. Ogino S, Noshro K, Kirkner GJ, Kawasaki T, Meyerhardt JA, Loda M, et al. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. *Gut.* 2009; 58:90–96. [PubMed: 18832519]
3. Tol J, Nagtegaal ID, Punt CJ. BRAF mutation in metastatic colorectal cancer. *N Engl J Med.* 2009; 361:98–99. [PubMed: 19571295]
4. Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell.* 2004; 116:855–867. [PubMed: 15035987]
5. Ikenoue T, Hikiba Y, Kanai F, Tanaka Y, Imamura J, Imamura T, et al. Functional analysis of mutations within the kinase activation segment of B-Raf in human colorectal tumors. *Cancer Res.* 2003; 63:8132–8137. [PubMed: 14678966]
6. Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet.* 2006; 38:787–793. [PubMed: 16804544]
7. Shen L, Toyota M, Kondo Y, Lin E, Zhang L, Guo Y, et al. Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. *Proc Natl Acad Sci U S A.* 2007; 104:18654–18659. [PubMed: 18003927]
8. Kambara T, Simms LA, Whitehall VL, Spring KJ, Wynter CV, Walsh MD, et al. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. *Gut.* 2004; 53:1137–1144. [PubMed: 15247181]
9. Tran B, Kopetz S, Tie J, Gibbs P, Jiang ZQ, Lieu CH, et al. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. *Cancer.* 2011
10. Tsai J, Lee JT, Wang W, Zhang J, Cho H, Mamo S, et al. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. *Proc Natl Acad Sci U S A.* 2008; 105:3041–3046. [PubMed: 18287029]

11. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010; 363:809–819. [PubMed: 20818844]
12. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*. 2011; 364:2507–2516. [PubMed: 21639808]
13. Kopetz S, Desai J, Chan E, Hecht JR, O'Dwyer P, Lee RJ, et al. PLX4032 in metastatic colorectal cancer patients with mutant BRAF tumors. *Journal of Clinical Oncology, 2010 ASCO Annual Meeting Proceedings (Post-Meeting Edition)*. 2010; 28:2534.
14. Wagle N, Emery C, Berger MF, Davis MJ, Sawyer A, Pochanard P, et al. Dissecting Therapeutic Resistance to RAF Inhibition in Melanoma by Tumor Genomic Profiling. *J Clin Oncol*. 2011
15. Cory MJ, Jesse SB, So Young K, Sapana RT, Leslie W, Laura AJ, et al. COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature (London)*. 2010; 468:968–972. [PubMed: 21107320]
16. Nazarian R, Shi H, Wang Q, Kong X, Koya RC, Lee H, et al. Melanomas acquire resistance to B-Raf(V600E) inhibition by RTK or N-RAS upregulation. *Nature (London)*. 2010; 468:973–977. [PubMed: 21107323]
17. Corcoran RB, Ebi H, Turke AB, Coffee EM, Nishino M, Cogdill AP, et al. EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib. *Cancer Discov*. 2012; 2:227–235. [PubMed: 22448344]
18. Prahallad A, Sun C, Huang S, Di Nicolantonio F, Salazar R, Zecchin D, et al. Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. *Nature*. 2012; 483:100–103. [PubMed: 22281684]
19. Davies MA, Stemke-Hale K, Lin E, Tellez C, Deng W, Gopal YN, et al. Integrated Molecular and Clinical Analysis of AKT Activation in Metastatic Melanoma. *Clin Cancer Res*. 2009; 15:7538–7546. [PubMed: 19996208]
20. Park ES, Rabinovsky R, Carey M, Hennessy BT, Agarwal R, Liu W, et al. Integrative Analysis of Proteomic Signatures, Mutations, and Drug Responsiveness in the NCI 60 Cancer Cell Line Set. *Mol Cancer Ther*. 2010; 9:257–2567. [PubMed: 20124458]
21. Gopal YN, Deng W, Woodman SE, Komurov K, Ram P, Smith PD, et al. Basal and treatment-induced activation of AKT mediates resistance to cell death by AZD6244 (ARRY-142886) in Braf-mutant human cutaneous melanoma cells. *Cancer Res*. 2010; 70:8736–8747. [PubMed: 20959481]
22. Ji H, Kumm J, Zhang M, Farnam K, Salari K, Faham M, et al. Molecular inversion probe analysis of gene copy alterations reveals distinct categories of colorectal carcinoma. *Cancer Res*. 2006; 66:7910–7919. [PubMed: 16912164]
23. Wang Y, Moorhead M, Karlin-Neumann G, Wang NJ, Ireland J, Lin S, et al. Analysis of molecular inversion probe performance for allele copy number determination. *Genome Biol*. 2007; 8:R246. [PubMed: 18028543]
24. Schiffman JD, Hodgson JG, VandenBerg SR, Flaherty P, Polley MY, Yu M, et al. Oncogenic BRAF mutation with CDKN2A inactivation is characteristic of a subset of pediatric malignant astrocytomas. *Cancer Res*. 2010; 70:512–519. [PubMed: 20068183]
25. Massarelli E, Varella-Garcia M, Tang X, Xavier AC, Ozburn NC, Liu DD, et al. KRAS Mutation Is an Important Predictor of Resistance to Therapy with Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors in Non-Small-Cell Lung Cancer. *Clinical Cancer Research*. 2007; 13:2890–2896. [PubMed: 17504988]
26. Varella-Garcia M. Stratification of non-small cell lung cancer patients for therapy with epidermal growth factor receptor inhibitors: the EGFR fluorescence in situ hybridization assay. *Diagn Pathol*. 2006; 1:19. [PubMed: 16911776]
27. Tsai HC, Li H, Van Neste L, Cai Y, Robert C, Rassool FV, et al. Transient low doses of DNA-demethylating agents exert durable antitumor effects on hematological and epithelial tumor cells. *Cancer Cell*. 2012; 21:430–446. [PubMed: 22439938]
28. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul*. 1984; 22:27–55. [PubMed: 6382953]

