

Context-dependent roles of mutant B-Raf signaling in melanoma and colorectal carcinoma cell growth

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Abstract

Mutational activation of Ras and a key downstream effector of Ras, the B-Raf serine/threonine kinase, has been observed in melanomas and colorectal carcinomas. These observations suggest that inhibition of B-Raf activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase kinase (MEK) and the extracellular signal-regulated kinase MAPK cascade may be an effective approach for the treatment of *RAS* and *B-RAF* mutation-positive melanomas and colon carcinomas. Although recent studies with interfering RNA (RNAi) and pharmacologic inhibitors support a critical role for B-Raf signaling in melanoma growth, whether mutant B-Raf has an equivalent role in promoting colorectal carcinoma growth has not been determined. In the present study, we used both RNAi and pharmacologic approaches to further assess the role of B-Raf activation in the growth of human melanomas and additionally determined if a similar role for mutant B-Raf is seen for colorectal carcinoma cell lines. We observed that RNAi suppression of mutant B-Raf (V600E) expression strongly suppressed the anchorage-dependent growth of *B-RAF* mutation-positive melanoma, but not colorectal carcinoma, cells. However, the anchorage-independent and tumorigenic growth of *B-RAF* mutation-positive colorectal carcinomas was dependent on mutant B-Raf function. Finally, pharmacologic inhibition of MEK and Raf was highly effective at inhibiting the growth of *B-RAF* mutation-positive melanomas and colorectal carci-

noma cells, whereas inhibitors of other protein kinases activated by Ras (AKT, c-Jun NH₂-terminal kinase, and p38 MAPK) were less effective. Our observations suggest that Raf and MEK inhibitors may be effective for the treatment of *B-RAF* mutation-positive colorectal carcinomas as well as melanomas. [Mol Cancer Ther 2007;6(8):2220–9]

Introduction

Mutational activation of Ras (H-Ras, K-Ras, and N-Ras) is associated with a diverse spectrum of human cancers (1). For example, 50% of colorectal carcinomas harbor mutated *K-RAS*, and 25% of melanomas contain mutated *N-RAS* alleles. Consequently, there is considerable interest and effort in the development of anti-Ras strategies for cancer treatment (2, 3). One approach involves the inhibition of Ras-mediated signal transduction. Of these efforts, inhibitors of signaling mediated by the Ras effectors, the Raf serine/threonine kinases (c-Raf-1, A-Raf, and B-Raf), have attracted the most interest. Ras promotes Raf activation, which in turn, activates the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK) 1 and MEK2 dual-specificity protein kinases. MEK1/2 kinases then activate the ERK1 and ERK2 mitogen-activated protein kinases and inhibitors of this cascade have been developed and are currently under evaluation in clinical trials (4). These include kinase inhibitors of MEK [PD0325901 and ARRY-142886 (also called AZD6244)] and Raf (BAY 43-9006; sorafenib), with sorafenib approved recently for the treatment of renal cell cancers, a cancer where neither *RAS* or *B-RAF* mutation is seen (5). Although the Raf-MEK-ERK cascade represents an attractive therapeutic target, one possible complication of targeting Ras-Raf-MEK-ERK downstream signaling is the fact that Ras also uses non-Raf effectors [phosphatidylinositol 3-kinases (PI3K) and Ral guanine nucleotide exchange factors, Tiam1 and phospholipase C ϵ] to mediate transformation (6). Hence, whether blocking both Raf-dependent and Raf-independent signaling may be required to effectively block Ras-mediated oncogenesis remains to be determined.

The importance of Raf signaling in Ras transformation is supported by recent identification of mutationally activated and transforming *B-RAF* in human cancers (7). The highest frequency of *B-RAF* mutations is found in melanomas (30–60%) and colorectal adenocarcinomas (4–22%; refs. 8–17). Interestingly, *RAS* mutations are also found in melanomas (~25%) and colorectal carcinomas (34–51%), but in nonoverlapping occurrence with *B-RAF* mutations. This suggests that mutational activation of Ras and Raf may serve functionally equivalent roles in these tumor types. However, differences have been reported for the tumor types associated with each mutant gene. For

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example, *B-RAF* mutations are more commonly seen in colorectal carcinomas with mismatch repair-deficient tumors (MSI; 31–45%) than with mismatch repair-proficient tumors (MSS; 5–12%; refs. 8–18). In contrast, *K-RAS* mutations are associated with both MSI (7–43%) and MSS (32–59%). Colorectal carcinomas with *B-RAF* mutations are associated with early Duke's tumor stages, but this association was not seen with *K-RAS* mutations (10). Other studies also associated *B-RAF* mutations with distinct clinical features or outcome (16, 19). A recent study using defined genetic alterations found that B-Raf activation alone did not substitute for Ras activation to cause growth transformation of primary human melanocytes (20). Another study found that *B-RAF*, but not *RAS*, mutation status of human tumor cell lines predicted sensitivity to MEK inhibition of growth (21). Hence, whether mutational activation of Ras and B-Raf serves functionally equivalent roles in tumor growth and development remains unresolved.

Cancer development is a multistep process involving genetic alteration of six or more genes (22). For example, mutations in *K-Ras* or *B-Raf* occur early in colon cancer progression, at the adenoma stage (10), with subsequent genetic mutations required for full progression to the malignant state. Therefore, a key issue in the use of anti-Ras or anti-Raf strategies is whether these mutant genes serve a hit-and-run role, or alternatively, are still required for supporting the aberrant growth of *RAS* or *B-RAF* mutation-positive tumor cells. There is strong evidence that aberrant Ras and Raf functions are required for tumor maintenance. For example, one study used homologous recombination to delete the mutant *K-RAS* allele from DLD-1 and HCT-116 colorectal carcinoma cell lines (23). Loss of mutant Ras expression impaired anchorage-independent growth and tumor formation in nude mice. Similarly, interfering RNA (RNAi) suppression of mutant *N-RAS* expression caused a reduction in the anchorage-dependent growth in *N-RAS* mutation-positive melanomas cells (24). Likewise, RNAi suppression of mutant B-Raf(V600E) expression showed that continued mutant B-Raf protein expression is required to maintain the transformed and tumorigenic growth of human melanomas. Hingorani et al. (25) showed that use of short hairpin RNAi for stable suppression of mutant B-Raf expression in the WM793 melanoma cell line caused a reduction in ERK activity, morphologic reversion to a flatter, less refractile morphology, a reduced rate of DNA synthesis, and increased levels of apoptosis. Similarly, transient RNAi suppression of mutant B-Raf(V600E) in UACC 903 and 1205 Lu human melanoma cell lines reduced ERK activity and tumor formation when inoculated into nude mice (26). Transient RNAi expression using lentivirus vectors to suppress mutant B-Raf(V600E) expression in eight *B-RAF*(V600E) mutation-positive human melanoma cell lines reduced ERK activity, growth on plastic, invasion through Matrigel (A375mel and 928mel), and tumor formation in severe combined immunodeficient mice (A375mel) but did not significantly affect apoptosis (27). Finally, a recent study showed that inducible RNAi

suppression of BRAF in melanoma xenografts caused inhibition of tumor progression and/or regression (17). Whether mutant B-Raf is also essential for colorectal carcinoma growth has not been determined.

