Extracellular Signal-regulated Kinase-dependent Proliferation Is Mediated through the Protein Kinase A/B-Raf Pathway in Human Uveal Melanoma Cells*

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Mutated B-Raf-mediated constitutive activation of ERK1/2 is involved in about 66% of cutaneous melanoma. By contrast, activating mutations in B-RAF are rare in uveal melanoma. This study aimed to determine the role of wild-type B-Raf (WT-B-Raf) in uveal melanoma cell growth. We used cell lines derived from primary tumors of uveal melanoma to assess the role of WT-B-Raf in cell proliferation and to characterize its upstream regulators and downstream effectors. Melanoma cell lines expressing WT-B-Raf and WT-Ras grew with similar proliferation rates, showed constitutive activation of ERK1/2, and had similar levels of B-Raf expression and B-Raf kinase activity as melanoma cell lines expressing the activating V600E mutation (V600E-B-Raf). They were equally as sensitive to pharmacological inhibition of MEK1/2 for cell proliferation and transformation as V600E-B-Raf cells. siRNA-mediated depletion of Raf-1 did not affect either ERK1/2 activation, whereas siRNA-mediated depletion of B-Raf reduced cell proliferation by up to 65% through the inhibition of ERK1/2 activation, irrespective of the mutational status of B-Raf. Pharmacological inhibition of cAMP-dependent protein kinase (PKA) and siRNA-mediated depletion of PKA greatly reduced B-Raf activity, ERK1/2 activation, and cell proliferation in WT-B-Raf cells, whereas it did not affect V600E-B-Raf cells, demonstrating a key role of PKA in mediating WT-B-Raf/ERK signaling for uveal melanoma cell growth. Moreover, inactivation or depletion of PKA did not affect Rap-1 activity, and Rap-1 depletion did not affect either B-Raf activity or ERK1/2 activation. This ruled out a role for Rap1 in the PKA-mediated B-Raf/ERK activation in WT-B-Raf cells. Finally, we demonstrated the importance of cyclin D1 in mediating PKA/WT-B-Raf signaling for cell proliferation. Altogether, our results suggest that the PKA/B-Raf pathway is a potential target for therapeutic strategies against WT-B-Raf-expressing uveal melanoma.

Constitutive activation of the Ras/Raf/extracellular signal-regulated kinase (ERK)3 signaling pathway has long been associated with cancers.

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¶ The abbreviations used are: ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase extracellular signal-regulated kinase; PKA, cAMP-dependent protein kinase; FCS, fetal calf serum; B-BCAMP; b-bromo-cyclic AMP; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PI, propidium iodide; MOPS, 4-morpholinepropanesulfonic acid; AS, antisense; ODN, oligonucleotide; GST, glutathione S-transferase.

Recently, activating point mutations in B-RAF were detected in a large scale screen for genes mutated in human cancer (1). About 80% of the B-Raf mutations seen in human cancers have the valine at position 600 in the activation segment substituted by a glutamic acid, which corresponds to the hot spot transversion T1799A mutation. Reconstitution experiments have shown that this mutation increases the activity of ectopically expressed B-Raf kinase and induces cell transformation (1, 2), whereas siRNA-mediated suppression of V600E-B-Raf abolishes cell transformation (3). This gain of function mutation in B-RAF (V600E-B-Raf) is largely responsible for the constitutive activation of ERK1/2 and is therefore for the acquisition of self-sufficiency in the growth signal in human cutaneous melanoma cells (1, 3, 4). Activating mutations in B-RAF and RAS are associated with about 60 and 20% of cutaneous melanoma, respectively (1, 5, 6). However, about 20% of cutaneous melanoma have no mutations in either B-RAF or RAS. This suggests that other molecular mechanisms activate the ERK signaling pathway. Consistent with this, the overexpression of B-Raf without gene amplification has been shown to be one of the mechanisms by which the constitutive activation of ERK1/2 stimulates cell growth in cutaneous melanoma cell lines expressing WT-B-Raf and WT-N-Ras (7). However, in melanoma cell lines that express normal levels of WT-B-Raf, no constitutive activation of ERK1/2 and no implication of ERK1/2 in cell proliferation have been seen (8). The precise effect of WT-B-Raf overexpression on the constitutive activation of ERK1/2 is still unknown, since reconstitution experiments showed that ectopically overexpressed WT-B-Raf did not activate ERK1/2 in COS cells (9). During malignant transformation, cutaneous melanoma cells also acquire the ability to overexpress growth factor receptors and to express and secrete growth factors that together activate the ERK1/2 signaling pathway. It is therefore likely that autocrine growth factor activation loops are also involved in the acquisition of autonomous growth via the constitutive activation of ERK1/2 in cutaneous melanoma (6).