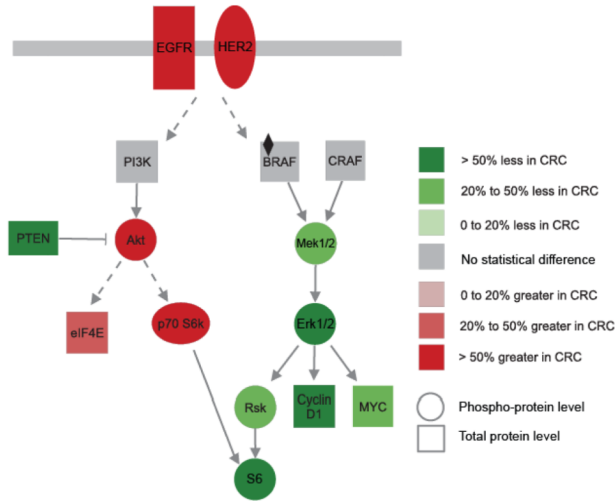
29. Hanafusa T, Shinji T, Shiraha H, Nouse K, Iwasaki Y, Yumoto E, et al. Functional promoter upstream p53 regulatory sequence of IGFBP3 that is silenced by tumor specific methylation. *BMC Cancer*. 2005; 5:9. [PubMed: 15661074]
30. Goel A, Arnold CN, Niedzwiecki D, Carethers JM, Dowell JM, Wasserman L, et al. Frequent inactivation of PTEN by promoter hypermethylation in microsatellite instability-high sporadic colorectal cancers. *Cancer Res*. 2004; 64:3014–3021. [PubMed: 15126336]
31. Little AS, Balmanno K, Sale MJ, Newman S, Dry JR, Hampson M, et al. Amplification of the Driving Oncogene, KRAS or BRAF, Underpins Acquired Resistance to MEK1/2 Inhibitors in Colorectal Cancer Cells. *Science Signaling*. 2011; 4:ra17-ra. [PubMed: 21447798]
32. Tentler JJ, Nallapareddy S, Tan AC, Spreafico A, Pitts TM, Morelli MP, et al. Identification of predictive markers of response to the MEK1/2 inhibitor selumetinib (AZD6244) in K-ras-mutated colorectal cancer. *Mol Cancer Ther*. 2010; 9:3351–3362. [PubMed: 20923857]
33. Paraiso KH, Xiang Y, Rebecca VW, Abel EV, Chen A, Munko AC, et al. PTEN loss confers BRAF inhibitor resistance to melanoma cells through the suppression of BIM expression. *Cancer Res*. 2011
34. Tol J, Dijkstra JR, Klomp M, Teerenstra S, Dommerholt M, Vink-Borger ME, et al. Markers for EGFR pathway activation as predictor of outcome in metastatic colorectal cancer patients treated with or without cetuximab. *Eur J Cancer*. 2010; 46:1997–2009. [PubMed: 20413299]
35. Ogino S, Nosho K, Kirkner GJ, Shima K, Irahara N, Kure S, et al. PIK3CA mutation is associated with poor prognosis among patients with curatively resected colon cancer. *J Clin Oncol*. 2009; 27:1477–1484. [PubMed: 19237633]
36. Ihle NT, Powis G, Kopetz S. PI-3-Kinase inhibitors in colorectal cancer. *Curr Cancer Drug Targets*. 2011; 11:190–198. [PubMed: 21158718]
37. Bamford S, Dawson E, Forbes S, Clements J, Pettett R, Dogan A, et al. The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *Br J Cancer*. 2004; 91:355–358. [PubMed: 15188009]
38. Yang H, Higgins B, Kolinsky K, Packman K, Bradley WD, Lee RJ, et al. Antitumor activity of BRAF inhibitor vemurafenib in preclinical models of BRAF-mutant colorectal cancer. *Cancer Res*. 2012; 72:779–789. [PubMed: 22180495]
39. Oikonomou E, Koc M, Sourkova V, Andera L, Pintzas A. Selective BRAFV600E inhibitor PLX4720, requires TRAIL assistance to overcome oncogenic PIK3CA resistance. *PLoS ONE [Electronic Resource]*. 2011; 6:e21632.
40. Xing YP F, Pratilas CA, Taylor BS, Janakiraman M, et al. Concurrent loss of the PTEN and RB1 tumor suppressors attenuates RAF dependence in melanomas harboring V600EBRAF. *Oncogene*. 2012; 31:446–457. [PubMed: 21725359]
41. Windham TC, Parikh NU, Siwak DR, Summy JM, McConkey DJ, Kraker AJ, et al. Src activation regulates anoikis in human colon tumor cell lines. *Oncogene*. 2002; 21:7797–7807. [PubMed: 12420216]
42. Kopetz S, Lesslie DP, Dallas NA, Park SI, Johnson M, Parikh NU, et al. Synergistic activity of the SRC family kinase inhibitor dasatinib and oxaliplatin in colon carcinoma cells is mediated by oxidative stress. *Cancer Res*. 2009; 69:3842–3849. [PubMed: 19383922]
43. Bettstetter M, Dechant S, Ruemmele P, Grabowski M, Keller G, Holinski-Feder E, et al. Distinction of hereditary nonpolyposis colorectal cancer and sporadic microsatellite-unstable colorectal cancer through quantification of MLH1 methylation by real-time PCR. *Clin Cancer Res*. 2007; 13:3221–3228. [PubMed: 17545526]
44. Semba S, Itoh N, Ito M, Youssef EM, Harada M, Moriya T, et al. Down-regulation of PIK3CG, a catalytic subunit of phosphatidylinositol 3-OH kinase, by CpG hypermethylation in human colorectal carcinoma. *Clin Cancer Res*. 2002; 8:3824–3831. [PubMed: 12473596]
45. Lin J, Lai M, Huang Q, Ma Y, Cui J, Ruan W. Methylation patterns of IGFBP7 in colon cancer cell lines are associated with levels of gene expression. *J Pathol*. 2007; 212:83–90. [PubMed: 17334979]
46. Dankort D, Curley DP, Cartledge RA, Nelson B, Karnezis AN, Damsky WE Jr, et al. BrafV600E cooperates with Pten loss to induce metastatic melanoma. *Nat Genet*. 2009; 41:544–552. [PubMed: 19282848]