Pharmacologic inhibitors of Raf or MEK have also been used to assess the importance of mutant B-Raf in melanoma growth. Sharma et al. (26) found that treatment of melanoma cells with the BAY 43-9006 Raf kinase inhibitor in culture, followed by inoculation into mice, caused a reduced rate of tumor formation. *In vivo* treatment also reduced the rate of tumor growth of UACC 903 and 1205 Lu cells. Another study found that BAY 43-9006 treatment of melanoma cells (A375, A2058, and SKMEL5) induced apoptosis (28). In an additional study, BAY 43-9006 treatment also effectively blocked the tumorigenic growth of *B-RAF* (COLO 205 and HT-29) as well as *K-RAS* mutation-positive colon carcinoma cell lines (29). However, because BAY 43-9006 is a potent inhibitor of a variety of other kinases, the results with this inhibitor may not accurately define the role of mutant B-Raf activation of ERK in tumor growth. Nevertheless, studies with CI-1040, a highly specific inhibitor of MEK1/2, also support the critical role of mutant B-Raf in melanoma growth (21). All *B-RAF*(V600E) mutation-positive melanoma (SK-MEL-28, SK-MEL-3, SK-MEL-19, SK-MEL-1, and MALME-3M) and one colon carcinoma (COLO 205) cell line showed reduced viability on plastic after a 24-h treatment with CI-1040 that corresponded with a reduction in ERK activity. In contrast, tumor cell lines that lacked *B-RAF* mutations show limited or no inhibition by CI-1040 treatment. However, this study did not evaluate the consequence of MEK inhibition on other growth properties of tumor cells. Therefore, the effectiveness of MEK inhibition in blocking the growth of *B-RAF* mutation-positive tumor cells remains to be rigorously evaluated.

Establishing the role of mutant Ras and Raf in melanoma and colorectal carcinoma growth is important to validate the use of pharmacologic inhibitors of Raf-MEK-ERK signaling for the treatment of these cancers. In the present study, we have used both RNAi and pharmacologic inhibitors to evaluate the role of aberrant Raf signaling in the growth of *B-RAF* mutation-positive melanomas and colorectal carcinoma cell lines. We found that mutant B-Raf contributes to different facets of growth of these two types of cancer cells and that inhibition of MEK and Raf was effective at inhibiting the anchorage-independent growth of *B-RAF* mutation-positive melanomas and colorectal carcinoma cells.

Materials and Methods

Constructs and Cell Lines

B-RAF cDNA sequences encoding V600E, V600R, V600K, and D594E mutations were generated by site-directed mutagenesis with the Quick Change kit (Stratagene) using pcDNA3 B-Raf [wild-type (WT); provided by P. Stork, Oregon Health Sciences University, Portland, OR] as template. All *B-RAF* cDNA sequences were verified by

sequence analyses and subcloned into the retroviral expression vector pBabe-puro. Short hairpin RNAi constructs were generated in the mammalian expression vector pSUPER.retro.puro (OligoEngine) as follows: N19 = 5'-GCTACAGAGAAATCTCGAT-3' (for RNAi specific for the mutant *B-Raf* V600E transcript) and N19 = 5'-GGCT-TCCCGAGACTGATAA-3' (for a random negative control sequence). Each N19 sequence is separated by a nine-nucleotide noncomplementary spacer (TTCAAGAGA) followed by the reverse complement of the same N19 sequence.

293T cells were grown in DMEM supplemented with 10% calf serum and transfected by the calcium phosphate precipitation method (30). Melanoma cell lines were as follows: SK-MEL-24, SK-MEL-28, and SK-MEL-31 cells were purchased from American Type Culture Collection and grown in MEM supplemented with 10% FCS and 1× nonessential amino acids; MEL 505 cells (kindly provided by A. Sakko, Karolinska Institute, Stockholm, Sweden) were grown in RPMI 1640 containing 10% FCS. It should be noted that the SK-MEL-31 cells were reported previously by Davies et al. (8) as B-RAF WT. However, our sequence analysis of this cell line with DNA isolated from a P2 passage from cells obtained directly from American Type Culture Collection showed it to be *B-Raf* V600E mutation positive as reported by others (31). Colorectal carcinoma cell lines obtained from American Type Culture Collection (provided by L. Tillitson, Rapid City Regional Hospital, Rapid City, SD) were as follows: COLO 205 and COLO 320 HSR cells were grown in RPMI 1640 and Caco-2 cells in DMEM and supplemented with 10% FCS. The mutational status of *B-RAF* exon 15 and *N-RAS* at codons 12,13, 18, and 61 was determined in all melanoma cell lines as described previously (32). *K-RAS* mutational status of all colorectal carcinoma cell lines was determined previously (33).

Growth Assays

For growth assays, mass populations of cells stably infected with the pSUPER.retro.puro empty vector, or expressing RNAi for *B-Raf* V600E, or a random sequence were generated by retroviral infection using the triple transfection pVPack vector system of Stratagene. Briefly, 293T cells were transfected with 3 µg each pVPack-GagPol, pVPack-Ampho, and the indicated pSUPER.retro.puro construct and the resulting retrovirus was used to infect target cells, which were then selected with 1 µg/mL puromycin to establish mass populations of stably infected cells. To assess growth rates, cells were seeded at 1×10^5 cells/60-mm plate in triplicate and counted with a hemacytometer on days 1 to 5 after plating. To assess colony formation in soft agar, cells were seeded at 2×10^3 to 5×10^3 cells per six-well plate in growth medium containing 0.3% agar over a base layer of 0.6% agar. Verification that colonies were viable at the time of quantification was done by staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Thiazolyl blue, Sigma) after 21 days in culture. Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

was dissolved at 2 mg/mL in PBS and 0.5 mL was gently washed over colonies that were counted and photographed after incubation at 37°C for 15 min.