Unlike cutaneous melanoma, which has been extensively studied, little is known about the molecular pathogenesis of ocular (uveal and conjunctival) melanoma. Uveal melanoma is the most common primary ocular neoplasm in adult humans in developed countries. Although cutaneous and uveal melanomas share similar histological features, mutations in the genes encoding the major signaling pathway proteins that control cell proliferation, including those affected in cutaneous melanoma, are infrequent in uveal melanoma (10–17). This is consistent with the differences in the epidemiological and cytogenetic aspects of cutaneous and uveal melanomas. We recently demonstrated a large increase in the activity of endogenous B-Raf kinase and a constitutive activation of the MEK/ERK pathway in the rare uveal melanoma cell lines that express V600E-B-Raf (10). siRNA-mediated depletion of V600E-B-Raf reduced ERK1/2 activation, cell proliferation, and transformation in
uveal melanoma cell lines, showing that oncogenic V600E-B-Raf activates a similar signaling pathway for tumorigenesis in both uveal and cutaneous melanomas (10). However, constitutive activation of ERK1/2 in the absence of mutations in RAS and B-RAF has been shown in primary tumors and cell lines of uveal melanoma, demonstrating a large difference between cutaneous and uveal melanomas (18, 19). However, the intracellular signaling responsible for the constitutive activation of ERK1/2 and the role of ERK1/2 in proliferation and transformation have not been characterized. It has been recently suggested that in the absence of mutations in RAS or B-RAF, the Ras/B-Raf signaling pathway could not be involved in uveal melanoma (20). We showed in a recent study that the c-Kit/ERK1/2 autocrine loop was activated and participated in cell proliferation and transformation of uveal melanoma cell lines, showing the potential role of ERK1/2 in uveal melanoma tumorigenesis (21). However, the contribution of the Ras and Raf signaling in controlling ERK1/2 activation and proliferation in the absence of RAS and B-RAF mutations has not been studied in uveal melanoma cells. Moreover, the heterogeneity of WT B-Raf uveal melanoma cell lines with respect to c-Kit expression and the response to glivec suggests a role for other growth factors/chemokines for cell proliferation. However, proliferation of normal uveal melanocytes is tightly controlled by exogenous growth factors, such as fibroblast growth factor 2 and hepatocyte growth factor, that exert their mitogenic activity, at least in part, through the activation of ERK1/2. Endothelins, adrenergic β2-receptor agonists such as isoproterenol and epinephrine, some prostaglandins, and various cAMP-elevating agents have also been shown together with growth factors to be growth-promoting agents of normal uveal melanocytes (22, 23). This supports the suggestion that signaling pathways other than ERK1/2, such as cyclic AMP (cAMP) and cAMP-dependent protein kinase (PKA), stimulate the proliferation of normal uveal melanocytes and possibly the autonomous growth of uveal melanoma cells. The high complexity of the cross-talk between ERK signaling and both the PKA/B-Raf pathway may be a potential target for therapeutic strategies against WT B-Raf expressing uveal melanoma.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—92.1, Mel270 (provided by Dr. M. Jager, University of Leiden, The Netherlands), OCM-1 (provided by Dr. F. Malecaze, Ophthalmology Department, CHU Toulouse, France), and TP3 (provided by Dr. S. Guerin, Centre Hospitalo-Universitaire de Quebec, Canada) cell lines were grown in RPMI 1640 medium supplemented with 5% FCS, 2.5 μg/ml fungizone/amphotericin B, 50 μg/ml gentamycin, and 2 mM l-glutamine (Invitrogen), as previously described (10, 21). Cells were cultured at 37 °C in a humidified air/CO2 (19:1) atmosphere.

**Cell Proliferation Assay**—We investigated cell proliferation by treating cells with specific pharmacological inhibitors and activators of signaling pathways. Stock solutions were made up in Me2SO with a final concentration of Me2SO in the culture media not exceeding 0.1%; this concentration has been shown to have no effect on melanoma cell proliferation. Cells were seeded in triplicate in 24-well plates at a density of 1.5 × 10^4 cells/well. The plates were incubated for 3 days and then treated with 1) the MEK inhibitor, UO126; 2) the Raf inhibitor, Raf kinase inhibitor II/BAY43-9006; 3) the Ras farnesylation inhibitor, FPT III, and FTS; 4) the PKA inhibitor, (R)-8-Br-cAMP-S; 5) the activator of Epac, 8CPT-2Me-cAMP; and 6) the cAMP analog, 8-Br-cAMP. The inhibitors were added both 2 h before induction of cell proliferation and at induction, and the activator was then added for the indicated times. The number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric method after 3 days of culture in either low serum conditions (0.5% FCS) or normal serum conditions (5% FCS). The percentage growth inhibition was calculated with respect to control Me2SO-treated cells.

**Cell Cycle Progression Analysis**—We analyzed cell cycle progression by determining the DNA content of the cells with propidium iodide (PI). Cells were washed in phosphate-buffered saline and fixed in ice-cold 70% ethanol by incubation at 4 °C for at least 2 h. The cells were rehydrated in cold phosphate-buffered saline, treated with 1 mg/ml RNase A (Roche Applied Science), and stained with 50 μg/ml PI by incubation at 4 °C for at least 15 min. The stained cells were analyzed by flow cytometry (Epics ALTRA; Beckman Coulter).

**Transformation Assay**—We analyzed cell transformation using a clonogenic assay by determining the ability of cells to form colonies in soft agar under anchorage-independent conditions. Melanoma cells were suspended in complete medium containing 0.3% agar and either pharmacological inhibitors or vehicle. The cells were then plated on a layer of 0.7% agar in complete medium in 6-well culture plates at a density of 3 × 10^3 cells/well and incubated at 37 °C for 2 weeks. Pharmacological inhibitors or vehicle were added every 3 days during the culture period. Colonies in three randomly chosen 9-cm² areas were counted on day 10 of the culture period.

**Genomic DNA Purification, PCR, and Mutation Screening**—Genomic DNA was prepared from melanoma cell lines according to standard procedures, checked by electrophoresis in 0.8% agarose gel, and quantified by spectrophotometry. We analyzed exons 1, 2, and 12 of the human HRAS, KRAS, and NRAS and exons 11 and 15 of B-Raf using specific PCR as previously described (10, 16). Briefly, PCR amplification was carried out in a total volume of 50 μl, containing 250 ng of genomic DNA, a 0.5 μM concentration of each primer, 0.2 mM each dNTP, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 1.5 mM MgCl2, and 1.25 IU of Taq polymerase (Invitrogen). DNA was denatured by heating at 95 °C for 5 min and was then amplified by 35 cycles of denaturing at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 60 s. The last cycle contained a 10-min elongation step at 72 °C. The amplified products were separated on a 2.5% agarose gel and purified with the Qiagen MiniElute purification kit before sequencing (MWG Biotech).