47. McGarvey KM, Fahrner JA, Greene E, Martens J, Jenuwein T, Baylin SB. Silenced tumor suppressor genes reactivated by DNA demethylation do not return to a fully euchromatic chromatin state. *Cancer Res.* 2006; 66:3541–3549. [PubMed: 16585178]
48. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature.* 2012; 486:532–536. [PubMed: 22722830]
49. Ebi H, Corcoran RB, Singh A, Chen Z, Song Y, Lifshits E, et al. Receptor tyrosine kinases exert dominant control over PI3K signaling in human KRAS mutant colorectal cancers. *J Clin Invest.* 2011; 121:4311–4321. [PubMed: 21985784]

### Translational Relevance

Activating mutations in BRAF are present in several tumor types, including melanoma and colorectal cancer. Vemurafenib (PLX4032), a selective inhibitor of BRAF, has shown notable clinical benefit in BRAF<sup>Fmut</sup> melanoma patients, but had substantially less benefit in BRAF<sup>Fmut</sup> colorectal cancer. In this study, we show that the PI3K/AKT pathway is activated to a greater extent in BRAF<sup>Fmut</sup> colorectal cancer tumors than in melanoma. We demonstrate that PI3K/AKT pathway activation results in de novo and acquired resistance to BRAF inhibition. This activation occurs through PIK3CA mutation or PTEN loss, and is associated with the inherent CpG island phenotype associated with BRAF<sup>Fmut</sup> CRC through epigenetic silencing. We show that PI3K/AKT inhibitors or epigenetic therapy are synergistic with a BRAF inhibitor. These results provide rationale therapeutic strategies for clinical studies in this poor prognosis subtype of colorectal cancer.