Analyses of the ability of pharmacologic inhibitors of Raf or MEK1/MEK2 to inhibit colony formation were done by plating single-cell soft agar suspensions of COLO 205 or SK-MEL-28 cells in 0.3% agar-containing growth medium supplemented with vehicle (DMSO) or with different inhibitors of the Raf-MEK-ERK and related mitogen-activated protein kinase cascades: 30 µmol/L of the MEK1 and MEK2 kinase inhibitor U0126 (Promega), 20 µmol/L of the Raf kinase inhibitor BAY 43-9006, 10 µmol/L of the c-Jun NH₂-terminal kinase inhibitor SP600125, 10 µmol/L of the p38 kinase inhibitor SB203580, and 20 µmol/L of the MCP110 inhibitor of Ras interaction with Raf (29, 34, 35). Although the exact mechanism of action of MCP110 has not been established, the inability of this compound to block Ras activation of AKT supports the possibility that it interacts with Raf (36). MCP122 is a weakly active derivative of MCP110 that was included as a control (20 µmol/L) for specificity. Additionally, because we have found that the PI3K-AKT serine/threonine kinase pathway is frequently activated in *B-Raf* mutation-positive melanomas,⁵ we also evaluated the activity of the PI3K lipid kinase inhibitor LY294002 at 10 µmol/L. Colony formation was quantified after 3 weeks and the average number of colonies from plates in triplicate was determined.

Tumorigenicity Assays

Tumorigenicity was determined by generation of a single-cell suspension of 2×10^6 cells in 100 µL PBS that was inoculated s.c. into each flank of athymic nude mice. Tumor volume was measured at intervals by caliper measurement in three dimensions and calculated by multiplying length × width × height × 0.5236. On reaching a tumor size of 1 cm³, animals were sacrificed by cervical dislocation and the tumors were excised and snap frozen in liquid nitrogen.

Western Blot Analysis

Subconfluent, exponentially growing cultures of cells were harvested in a buffer containing 0.5× PBS, 1% NP40, 50 mmol/L sodium fluoride, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium pyrophosphate, 1 mmol/L EDTA, phosphatase inhibitor cocktail 1 and 2 (Sigma) at 1 µg/mL, and 25 µg/mL each of aprotinin and leupeptin. Protein concentrations were measured by Lowry assay (Bio-Rad Laboratories). Unless otherwise noted, all blots were analyzed with 30 µg of soluble lysate separated over SDS-polyacrylamide gels, transferred to Immobilon-P (Millipore) membranes, and immunoblotted with antibody to *B-Raf* (Upstate), *A-Raf* (Calbiochem), *c-Raf-1* (Santa Cruz Biotechnology), phospho-ERK1/2 (Cell Signaling), ERK1/2 (Santa Cruz Biotechnology), β-actin (clone AC-15; Sigma),

⁵ Unpublished data.

or Ras (Calbiochem) and detected with appropriate anti-mouse or antirabbit horseradish peroxidase-conjugated secondary antibody (Amersham) by enhanced chemiluminescence (Amersham).

Results

Persistent B-Raf(V600E) Expression Is Critical for the Anchorage-Dependent Growth of Melanoma but not Colorectal Carcinoma Cells

Recent studies showed that suppression of B-Raf V600E expression by RNAi inhibited the proliferation of *B-RAF* mutation-positive melanoma cells (25–27), where *B-RAF* mutations are more prevalent than *RAS* mutations. Whether mutant B-Raf is also critical for colorectal

carcinoma cell growth, where *RAS* mutations are more prevalent than *B-RAF* mutations, has not been determined. Because different consequences for cell growth were observed in previous melanoma studies, where different modes of RNAi delivery were used, we also did parallel analyses of two additional melanoma cell lines (SK-MEL-28 and SK-MEL-31), to compare the role of mutant B-Raf in these two different tumor types. Additionally, this allowed us to establish the role of mutant B-Raf in cell lines for comparison with the biological consequences seen with pharmacologic inhibitors of the Raf-MEK-ERK pathway.

Short hairpin RNAi constructs were generated in the mammalian retroviral expression vector pSUPER.retro for suppression of mutant B-Raf(V600E) expression (*BRAF*V600E RNAi). We first evaluated the ability and