**Western Blot Analysis**—Cells were washed twice in phosphate-buffered saline, lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Nonidet P-40, 1% deoxycholate, 50 mM β-glycerophosphate, 0.2 mM sodium orthovanadate, 50 mM sodium fluoride, 1 μM leupeptin, 5 μM pepstatin, 20 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride) and then centrifuged for 10 min at 10,000 × g at 4 °C. Protein concentrations were determined using the Bio-Rad kit. Cell lysates were mixed with 3× Laemmli buffer and heated for 5 min at 95 °C. They were then resolved by SDS-PAGE (10 or 15% polyacrylamide gels), transferred to polyvinylidene difluoride
membrane (Immobilon™, Millipore Corp.) by electroblotting, and probed with polyclonal antibodies directed against ERK1/2 (dilution 1:1000; Cell Signaling Technology), B-Raf (dilution 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), PTEN (dilution 1:250; BD Biosciences), Sprouty2 (dilution 1:500; Upstate Biotechnology, Inc., Lake Placid, NY), Rap1 (dilution 1:600; Santa Cruz Biotechnology), RKIP (dilution 1:1000; Cell Signaling Technology), the α catalytic subunit of PKA (PKαcat), and the γ catalytic subunit of PKA (PKγcat) (dilution 1:1000; Santa Cruz Biotechnology). We used a polyclonal antibody directed against phospho-ERK1/2 (Thr202/Tyr204) (dilution 1:1000; Cell Signaling Technology) to analyze the activation of these kinases during melanoma cell proliferation. Membranes were probed with a rat monoclonal antibody directed against β-tubulin (dilution 1:4000; Serotec) or with a goat antibody directed against actin (dilution 1:1000; Santa Cruz) to control for equal loading. The primary antibodies were tagged with specific secondary horseradish peroxidase-conjugated antibodies. Antibody complexes were detected by ECL (Amersham Biosciences), and the membrane was placed against BioMax Light-1 film (Eastman Kodak Co.). Quantification was carried out using the Eastman Kodak Co. image station 2000 MM and 1D3.6 software.

Gene Silencing—Uveal melanoma cells were plated at 50–60% confluence in complete melanoma cell culture medium in 12-well plates and incubated for 24 h. Cells were then transiently transfected in Opti-MEM I medium (Invitrogen) with Lipofectamine 2000 reagent (Invitrogen) and siRNA for either WT 1-B-Raf (Eurogentec) (forward, 5′-GUG AAA UCU CGA UGG AGU GdTdT-3′; reverse, 5′-CAC UCC AUC GAG AUU UCA CdTdT-3′) targeting 1798 nucleotides downstream from the start codon or a scrambled sequence of WT B-Raf as a control (forward, 5′-AAA UGG GUG GAG CUC UUG AdTdT-3′; reverse, 5′-UCA AGA GCU CCA CCC AUU UdTdT-3′). The sequences targeting WT 1-B-Raf were 5′-GAG AAA UCU CGA UGG AGU GdTdT-3′ (forward) and 5′-CAC UCC AUC GAG AUU UCA CdTdT-3′ (reverse), and those targeting Rap1 were 5′-UGU GGC AAA UGG AUU GAG CdTdT-3′ (forward) and 5′-GCU CAU UCC AUU UCG CAC AdTdT-3′ (reverse) (Eurogentec). As a control, a nonspecific siRNA duplex containing the same nucleotides but in irregular sequence (scrambled) was used (forward, 5′-AAA UGG GUG GAG CUC UUG AdTdT-3′; reverse, 5′-UCA AGA GCU CCA CCC AUU UdTdT-3′). Cells were also transiently transfected with a mixture of siRNAs for the PKαcat and PKγcat (siRNA gene silencers; Santa Cruz Biotechnology) and siRNAs for Rap1 (siRNA gene silencers; Santa Cruz Biotechnology). After 5 h, the Opti-MEM I medium was replaced by complete culture medium. We assessed the effects on cell proliferation, cell cycle progression, and WT 1-B-Raf, v600B-Raf, Rap1, and PKα down-regulation 72 h after transfection using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, fluorescence-activated cell sorting analysis, and Western blotting, as previously described (10).

B-Raf Expression and B-Raf Kinase Activity—Uveal melanoma cell lines were cultured in overnight serum depletion medium. B-Raf was immunoprecipitated with 2.5 μl of monoclonal B-Raf antibody (dilution 1:1000; Santa Cruz Biotechnology) from extracts containing equal amounts of protein in lysis buffer supplemented with 25 mM sodium pyrophosphate. Test kinase buffer containing 20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 500 μM cold ATP, 75 mM magnesium chloride, and inactive MEK1 (Upstate Biotechnology) was added. After a 30-min incubation at 30°C, the enzymatic reaction was stopped by adding 2× Laemmli buffer. A SDS-polyacrylamide gel electrophoresis was carried out, and the proteins were then transferred to a polyvinylidene difluoro-
1D3.6 software. The relative kinase activity was determined after quantifying the amount of immunoprecipitated B-Raf by Western blotting by probing the membrane with the B-Raf antibody, as described above using the 1D3.6 software (Kodak).

PKA Activity—PKA activity was measured using a fluorescent Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) assay (PepTag nonradioactive cAMP-dependent protein kinase assay; Promega) on whole-cell extracts containing equal amounts of protein according to the supplier's instructions. Kemptide is a specific substrate of PKA. As kemptide is phosphorylated, it becomes negatively charged and migrates toward the anode on an agarose gel run at neutral pH. The phosphorylated Kemptide was visualized by UV light, and the fluorescent intensity of phosphorylated Kemptide was quantified spectrophotometrically at 570 nm.

Determination of Rap1 Activation—A GST fusion protein of the Rap-1 binding domain of RalGDS (GST-RalGDS-RBD) was a gift from Dr. J. De Gunzburg (INSERM 528, Institut Curie, France). The cell lines were cultured in serum-depleted medium for 18 h and were washed with cold phosphate-buffered saline and lysed in ice-cold lysis buffer (10% glycerol, 1% Nonidet P-40, 50 mM HEPES-Na, pH 7.4, 2.5 mM MgCl2, 200 mM NaCl, 1 mM aprotinin, 10 μM leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride). Lysates were cleared by centrifugation, and equal amounts of protein according to the supplier's instructions. Kemptide is a specific substrate of PKA. As kemptide is phosphorylated, it becomes negatively charged and migrates toward the anode on an agarose gel run at neutral pH. The phosphorylated Kemptide was visualized by UV light, and the fluorescent intensity of phosphorylated Kemptide was quantified spectrophotometrically at 570 nm.