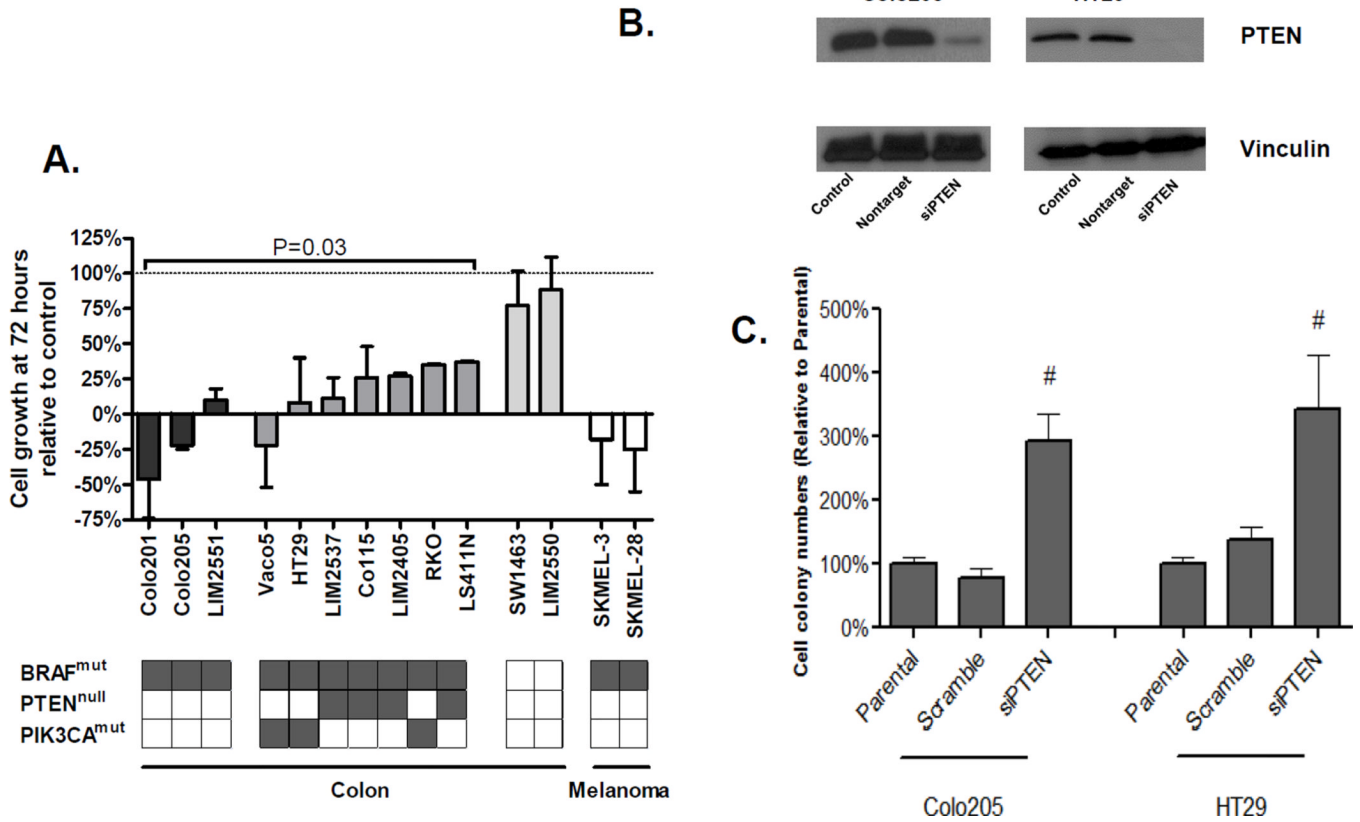
**A.**



**B.**

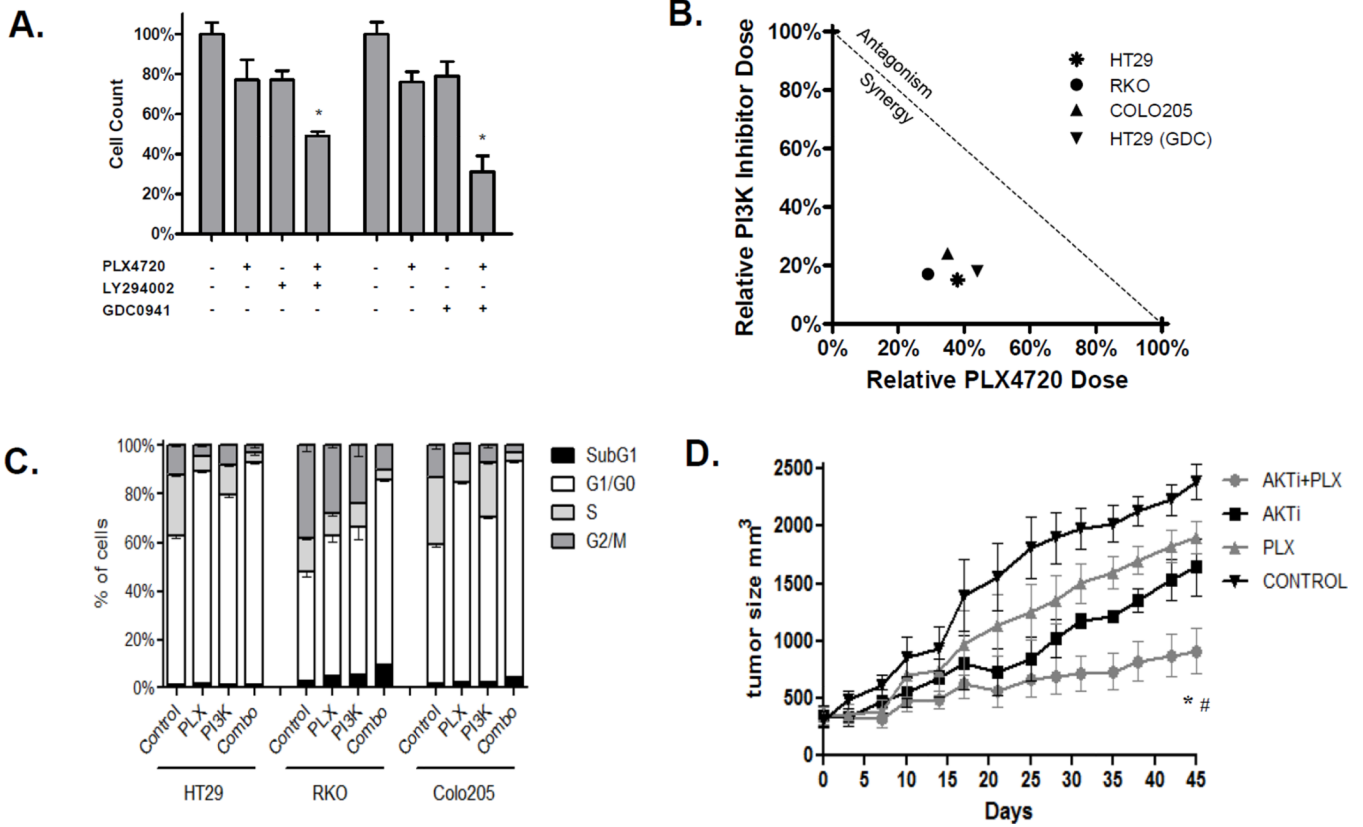
Protein	Normalized to*	Colorectal expression as percent of melanoma expression	p-value
Akt.pT308	Total Akt	556%	<0.00001
Akt.pS473	Total Akt	421%	0.010
Cyclin D1	Total protein	57%	0.018
c.Myc	Total protein	64%	0.010
eIF4E	Total protein	134%	0.037
EGFR	Total protein	210%	<0.00001
HER2.pY1248	Total protein	322%	0.0001
ERK1/2.pT202.Y204	Total protein	27%	<0.00001
MEK1.2.pS217.S221	Total MEK1	70%	0.123
p70S6K.pT389	Total p70S6K	179%	0.003
PI3K.p110a	Total protein	115%	0.204
PTEN	Total protein	31%	0.002
p90.RSK.pT389.S363	Total p90.RSK	61%	0.008
S6.pS240.S244	Total S6	46%	0.079
S6.pS235.S236	Total S6	30%	0.013

**Figure 1. Comparison of molecular pathways between CRC and melanoma cell lines**  
 RPPA analysis was performed using lysates prepared from CRC (n= 11) or melanoma cell lines (n=30), and demonstrated more PI3K/AKT pathway activation and less MEK/ERK pathway activation in BRAF mutant CRC compared to melanoma. **A.** Mapped expression data. Circles represent phosphorylated epitopes on the proteins (normalized to total protein) and squares represent total proteins. Percentages indicate relative expression levels of CRC cells compared with melanoma cells. Grey indicates no change. Diamond represents the V600E mutation of BRAF that is present in all cell lines. **B.** Relative expression levels of CRC cells above or below expression in melanoma, with p-value for comparison. Phospho-protein expression was normalized to total levels of expression of the specific protein, if available, or to total protein loading levels.