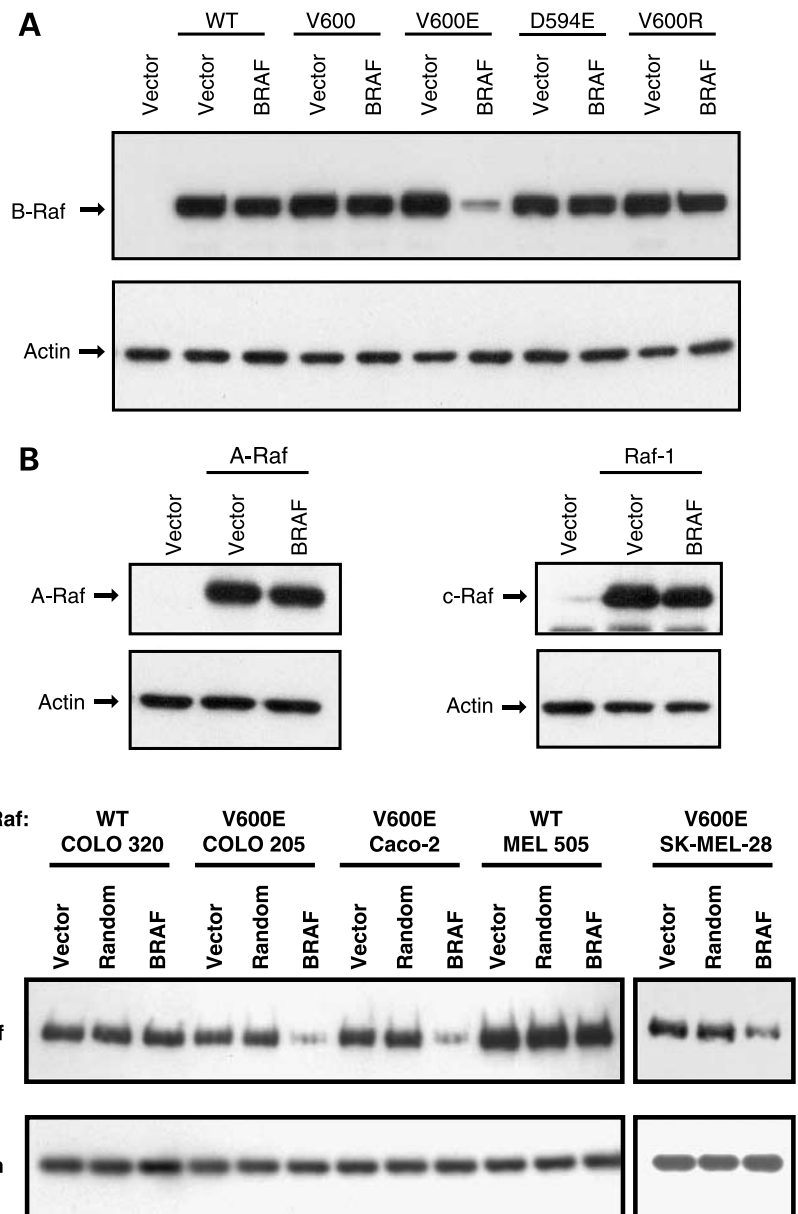


Figure 1. Preferential suppression of mutant B-Raf(V600E) protein expression by retrovirus expression of short hairpin RNAi. 293T cells were transiently cotransfected with pSUPER.retro.puro empty vector (*Vector*) or pSUPER.retro.puro expressing B-RAF V600E RNAi (*BRAF*) along with expression vectors of pBabe-puro encoding (A) WT or mutant (V600K, V600E, D594E, or V600R) B-Raf or (B) WT A-Raf, c-Raf-1, or empty vector (*Vector*). Total cell lysates (30 μ g) were resolved by SDS-PAGE and immunoblotted with (A) anti-B-Raf serum or (B) anti-A-Raf or anti-c-Raf-1 serum. Filters were reblotted with anti-actin serum to verify equivalent levels of total protein. To evaluate stable suppression of endogenous B-Raf(V600E) expression, total cell protein lysates (30 μ g) from (C) colorectal carcinoma and melanoma cells stably infected with the pSUPER.retro.puro empty vector (*Vector*) or containing a random sequence (*Random*) or specific for B-RAF V600E RNAi (*BRAF*) were resolved by SDS-PAGE and immunoblotted with anti-B-Raf or anti-actin serum as a control for loading of equivalent total protein. Data are representative of three independent experiments.

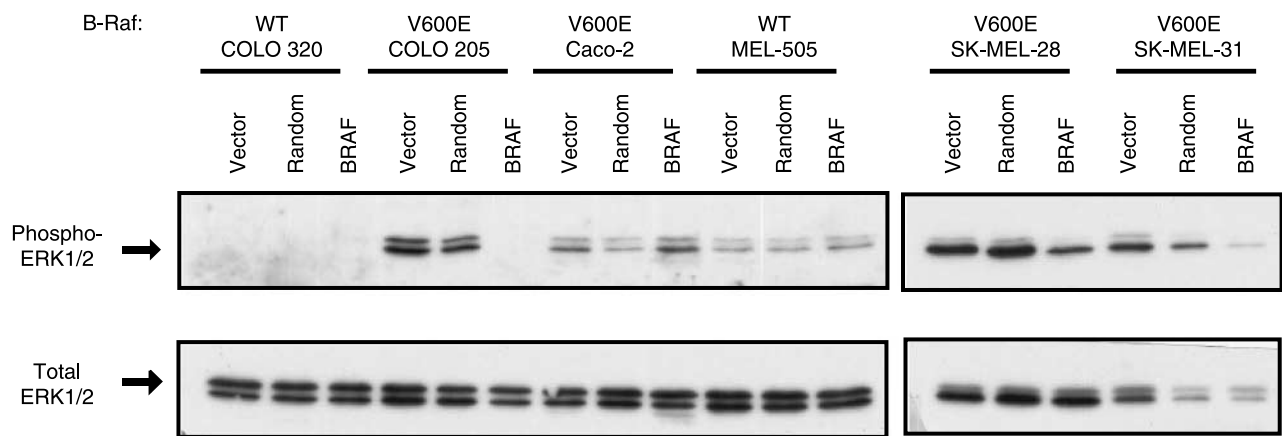


Figure 2. ERK activation is dependent on mutant B-Raf(V600E) expression in some, but not all, human tumor cell lines. Mass populations of *B-RAF* V600E mutation-positive human colorectal carcinoma (COLO 205 and Caco-2) and melanoma (SK-MEL-28 and SK-MEL-31) stable cell lines were generated by infection with pSUPER.retro.puro empty vector (*Vector*), pSUPER.retro.puro expressing *B-RAF* V600E RNAi (*BRAF*), or a randomized insert (*Random*). Parallel cultures of *B-RAF* mutation-negative colorectal (COLO 320 HSR) and melanoma (MEL-505) cell lines were infected with retroviruses in a similar fashion. All cell lines were serum starved in 0.1% FCS for 24 h and protein lysates (30 μ g) were resolved over SDS-PAGE and immunoblotted with anti-phospho-ERK1/2 or anti-total ERK1/2 serum as a control for equivalent total protein loading.

specificity of the BRAFV600E RNAi to suppress B-Raf (V600E) protein expression in 293T cells. As shown in Fig. 1, coexpression of BRAFV600E RNAi with WT or mutant B-Raf expression vectors showed significant inhibition of mutant B-Raf(V600E), but not WT B-Raf, protein expression. Furthermore, no suppression of other B-Raf mutant proteins (V600K, D594E, and V600R) or the closely related WT A-Raf or c-Raf-1 isoforms was seen. These data show the specificity of the BRAFV600E RNAi construct for selective suppression of mutant B-Raf(V600E) protein expression.

To determine the dependence of mutation-positive tumor cell lines on the continued expression of B-Raf(V600E), we established mass populations of cell lines stably infected with the empty pSUPER.retro vector, or expressing RNAi specific for B-Raf(V600E), or a scrambled, random control sequence in three melanoma (SK-MEL-28, SK-MEL-31, and MEL 505) and three colorectal carcinoma (COLO 205, Caco-2, and COLO 320 HSR) tumor cell lines. Two cell lines in each group were *B-RAF*(V600E) mutation positive with the third being *B-RAF* WT to serve as a control. After infection and selection with puromycin, mass populations of drug-resistant cells were pooled together for analyses. Efficient selective suppression of endogenous mutant (V600E) but not WT B-Raf protein expression was seen in colorectal carcinoma and melanoma cells expressing BRAFV600E RNAi, but not a randomized insert or the empty vector (Fig. 1C; data not shown).