RESULTS
Constitutive Activation of ERK1/2 and Key Role of the MEK/ERK Module in the Proliferation of Uveal Melanoma Cells Expressing WT-B-Raf in the Absence of Mutated RAS—First, we screened for mutations in exons 11 and 15 of the B-Raf gene in four uveal melanoma cell lines. We detected no mutations in exon 11 in any of the four cell lines. By contrast, we detected a single-base substitution in exon 15 in the OCM-1 and TP31 cell lines. This T1799A substitution led to the replacement of a valine by a glutamic acid at position 600 (V600E) in B-Raf. In the 92.1 and Mel270 cell lines, we did not detect mutations in exon 15. Therefore, in our subsequent experiments, we considered the OCM-1 and TP31 cell lines to be V600EB-Raf melanoma cells and the 92.1 and Mel270 cell lines to be WT-B-Raf melanoma cells. We then screened exons 1 and...
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2 of HRAS, KRAS, and NRAS for mutations in the four melanoma cell lines. We detected no mutations in any of these exons in the V600E-B-Raf-expressing melanoma cells, confirming that V600E-B-Raf and RAS mutations are mutually exclusive (data not shown). Similarly, in the two WT-B-Raf expressing melanoma cell lines, we detected no mutations in these exons (data not shown).

Cutaneous melanoma cell lines ectopically or endogenously expressing WT-B-Raf and V600E-B-Raf contained serum-inducible and constitutively activated ERK1/2, respectively (2, 8). We have previously shown that V600E-B-Raf uveal melanoma cell lines display constitutively high levels of ERK1/2 phosphorylation, and the inhibition of ERK1/2 leads to a large reduction in cell proliferation, as seen for V600E-B-Raf cutaneous melanoma cells (10). By contrast, it has been recently shown that WT-B-Raf uveal melanoma cell lines display constitutively high levels of ERK1/2 phosphorylation, although the effects of ERK1/2 inhibition have not been investigated (19). We confirmed by Western blot analysis that, irrespective of the expression of V600E-B-Raf, all of the melanoma cell lines showed high levels of phosphorylated ERK1/2 in the absence of serum stimulation (Fig. 1A). Serum stimulation over a 6-h period did not significantly increase ERK1/2 phosphorylation in the four melanoma cell lines (Fig. 1A). We used fast activated cell-based enzyme-linked immunosorbent assay to quantify ERK1/2 phosphorylation relative to the total ERK1/2. Analysis at different times during serum stimulation confirmed that WT-B-Raf melanoma cells constitutively activated ERK1/2 (Fig. 1B). In addition, the kinetics of cell proliferation in the presence of low serum concentrations confirmed that a major mitogenic signaling pathway is constitutively activated in all four melanoma cell lines, irrespective of the expression of V600E-B-Raf (Fig. 1C).

We then treated melanoma cells with UO126, a pharmacological inhibitor of MEK1/2, and thus ERK1/2 phosphorylation, to establish that constitutive ERK1/2 activation is involved in the cell proliferation signaling pathway. Inhibition of MEK1/2 strongly reduced ERK1/2 activation/phosphorylation in the four melanoma cell lines after culturing for 24 h in the presence of serum (Fig. 2A). Inhibition of MEK1/2 also greatly reduced proliferation of the four melanoma cell lines (Fig. 2B). We quantified the efficiency of UO126 by determining the concentration of UO126 necessary to inhibit cell proliferation by 50% (IC50). After MEK1/2 inhibition, the IC50 values were similar in both V600E-B-Raf and WT-B-Raf melanoma cells after culturing for 48 h in the presence of serum (Fig. 2C). In the presence of low serum concentrations, the four melanoma cell lines were also equally sensitive to UO126 (Fig. 2C), demonstrating that the MEK/ERK module is a major signaling component for melanoma cell proliferation, irrespective of V600E-B-Raf expression. We have previously shown that the MEK/ERK module controls cell transformation of uveal melanoma cells harboring V600E-B-Raf (10). Therefore, we analyzed the role of the MEK/ERK pathway in the cell transformation of WT-B-Raf melanoma cells by examining the ability of 92.1 cells to grow under anchorage-independent conditions. The ability of cells to form colonies in soft agar, a property closely associated with the malignant phenotype, was used to assess cell transformation. The 92.1 cells formed numerous and large colonies in soft agar similar to the V600E-B-Raf melanoma cells (Fig. 2D). Inhibition of MEK1/2 by UO126 markedly inhibited colony formation, and the efficiency of UO126 to inhibit WT-B-Raf melanoma cell transformation was similar to that observed in the V600E-B-Raf melanoma cell line, TP31 (Fig. 2D). Together, these data showed that the constitutive activation of the MEK/ERK signaling pathway is necessary for tumorigenesis of uveal melanoma cells expressing V600E-B-Raf and for uveal melanoma cells expressing WT-B-Raf.

There are many mechanisms that may explain the surprisingly high levels of ERK1/2 activation in WT-B-Raf uveal melanoma cells. These mechanisms may include 1) a specific increase in the expression of one of the components of the B-Raf/MEK/ERK signaling pathway (7); 2) a decrease in the expression of a negative regulator of this pathway, such as SPRY2 (27), RKIP (28, 29), or PTEN (30); or 3) the constitutive activation of a RAS and/or B-Raf mutation-independent ERK signaling.