**Figure 2. Sensitivity of CRC cell lines is associated with presence of activating mutations in PI3K or loss of PTEN**

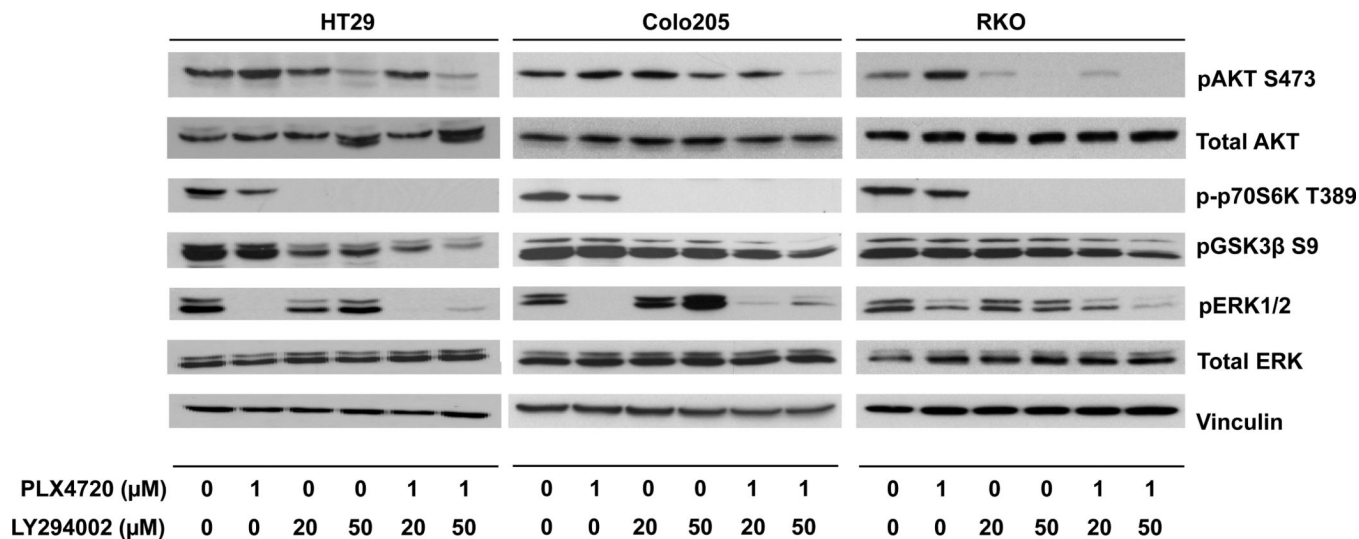
**A.** Panel of cell lines was treated with 1  $\mu$ M PLX4720, and growth relative to baseline was assessed at 72 h. Zero percent represents no growth of the treated cells from 0 h to 72 h, while 100% represents the same number of treated cells as untreated cells after 72 h. Values less than zero represent a reduction in treated cell number from 0 h to 72 h. CRC cell lines with intact PIK3CA and PTEN are shown in black, while lines with either PTEN null and/or PIK3CA mutations are shown in dark gray. Cell lines with either PTEN null or PIK3CA are less sensitive to PLX4720 than those cell lines without these PI3K/AKT pathway activating mutations ( $P = 0.03$  by Mann-Whitney signed-rank test). CRC cell lines without BRAF mutation are insensitive to this PLX4720, as shown in light gray. Melanoma cell lines are shown for reference. **B.** HT29, Colo205, and Vaco5 cells were treated with transfection reagent alone (control) or together with PTEN siRNA (50 nM) or nontarget siRNA for 48 h and then lysed for immunoblotting. **C.** Cells were treated with PTEN siRNA for 48 h, and then treated with 0.25  $\mu$ M PLX4720 for two weeks. HT29 contains an atypical and poorly characterized mutation (P449T) outside of the hotspots in the kinase and helical domains.