We first determined the consequences of suppression of mutant B-Raf(V600E) expression on ERK activation under reduced serum conditions. BRAF(V600E) RNAi expression caused a selective reduction in the level of phosphorylated and activated ERK in the *B-RAF*(V600E) mutation-positive, but not WT, melanomas (Fig. 2). BRAF(V600E) RNAi also reduced ERK activity in the *B-RAF*(V600E) mutant COLO 205 cells but, surprisingly, not in the *B-RAF*(V600E)

mutant Caco-2 cells. Because mutant B-Raf protein expression was suppressed in these cells, this suggests that the ERK activation is not dependent on B-Raf activation in the Caco-2 cells. Finally, the COLO 320 HSR cells, which contain WT *B-Raf* and WT *K-Ras*, lacked detectable ERK activity under serum starvation conditions.

We found that suppression of mutant B-Raf expression in the melanoma cells caused a more flattened and less refractile morphology and the cells were strongly inhibited in their ability to grow on plastic (Fig. 3A). We were not successful in establishing proliferating populations of SK-MEL-28 or SK-MEL-31 cells where B-Raf(V600E) expression was stably suppressed. In comparison, the morphology and growth rate of the *B-RAF* WT control cell line (MEL 505) were not significantly affected by the expression of BRAFV600E RNAi, indicating that these effects were specific to suppression of mutant B-Raf(V600E) expression. In contrast, the morphology and growth of the *B-RAF* mutation-positive colorectal tumor cell lines (COLO 205 and Caco-2) were unchanged by the expression of BRAFV600E RNAi. The morphology and growth rate of the COLO 320 HSR *B-RAF* WT control cell line were also not significantly affected by the expression of BRAFV600E RNAi. A comparison of the growth rates of the colorectal carcinoma cell lines was done and we found that suppression of mutant B-Raf expression did not alter the growth rate of either WT or mutant *B-RAF* colorectal carcinoma cells (Fig. 3B). These data suggest that melanoma cells are significantly more dependent than colorectal carcinoma cells on the continued expression of B-Raf (V600E) for anchorage-dependent growth.

Persistent B-Raf(V600E) Expression Is Critical for the Anchorage-Independent and Tumorigenic Growth of Melanoma and Colorectal Carcinoma Cells

We next determined the contribution of mutant B-Raf(V600E) expression to the anchorage-independent

growth of colorectal carcinoma and melanoma cells. Consistent with the impaired anchorage-dependent growth of melanoma cells, the anchorage-independent colony formation of the *B-Raf* mutant SK-MEL-28 cells was also reduced (91%) when B-Raf(V600E) expression was suppressed (Fig. 4). In contrast to anchorage-dependent growth, the anchorage-independent growth of *B-RAF* mutant COLO 205 and Caco-2 colorectal carcinoma cells in the presence of B-Raf(V600E) RNAi reduced colony formation by 58% and 90%, respectively, whereas the *B-Raf* WT control colorectal carcinoma cell line (COLO 320 HSR) showed no change in growth when compared with control cells. Thus, B-Raf activity is critical for the anchorage-independent growth of both colorectal carcinoma and melanoma cells.

We next evaluated the importance of mutant *B-Raf* for the tumorigenic growth potential of colorectal carcinoma cells. As shown in Fig. 5, the flanks of mice receiving COLO 205 cells infected with the empty vector showed rapid, linear

growth of tumors, whereas COLO 205 cells expressing BRAFV600E RNAi showed no evidence of tumor growth for up to 21 days. In contrast, although the tumorigenic growth of Caco-2 cells was not abolished by expression of BRAFV600E RNAi, a significantly reduced rate of tumor formation was seen. These data strongly suggest that, as reported previously for melanomas (26), mutant B-Raf activity contributes significantly to the tumorigenic growth of *B-RAF*(V600E) mutation-positive colorectal carcinoma cells.

Pharmacologic Inhibition of Raf-MEK-ERK Signaling Impairs the Anchorage-Independent Growth of Colorectal Carcinoma and Melanoma Cells

The analyses with RNAi showed that mutant B-Raf expression was critical for the anchorage-independent growth of COLO 205 and SK-MEL-28 cells. Therefore, we next determined if pharmacologic inhibition of B-Raf signaling would effectively impair the soft agar growth of these two *B-RAF* mutation-positive cell lines. We used the