WT-B-Raf Presents a Basal Kinase Activity Similar to the Constitutive V600E-B-Raf Mutant in Uveal Melanoma Cells—The high activation rate of ERK1/2 in WT-B-Raf-expressing melanoma cells may be explained by the expression of either B-Raf, MEK1/2, or ERK1/2 being higher in these cells than in V600E-B-Raf melanoma cells. Therefore, we compared the expression levels of these three kinases in the 92.1, Mel270, OCM-1, and TP31 cell lines. We detected Western blot analysis of B-Raf, MEK1/2, and ERK1/2. We found no difference in the protein levels of B-Raf, MEK1, and ERK1/2 in the four melanoma cell lines (Fig. 3A). We also observed similar levels of expression of the two components of the MEK/ERK module in the four melanoma cell lines (Fig. 3A). Therefore, overexpression of B-Raf, MEK, or ERK does not account for the constitutive ERK1/2 activation in the WT-B-Raf melanoma cells (Fig. 3A). In WT-B-Raf-expressing cutaneous melanoma cells, the level of SPRY2, an inhibitor of ERK1/2 signaling, is lower than in V600E-B-Raf-expressing cutaneous melanoma cells (27), which may also be true in uveal melanoma cells. However, we observed similar expression levels of SPRY2 in both the V600E-B-Raf and...
WTB-Raf uveal melanoma cells (Fig. 3A). Recently, it has been suggested that low expression levels of RKIP, an antagonist of Raf, leads to high levels of Raf/ERK signaling in cutaneous melanoma (28, 29). We found no difference in RKIP expression in V600EB-Raf uveal melanoma cells and WTB-Raf uveal melanoma cells by Western blot analysis (Fig. 3A), ruling out this explanation for the constitutive activation of ERK1/2 in WTB-Raf melanoma cells. It has been shown that ectopic expression of PTEN inhibits ERK1/2-mediated growth factor signaling and that loss of PTEN results in overactivation of ERK1/2 (30). Analysis of PTEN expression by Western blotting showed similar levels of the protein in the four melanoma cell lines, irrespective of V600EB-Raf expression, ruling out yet another explanation for the overactivation of ERK1/2 in WTB-Raf melanoma cells (Fig. 3A). Together, these data suggest that alteration of the expression of kinases of the B-Raf/MEK/ERK pathway or of its major negative regulators, including SPRY2, RKIP, and PTEN, are not involved in the constitutive activation of ERK1/2 in WTB-Raf melanoma cells.

That WTB-Raf is not overexpressed compared with V600EB-Raf in uveal melanoma cells does not rule out a high basal kinase activity for WTB-Raf, which may explain a key role of ERK1/2 activation in cell proliferation and transformation. Therefore, we measured the endogenous basal B-Raf kinase activity in the four WTB-Raf melanoma cells that have been previously shown to have 10 times higher B-Raf basal kinase activity than in normal uveal melanocytes (10). After B-Raf immunoprecipitation, we measured its kinase activity using an in vitro kinase assay that used recombinant MEK1 as a B-Raf substrate. We found that the basal kinase activity of B-Raf in WTB-Raf-expressing cells was similar to that in V600EB-Raf-expressing cells, including OCM-1 cells, suggesting that the constitutive activation of ERK1/2 may involve high levels of WTB-Raf kinase activity (Fig. 3B).

WTB-Raf Plays a Key Role in the Control of Cell Proliferation through the Activation of ERK1/2 in Uveal Melanoma Cells—BAY43-9006 was designed to inhibit Raf-1-dependent MEK phosphorylation and has
more recently been shown to also block B-Raf kinase (8, 9, 31, 32). Therefore, we investigated the effects of BAY43-9006 in WT-B-Raf uveal melanoma cells.

First, we assessed the effects of BAY43-9006 on cell proliferation. Cells were treated with various concentrations of BAY43-9006 in the presence of serum, and cell proliferation was analyzed using the MTT method. BAY43-9006 reduced cell proliferation in a concentration-dependent manner (Fig. 4A). Surprisingly, the IC_{50} values of BAY43-9006 to inhibit cell proliferation were similar in both V600E-B-Raf and WT-B-Raf melanoma cells (IC_{50} of 2.9 μM for V600E-B-Raf cells and IC_{50} of 3–3.2 μM for WT-B-Raf) (Fig. 4, A and B). This is in contrast with the suggestion that the inhibitor efficiency is lower in the mutated form of B-Raf (9). Therefore, we carried out similar experiments in serum-free conditions to avoid a potential inhibitory effect of serum on BAY43-9006. As for the results in the presence of serum, V600E-B-Raf melanoma cells were as sensitive to BAY43-9006 as the WT-B-Raf cells (IC_{50} of 0.44–0.66 μM for V600E-B-Raf melanoma cells and IC_{50} of 0.34–0.45 μM for WT-B-Raf melanoma cells) (Fig. 4B). We then investigated the precise mode of action of BAY43-9006 in uveal melanoma cells. We compared the effects of BAY43-9006 on the cell cycle of V600E-B-Raf and WT-B-Raf melanoma cells (Fig. 4C). Flow cytometry analysis showed that in V600E-B-Raf melanoma cells the cell cycle stopped at G1 after 24 h of treatment with BAY43-9006. We saw the same results in WT-B-Raf melanoma cells (Fig. 4C). Since inhibition of ERK1/2 activation with UO126 and treatment with BAY43-9006 induced similar effects on cell proliferation, we suspected that BAY43-9006 may mediate its effects by inhibiting ERK1/2 activation. We found that BAY43-9006 inhibited the phosphorylation of ERK1/2 in both V600E-B-Raf and WT-B-Raf melanoma cells, confirming that ERK1/2 play a central role in uveal melanoma cell proliferation (Fig. 4D). The inhibition of cell proliferation and ERK1/2 activation by BAY43-9006 greatly suggests that the Raf kinases, B-Raf and/or Raf-1, are involved in the control of ERK1/2 for cell proliferation in WT-B-Raf uveal melanoma cells.