**Figure 3. Combination therapy with BRAF and PI3K/AKT inhibitors is synergistic**

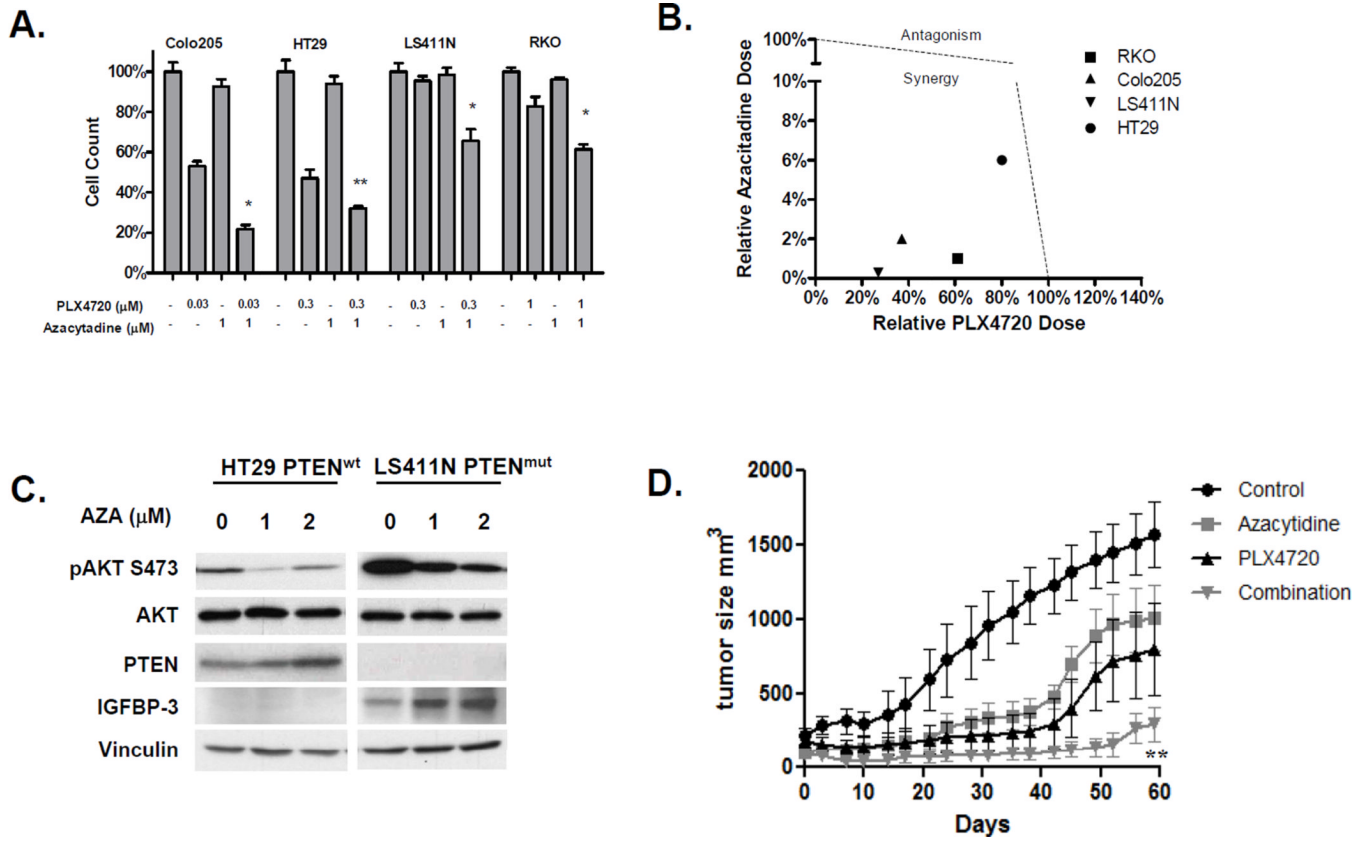
**A.** HT29 cells were treated with PLX4720 (0.15  $\mu$ M) alone, or in combination with the PI3K inhibitors LY294002 (5  $\mu$ M) or GDC0941 (0.5  $\mu$ M) for 72 h and cell count was assessed. The data represent mean values  $\pm$ SD (\*represents  $P < 0.001$  compared to either single agent). **B.** Isobologram demonstrates synergy of the combination of PLX4720 and PI3K inhibitors in CRC cell lines. Regions to the bottom left of the figure represent increasing degrees of synergy. **C.** Cells were incubated for 24 h with PLX4720 (1  $\mu$ M), LY294002 (15  $\mu$ M) or their combination, prior to cell cycle analysis. The data represent mean values from three independent experiments. **D.** Efficacy of MK2206 combined with PLX4720 on established LS411N xenografts is greater than either agent alone. Mice were administered PLX4720 chow (TGI 20%), MK2206 120 mg/kg by oral gavage three times a week (TGI 31%), or a combination (TGI 62%) (\* $P < 0.01$  compared to PLX4720 alone; # $P < 0.05$  compared to MK-2206 alone).