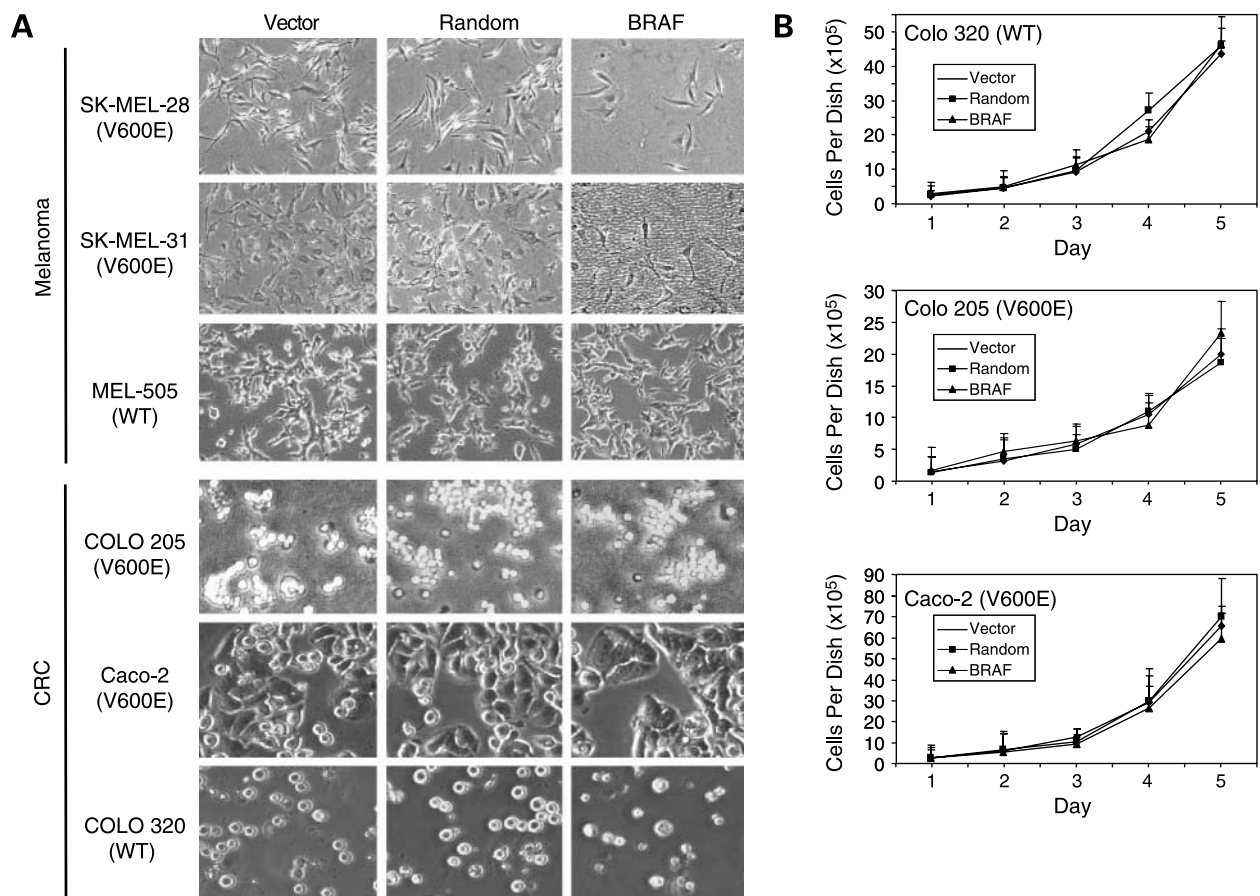


Figure 3. Melanoma, but not colorectal carcinoma, tumor cell lines are dependent on continued expression of mutant B-Raf(V600E) for anchorage-dependent growth. **A**, the growth and morphology of mass populations of *B-RAF*(V600E) mutation-positive colorectal carcinoma (COLO 205 and Caco-2) and melanoma (SK-MEL-28 and SK-MEL-31), or *B-RAF* WT colorectal carcinoma (COLO 320 HSR) and melanoma (MEL 505) cells stably infected with the pSUPER.retro.puro empty vector (*Vector*) or randomized insert (*Random*) were compared with those infected with pSUPER.retro.puro expressing B-RAF RNAi (*BRAF*). Inhibition of B-Raf(V600E) protein expression inhibits anchorage-dependent growth of *B-RAF*(V600E) mutation-positive melanoma cells but not mutation-positive colorectal carcinoma cells. Photomicrographs of stable cell lines at $\times 400$ magnification (**A**) and growth curves of colorectal carcinoma cell lines stably infected with the indicated vector (**B**). Data are representative of three independent experiments.

highly specific MEK1 and MEK2 inhibitor U0126 (37, 38) and two Raf inhibitors. Although sorafenib (BAY 43-9006) is an inhibitor of Raf, it also potently inhibits other protein kinases (26, 28, 29, 39). MCP110 has been shown to block Ras activation of Raf and a structurally related compound, MCP122, is only weakly active and serves as a negative control for this activity (36, 40, 41). We also evaluated the ability of pharmacologic inhibitors of the related p38 (SB203580) and c-Jun NH₂-terminal kinase (SP600125) mitogen-activated protein kinase cascades to impair the growth of these cells (42, 43). Finally, the PI3K-AKT pathway is frequently up-regulated in *B-RAF* mutation-positive melanomas (44). Therefore, we also determined if treatment with the PI3K inhibitor LY294002 would impair cell growth (45).

As shown in Fig. 6, colony formation of COLO 205 cells was completely blocked by U0126 inhibition of MEK1/2, whereas the growth of SK-MEL-28 cells was reduced by 63%. In contrast, sorafenib (BAY 43-9006) treatment caused complete inhibition of both COLO 205 and SK-MEL-28 soft agar growth. MCP110, but not the weakly active MCP122 compound, also strongly inhibited the growth of both cell lines. This result supports the possibility that MCP110 targets Raf, rather than Ras, and may be an effective inhibitor of mutant B-Raf function. In contrast, inhibition of PI3K, p38, or c-Jun NH₂-terminal kinase did not cause any significant growth inhibition with either cell line. Although inhibition of c-Jun NH₂-terminal kinase did reduce the size of the COLO 205 colonies, it only reduced colony numbers modestly.

Conclusion

The recent identification of mutationally activated and transforming *B-RAF* alleles in human cancers, in particular, melanomas and colorectal carcinomas, suggests that phar-

macologic inhibitors of Raf may be effective for the treatment of B-RAF mutation-positive cancers (46). Support for this is provided by RNAi studies showing that mutant B-Raf expression is critical for the growth of melanomas, where *B-RAF* mutations are seen at a frequency of approximately 30% to 60%. Whether the sustained expression of mutant *B-Raf* is also critical for colorectal carcinomas has not been determined. We used both RNAi and pharmacologic approaches to determine the importance of B-Raf activation in the growth of colorectal carcinoma and melanoma cell lines. RNAi suppression analyses determined that mutant B-Raf expression was critical for the anchorage-dependent and anchorage-independent growth of melanomas. In contrast, suppression of mutant B-Raf expression was critical for the anchorage-independent, but not anchorage-dependent, growth of colorectal carcinoma cells. Suppression of mutant B-Raf also either reduced or abolished colorectal carcinoma tumor formation. Finally, pharmacologic inhibitors of the Raf-MEK-ERK cascade also effectively impaired the growth of melanoma and colorectal carcinoma cells in soft agar. Taken together, these results support the feasibility of pharmacologic inhibitors of these signaling targets for the treatment of B-RAF mutation-positive melanomas and colorectal carcinomas.