To establish that B-Raf is responsible for the constitutive activation of ERK1/2, we used an siRNA-based approach to inhibit B-Raf protein expression. We first checked that the V600E mutation of B-Raf was responsible for the constitutive activation of ERK1/2 and proliferation of V600E-B-Raf uveal melanoma cells (Fig. 5, A and B). V600E-B-Raf-specific siRNA reduced proliferation by between 48 and 64% in V600E-B-Raf melanoma cells. We then studied the effects of WT-B-Raf-specific siRNA in WT-B-Raf melanoma cells. Transfection of WT-B-Raf uveal melanoma cells with WT-B-Raf-specific siRNA led to a marked decrease in ERK1/2 phosphorylation, whereas the total level of ERK1/2 in the cells was unaffected (Fig. 5A), demonstrating that B-Raf controlled ERK1/2 activation. We then studied the effects of WT-B-Raf depletion on cell proliferation. WT-B-Raf-specific siRNA reduced proliferation by between 52 and 65% in WT-B-Raf melanoma cells versus cell proliferation in the presence of scrambled B-Raf siRNA (Fig. 5B). Therefore, the efficiency of cell proliferation inhibition following B-Raf depletion was similar in both V600E-B-Raf and WT-B-Raf melanoma cells (Fig. 5B). Flow cytometry analysis showed that 3 days after transfection, B-Raf depletion by siRNA induced a significant increase in the G1 population of melanoma cells, irrespective of the mutational status of B-Raf (72% in OCM-1 cells and 48% in Mel270 cells) (Fig. 5C). This suggests that down-regulation of B-Raf expression stopped the cell cycle at G1. All of these data show that B-Raf plays a key role in the growth of uveal melanoma cells through the activation of ERK1/2.

Although B-Raf depletion greatly inhibits ERK1/2-mediated proliferation signaling in WT-B-Raf-expressing uveal melanoma cells, it does not completely rule out a role of Raf-1 in either ERK1/2 activation or cell proliferation in uveal melanoma cells. Therefore, we first used Western blotting to investigate the effect of siRNA-mediated WT-B-Raf depletion in the control of ERK1/2 activation. Transfection of WT-B-Raf uveal melanoma cells with Raf-1-specific siRNA down-regulated Raf-1 expression considerably, but it did not affect ERK1/2 phosphorylation (Fig. 6A). Similar results were observed in V600E-B-Raf-expressing cells (Fig. 6A), demonstrating that Raf-1 did not control ERK1/2 activation in uveal melanoma cells. We then studied the effect of Raf-1 depletion on
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cell proliferation. Raf-1-specific siRNA did not significantly reduce proliferation in WT-B-Raf and V600E-B-Raf melanoma cells versus the cell proliferation of cells transfected with scrambled Raf-1 siRNA (Fig. 6B). Altogether, these data demonstrate that B-Raf and not Raf-1 plays a key role in ERK1/2-mediated cell proliferation in both WT-B-Raf and V600E-B-Raf melanoma cells.

In cutaneous melanoma cells, Ras activates ERK1/2 through WT-B-Raf (33). Therefore, we suggest that Ras may also control ERK1/2 in WT-B-Raf uveal melanoma cells. We investigated whether the inhibition of Ras affected ERK1/2 activation in WT-B-Raf melanoma cells. Inhibition of Ras farnesylation with the Ras farnesylation inhibitor FPT-III did not affect ERK1/2 phosphorylation, suggesting that activation of ERK1/2 is independent of Ras activity (Fig. 7A). We then transfected cells with RasN17, an expression vector for a dominant negative mutant of Ras, rather than blocking Ras farnesylation, to confirm that Ras does not affect ERK1/2 activation. RasN17 overexpression did not affect ERK1/2 activation in both WT-B-Raf and V600E-B-Raf uveal melanoma cells, confirming the Ras-independent activation of ERK1/2 in uveal melanoma cells (Fig. 7A). We then investigated whether the inhibition of Ras, either through treatment with FPTIII or overexpression of RasN17, affected the levels of B-Raf kinase activity. Ras did not affect B-Raf kinase activity in both WT-B-Raf and V600E-B-Raf uveal melanoma cells (Fig. 7B), confirming that Ras and B-Raf/ERK1/2 are two independent signaling pathways. Altogether, these data showed that the B-Raf/ERK signaling is independent of Ras in uveal melanoma cells, irrespective of the mutational status of B-Raf.

cAMP/PKA Is the Upstream Effector of the B-Raf/ERK Signaling Pathway for Uveal Melanoma Cell Proliferation—PKA has been shown to activate cell proliferation in B-Raf-positive cells, whereas it inhibits proliferation in B-Raf-negative cells. cAMP/PKA controls cell proliferation by regulating ERK1/2 activation in a cell-specific manner and through multiple pathways (24, 25). Unlike normal uveal melanocytes, uveal melanoma cells express the α catalytic subunit or the type 1 regulatory subunit of PKA, suggesting that PKA may be involved in uveal melanoma cell proliferation (34). Therefore, we speculated that activation of PKA may stimulate B-Raf/ERK signaling for the proliferation of uveal melanoma cells. Analysis of PKA activity showed that, irrespective of V600E-B-Raf expression, all of the melanoma cell lines showed a similar level of basal kinase activity of PKA in the absence of serum stimulation (Fig. 8A). Serum stimulation over a 24-h period did not significantly increase the amount of PKA activity, suggesting a constitutive activation of PKA in the four melanoma cell lines (Fig. 8A). Cell stimulation with the cAMP-elevating agent, forskolin, did not increase the level of PKA activity, confirming the constitutive activation of PKA in uveal melanoma cells (Fig. 8A). By contrast, cell treatment with H89 greatly reduced PKA activation (Fig. 8A). We analyzed the role of the cAMP/PKA signaling in uveal melanoma cells by first investigating the effects on ERK1/2 activation and cell proliferation of treatment of uveal melanoma cells with \( \left( R_{ERK2} \right)_{-8-Br-cAMP} \), a competitive antagonist of cAMP binding to PKA, which therefore inhibits PKA. The inhibition of PKA had no effect on ERK1/2 activation in V600E-B-Raf uveal melanoma cells, whereas it greatly reduced ERK1/2 activation in WT-B-Raf uveal mela-
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The treatment of cells with (R)\_8-Br-cAMP also affected the proliferation of WT B-Raf uveal melanoma cells, which is consistent with ERK1/2 playing a key role in WT B-Raf uveal melanoma cell proliferation (Fig. 8C). By contrast, the inhibition of PKA did not affect the proliferation of V600E B-Raf uveal melanoma cells, which is consistent with PKA having no effect on activating oncogenic mutants of B-Raf-mediated ERK1/2 activation in these cells (Fig. 8D).