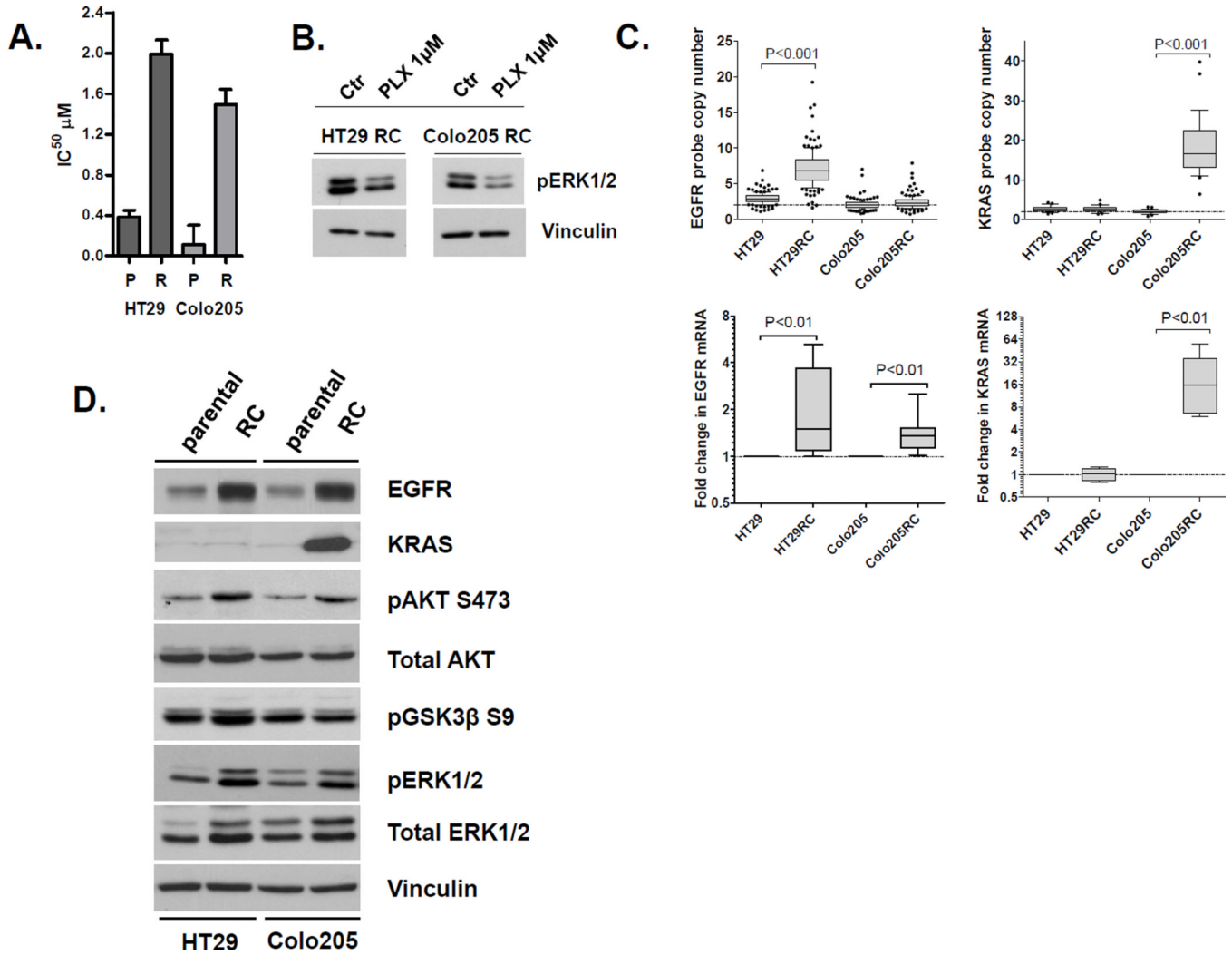
**Figure 4. Effect of PLX4720 and LY294002 on expression of signal proteins**

HT29, Colo205, and RKO cells were treated for 2 h with PLX4720, LY294002 or their combination at indicated concentrations. Cell lysates were subjected to western blot analysis with antibodies against phosphorylated or total forms of AKT or ERK1/2, and antibodies against p-P70S6K, pGSK3β or vinculin.



**Figure 5. Combination of PLX4720 and a demethylating agent**

**A.** Cell lines were treated as indicated for 72 h. Combination therapy with 5-azacytidine and PLX4720 resulted in greater cytotoxicity than either treatment alone. (\* P < 0.01 compared with PLX4720 alone) **B.** The combination of 5-azacytidine and PLX4720 demonstrates formal synergy for the four cell lines evaluated. **C.** HT29 and LS411N cells were treated with 5-azacytidine for 72 h before analysis. Phospho-AKT (S473), PTEN, and IGFBP-3 expression was assessed by immunoblot. **D.** Combination therapy with PLX4720 and 5-azacytidine demonstrates activity in HT29 xenografts. HT29 cells were pretreated with 1 μM 5-azacytidine or PBS (Control) prior to injection, followed by treatment of established tumor with PLX4720 chow, 5-azacytidine, or the combination, as described in the methods. Dual administration of PLX4720 and 5-azacytidine showed greater growth inhibition (TGI 83%) than either PLX4720 (TGI 55%, P<0.05) or 5-azacytidine alone (TGI 36%, P< 0.05).



**Figure 6. Secondary resistance to PLX4720 is associated with increased expression of EGFR and activated AKT**

**A.** HT29 and Colo205 parental (P) and resistant (R) cells were plated overnight and then treated with various doses of PLX4720. Cells were trypsinized 72 h later, and viable cells were counted. (See also Supplemental Figure 4) **B.** Resistant clones (RC) of HT29 and Colo205 were cultured in drug-free media for 24 hours, then treated with 1  $\mu M$  PLX4720 for 2 hours. **C.** Individual gene probes from a SNP tiling array for EGFR ( $n=145$  probes) and KRAS ( $n=27$  probes) were determined for HT29, Colo205, and their resistant clones. mRNA levels for EGFR and KRAS in the resistant clones were obtained as described in the methods and normalized to mRNA levels in the parental lines (which were set to a value of 1) **D.** Resistant clones of HT29 and Colo205 were cultured in drug-free medium for at least 24 h, and then cell lysates were prepared and subjected to immunoblot analysis to show expression of indicated proteins.