Similar to four recent studies that used three different approaches for RNAi delivery, we found that RNAi suppression of mutant B-Raf expression greatly impaired the anchorage-dependent growth of *B-RAF* mutation-positive melanoma cell lines (25–27, 47). Additionally, we found that the anchorage-independent growth of melanoma cells was also dependent on continued B-Raf activity. Because we found that the growth of melanoma cells was drastically impaired when mutant B-Raf expression was suppressed, we were unable to propagate sufficient numbers of cells for detailed growth rate or tumor

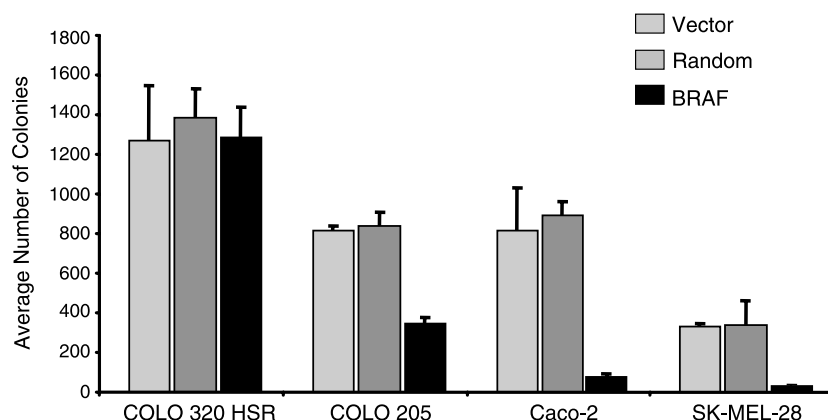


Figure 4. Human colorectal carcinoma and melanoma tumor cell lines are dependent on continued B-Raf(V600E) expression for anchorage-independent growth. Mass populations of *B-RAF*(V600E) mutation-positive human colorectal carcinoma (COLO 205 and Caco-2) and melanoma (SK-MEL-28), or *B-RAF* WT (COLO 320 HSR) cells, stably infected with the empty pSUPER.retro vector (*Vector*) or randomized insert (*Random*), were compared with those infected with pSUPER.retro.puro expressing *B-RAF* RNAi (*BRAF*) in soft agar colony formation assays and the growth of multicellular colonies was quantified on day 21 from plates in triplicate after staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Data are representative of three independent experiments. Columns, mean colony number per plate; bars, SD.

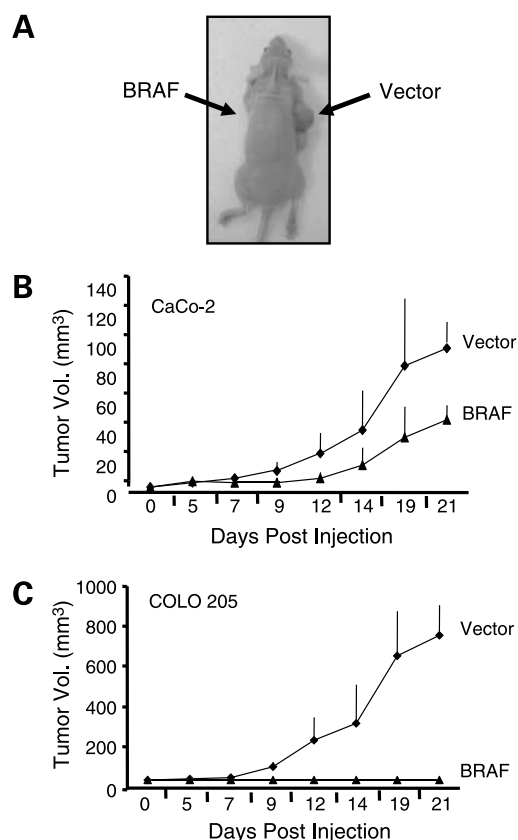


Figure 5. *B-RAF* mutation-positive colorectal carcinoma cells are dependent on continued B-Raf(V600E) expression for tumor formation in nude mice. COLO 205 or CaCo-2 cells stably infected with pSUPER.retro (*Vector*) or pSUPER.retro.puro expressing *B-RAF* RNAi (*BRAF*) were injected s.c. into the flanks of athymic nude mice and (A) monitored for the appearance of tumor growth (shown are COLO 205 cells), with (B) tumor volume determined by caliper measurement in three dimensions and calculated by multiplying length \times width \times height \times 0.5236 on the indicated days.

formation analyses. This loss of proliferative capacity may be attributed to both the inhibition of cell cycle progression and/or the increased rate of apoptosis (25, 26, 48) and may differ in different tumor cell lines (17). In contrast, we found that suppression of mutant B-Raf expression did not significantly alter the anchorage-dependent proliferation of colorectal carcinoma cells. Christensen et al. (31) showed that impaired growth on plastic due to the loss of B-Raf expression in melanoma cells could be rescued with exogenous supplementation with growth factors, suggesting that autocrine signaling is B-Raf dependent in B-Raf mutant melanomas. Thus, unlike melanoma cells, we speculate that colorectal carcinoma cells may harbor a B-Raf-independent signaling mechanism to stimulate an autocrine growth loop important for proliferation on plastic. Therefore, in contrast to melanomas, mutant B-Raf activity does not seem to be critical for the anchorage-dependent proliferation of colorectal carcinoma cells. However, suppression of B-Raf expression did impair the anchorage-independent growth *in vitro*, as well as tumor-

igenic growth *in vivo*, of both colorectal carcinoma cell lines. Thus, perhaps, mutant B-Raf contributes to a different aspect of colorectal carcinoma growth transformation, anchorage independence, which accounts for impaired tumor-forming capacity. Finally, suppression of B-Raf in CaCo-2 cells did not reduce the levels of activated ERK and this may be a consequence of activation of ERK through other upstream signaling events. Indeed, CaCo-2 cells have been reported to overexpress epidermal growth factor receptor, a known potent activator of the Ras-Raf-MEK-ERK pathway (49, 50). However, despite the persistent activation of ERK in these cells, growth in soft agar and nude mice was impaired by suppression of mutant B-Raf expression. Raf functions independent of MEK-ERK activation have been described (51–53), although the exact nature of these activities remain to be defined. Perhaps, the impaired growth of CaCo-2 cells observed with the loss of mutant B-Raf expression is a consequence of the loss of such ERK-independent functions.

Our results with RNAi suppression of mutant B-Raf (V600E) expression in COLO 205 colorectal carcinoma and SK-MEL-28 melanoma cells, which resulted in reduced ERK activity, showed that the anchorage-independent growth of these tumor cells is critically dependent on persistent ERK activation. Therefore, we used these two lines to evaluate the ability of various inhibitors of the Raf-MEK-ERK pathway to block soft agar growth. Treatment with the U0126 MEK inhibitor caused a complete block in the growth of COLO 205 cells, but only partial inhibition was seen with SK-MEL-28 cells. However, the BAY 43-9006 Raf kinase inhibitor caused a complete inhibition of soft agar growth for both cell lines. These results are consistent with studies with these inhibitors on other tumor cell types and suggest that Raf or MEK inhibitors may be effective therapeutic treatments for *B-RAF* mutation-positive colorectal carcinoma and melanomas. However, clinical trial analyses of BAY 43-9006 as monotherapy found that this inhibitor was not effective against melanomas (54). Similarly, phase II clinical trial analyses with the CI-1040 MEK inhibitor also did not show significant antitumor activity (55). Because neither inhibitor causes complete inhibition of ERK activation in patient tumors, the lack of patient response to these inhibitors may be due simply to insufficient potency.