We then used a siRNA-based approach that inhibits the expression of both the PKA \(\alpha\) and PKA \(\gamma\) catalytic subunits to confirm that PKA mediates the effects of cAMP on the B-Raf-stimulated ERK1/2 activation and cell proliferation. Down-regulation of the PKA \(\alpha\) and PKA \(\gamma\) catalytic subunits reduced ERK1/2 activation in WT B-Raf uveal melanoma cells (Fig. 9A). As expected, depletion of the PKA \(\alpha\) and PKA \(\gamma\) catalytic subunits by siRNAs also reduced cell proliferation in WT B-Raf uveal melanoma cells (decrease in cell proliferation by 48 and 58\% in 92.1 and Mel270 cells, respectively) (Fig. 9B). The reduction in cell proliferation was similar to that observed with siRNA-mediated B-Raf depletion (compare Figs. 5B and 9B). By contrast, siRNA-mediated PKA depletion did not affect ERK1/2 activation or proliferation of V600E B-Raf uveal melanoma cells (Fig. 9, A and B). This confirmed that V600E B-Raf stimulates ERK1/2 signaling independent of the normally required upstream activators of B-Raf. We confirmed that PKA controls ERK1/2-mediated cell proliferation signaling through B-Raf activation in WT B-Raf uveal melanoma cells by investigating the effects of siRNA-mediated PKA depletion on the levels of B-Raf kinase activity. PKA depletion reduced the kinase activity of B-Raf in WT B-Raf uveal melanoma cells, whereas it did not affect B-Raf kinase activity in V600E B-Raf uveal melanoma cells (Fig. 9C). Altogether, these data demonstrated that PKA lies upstream of B-Raf/ERK for the control of cell proliferation in WT B-Raf uveal melanoma cells and that V600E B-Raf does not require PKA for stimulating ERK1/2 signaling and proliferation.

PKA-stimulated WT B-Raf/ERK Activation Is a Rap1-independent Mechanism in Uveal Melanoma Cells—There are different mechanisms that may account for the cAMP/PKA-mediated B-Raf/ERK1/2 signaling pathway activation. Mechanisms involving cAMP-dependent and/or PKA-dependent stimulation of Ras can be ruled out, because inhibition of Ras did not affect ERK1/2 activation in WT B-Raf melanoma cells. PKA may also activate ERK1/2 by activating the small GTPase, Rap1, which can activate ERK1/2 through B-Raf. Rap1 is the closest homolog of Ras and has been shown to activate ERK1/2 in a wide range of biological processes, such as cell proliferation, differentiation, cell morphogenesis, and cell adhesion (35, 36). Therefore, we investigated the role of Rap1 in the activation of the B-Raf/ERK signaling pathway in WT B-Raf uveal melanoma cells.

Analysis of Rap1 activation shows that all melanoma cell lines expressed similar basal levels of Rap1 activity, irrespective of the mutational status of B-Raf (Fig. 10A). We investigated whether Rap1 mediated the cAMP/PKA signaling for ERK1/2 activation by first analyzing the effects of either PKA inhibition or PKA down-regulation on the activation of Rap1. siRNA-mediated reduction of the PKA \(\alpha\) catalytic subunit had no effect on Rap1 activity in all of the melanoma cell lines (Fig. 10). Similarly, inhibition of PKA with H89 did not affect Rap1 activity in all of the melanoma cell lines (Fig. 10A). In contrast, siRNA-mediated down-regulation of the PKA \(\alpha\) catalytic subunit or H89-mediated inhibition of PKA inhibited ERK1/2 phosphorylation in WT B-Raf melanoma cells, strongly suggesting that Rap1 was not required for PKA-mediated ERK1/2 activation in uveal melanoma cells (Fig. 10A). However, these results did not rule out a PKA-independent effect of Rap1 on ERK1/2 activation. Therefore, we used an siRNA-based approach inhibiting Rap1 expression to confirm that the activation of B-Raf/ERK signaling did not require Rap1 in uveal melanoma cells. Treatment of the cells with Rap1-specific siRNA resulted in a reduced Rap1 expression but had no significant effects on ERK1/2 activation in WT B-Raf melanoma cells (Fig. 10B). Consistent with these results, the kinase activity of B-Raf was not affected in WT B-Raf melanoma cells (Fig. 10C). These data demonstrate that Rap1 is not the upstream activator of the B-Raf/ERK signaling in WT B-Raf uveal melanoma cells. In control experiments, we found that siRNA-mediated Rap1 depletion did not affect either the levels of B-Raf kinase activity or ERK1/2 activation in V600E B-Raf melanoma cells, confirming that B-Raf/ERK activation occurs independently of Rap1 in V600E B-Raf melanoma cells (Fig. 10, B and C). As expected, siRNA-mediated Rap1 down-regulation did not significantly inhibit cell proliferation in uveal melanoma cells (reduction in cell proliferation of between 3 and 5.6\% in V600E B-Raf cells and between 12.7 and 19.6\% in WT B-Raf cells) (data not shown).