MCP compounds were identified in a yeast two-hybrid screen for inhibitors of Ras interaction with Raf-1 (36). MCP110 functions as an inhibitor of Ras activation of Raf, whereas the related analogue MCP122 is inactive and serves as a control for the specificity of MCP110 activity. Whether MCP110 functions by association with Ras or Raf to prevent effective Raf activation remains unclear. We found that MCP110, but not MCP122, treatment strongly inhibited the soft agar growth of both COLO 205 and SK-MEL-28 cells. Our results suggest that MCP compounds are effective inhibitors of mutant B-Raf activity and may provide a distinct approach from kinase inhibitors for therapeutic treatment of *B-RAF* mutation-positive cancers.

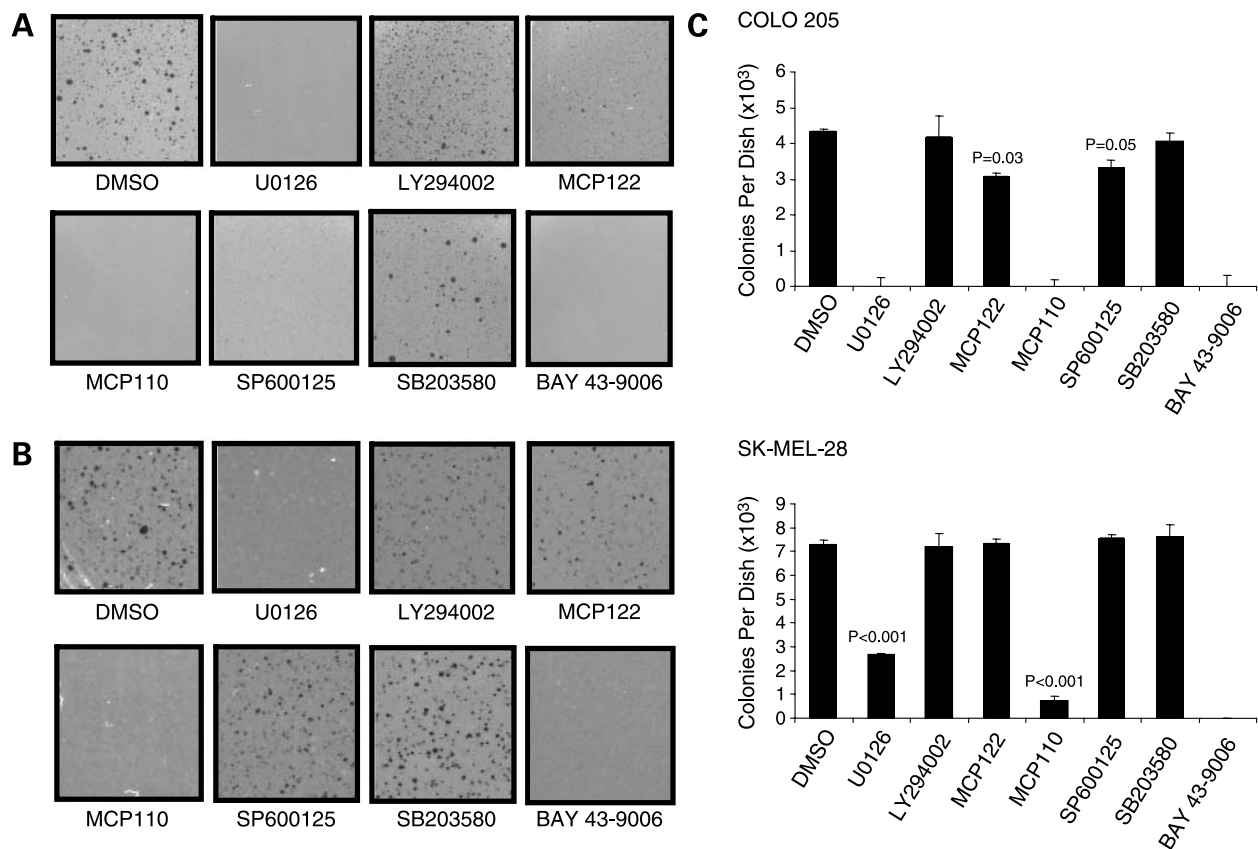


Figure 6. Differential consequences of pharmacologic inhibitors on melanoma and colorectal carcinoma growth in soft agar. Soft agar suspensions of (A) COLO 205 or (B) SK-MEL-28 were maintained in growth medium supplemented with vehicle (DMSO) or with the indicated inhibitors at concentrations described in Materials and Methods. C, colony formation was quantified after 3 wks and the average of plates in triplicate was determined. Data are representative of three independent experiments. Columns, mean colony number per plate; bars, SD.

In summary, we have used an RNAi approach to validate the importance of mutant B-Raf as a therapeutic target for colon cancer treatment. Like melanomas, B-Raf activity is important for colorectal carcinoma tumorigenic growth, although B-Raf may facilitate tumor formation by distinct mechanisms in these two cancer types. We recently determined a gene expression profile for ERK activation and dependency for melanoma cells (48). We identified 82 genes, including *TWIST1*, *HIF1 α* , and *IL-8*, which correlated with ERK levels across a panel of human melanoma cell lines, which decreased with pharmacologic MEK inhibition. We are currently doing the same analyses with colorectal carcinoma cell lines and our preliminary results suggest that the set of genes will be distinct but with overlapping patterns. These analyses may provide an indication of the mechanistic differences between B-Raf activation in these two cancer types. Additionally, whether inhibition of the Raf-MEK-ERK cascade will be equally effective for blocking the growth of Ras mutation-positive colorectal carcinomas is not clear. Our preliminary studies indicate that a subset of colorectal carcinoma cell lines is sensitive to CI-1040 MEK inhibitor treatment, as measured by growth in soft agar. However, unlike the report by Solit et al. (21), we did not find that B-RAF mutation status was

predictive of MEK inhibitor sensitivity. Therefore, our future analyses will determine if a gene expression signature may provide an accurate marker for colorectal carcinoma sensitivity to MEK inhibitor treatment.

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Context-dependent roles of mutant B-Raf signaling in melanoma and colorectal carcinoma cell growth

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