We confirmed that the PKA stimulation of ERK1/2 was effectively mediated by B-Raf and could not occur directly by analyzing the effects
on siRNA-mediated B-Raf-depleted uveal melanoma cells of cell treatment with either activators of PKA or activators of Epac. In a first set of experiments, we showed that activation of PKA with 8-Br-cAMP did not increase ERK1/2 activation in either WT B-Raf or V600E B-Raf uveal melanoma cells, confirming the constitutive activation of PKA in these cells (Fig. 11). Consistent with this, cell stimulation with 8-Br-cAMP did not increase cell proliferation (data not shown). Moreover, the specific activation of Epac with the selective activator, 8CPT-2Me-cAMP (37), had no effect on ERK1/2 phosphorylation in uveal melanoma cells, irrespective of the mutational status of B-Raf (Fig. 11). This is consistent with Rap1 having no effect on ERK1/2 activation. We then investigated the ability of PKA or Epac stimulation to reactivate ERK1/2 when B-Raf was down-regulated. Activation of PKA did not restimulate ERK1/2 phosphorylation in the absence of B-Raf in WT B-Raf uveal melanoma cells, showing that B-Raf is essential for PKA-mediated ERK1/2 activation in these cells (Fig. 11). Also, the selective activation of Epac could not induce ERK1/2 phosphorylation, confirming that Rap1 has no effect on ERK1/2 activation (Figs. 10B and 11). We then investigated the effects of isobutylmethylxanthine- and cholera toxin-stimulated increases in cAMP on ERK1/2 activation to determine whether cAMP stimulates ERK1/2 independently of B-Raf. Cell stimulation with isobutylmethylxanthine and cholera toxin did not activate ERK1/2 phosphorylation.
FIGURE 11. Effects of B-Raf depletion on the PKA/Rap1-dependent activation of ERK1/2. \( \text{V600E-B-Raf} \) cells (OCM-1 cells) and \( \text{WT-B-Raf} \) cells (92.1 cells) were transfected with \( \text{V600E-B-Raf} \)- and \( \text{WT-B-Raf} \)-specific siRNAs (+) or with scrambled siRNAs (−) as described under “Experimental Procedures.” Three days after transfection, cells were treated with or without 8-Br-cAMP (100 μM), 8CPT-2Me-cAMP (100 μM), or a combination of isobutylmethylxanthine (100 μM) and cholera toxin (10 ng/ml) for 15 min, and the expression of B-Raf, phospho-ERK1/2, and α-tubulin was analyzed by Western blotting. Similar results were observed after a 1-h cell treatment. The data are the mean of three independent experiments.

FIGURE 12. Regulation of the expression and role of cyclin D1 in the PKA/B-Raf/ERK signaling for uveal melanoma cell proliferation. \( \text{V600E-B-Raf} \) cells (OCM-1 cells) and \( \text{WT-B-Raf} \) cells (92.1 cells) were transfected with B-Raf-specific or with a mixture of PKA α and PKA γ catalytic subunit-specific siRNAs (PKACα + Cγ) or with scrambled siRNAs (Scr) as described under “Experimental Procedures.” The expression of cyclin D1 was analyzed by Western blotting 3 days after cell transfection. The results presented are representative of three independent experiments, B, the effect of cyclin D1 depletion on cell proliferation was measured by the MTT colorimetric assay on day 3. Scrambled siRNAs did not affect cell proliferation (data not shown). The results presented are representative of three independent experiments.

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The molecular basis of the role of B-Raf in melanoma biology is not fully understood. B-Raf is a serine/threonine kinase that is downstream of the Ras/Raf/MEK/ERK cascade, a major intracellular mediator of mitogenic signaling that regulates numerous biological processes (28, 29). Activating point mutations in \( \text{RAS} \) and \( \text{BRAF} \) are found in 9–15% and 60–66% of cutaneous melanoma, respectively (1). It has been suggested that a gain-of-function mutation in \( \text{BRAF}^{\text{V600E}} \) is primarily responsible for the constitutive activation of ERK1/2 and thus for the acquisition by cutaneous melanoma cells of self-sufficiency in the MEK/ERK signaling pathway, which partly controls cell proliferation by modulating the transcription of genes encoding proteins involved in regulating the cell cycle, such as cyclin D1 (38). We have previously reported that cyclin D1 is under the control of ERK1/2 in \( \text{V600E-B-Raf} \) uveal melanoma cells (10). However, the role of PKA/B-Raf signaling in controlling cyclin D1 has not been characterized in \( \text{WT-B-Raf} \) uveal melanoma cells. Therefore, we first investigated the role of B-Raf in controlling cyclin D1 expression using a siRNA-based strategy in \( \text{WT-B-Raf} \) cells. siRNA-mediated depletion of B-Raf substantially reduced the expression of cyclin D1 in \( \text{WT-B-Raf} \) cells (Fig. 12A). We detected a similar decrease in the expression of cyclin D1 in \( \text{V600E-B-Raf} \) cells, confirming the role of the B-Raf/ERK signaling on the control of cyclin D1 expression in these cells (Fig. 12A). We then investigated the role of PKA in cyclin D1 expression. siRNA-mediated depletion of PKA also substantially reduced the expression of cyclin D1 in \( \text{WT-B-Raf} \) cells, demonstrating that the PKA/B-Raf signaling pathway controls cyclin D1 expression in these cells (Fig. 12A). Unlike that observed with siRNA-mediated B-Raf depletion, we observed no decrease in the expression of cyclin D1 after siRNA-mediated PKA depletion in \( \text{V600E-B-Raf} \) cells, confirming the central role of the V600E mutation in B-Raf (Fig. 12A). Therefore, we studied whether the PKA/B-Raf-dependent control of cyclin D1 may play a key role in proliferation of \( \text{WT-B-Raf} \) uveal melanoma cells by investigating the role of cyclin D1 in uveal melanoma cells. siRNA-mediated cyclin D1 expression greatly affected the proliferation of \( \text{WT-B-Raf} \) uveal melanoma cells (decrease in cell proliferation by 53 and 47% in 92.1 and Mel270 cells, respectively) (Fig. 12B). The reduction in cell proliferation was similar in \( \text{V600E-B-Raf} \) uveal melanoma cells, strongly suggesting that \( \text{V600E-B-Raf} \) controls cell proliferation through a molecular mechanism similar to that of \( \text{WT-B-Raf} \) in uveal melanoma cells (Fig. 12B).

In conclusion, the PKA/B-Raf/ERK1/2 pathway is the major signaling for uveal melanoma cell proliferation through the control of cyclin D1 expression in \( \text{WT-B-Raf} \) uveal melanoma cells. It is therefore a key target for future pharmacological anti-tumor therapies in uveal melanoma. The effective inhibition of \( \text{WT-B-Raf} \) uveal melanoma cell proliferation by BAY43-9006 may be useful for the treatment of uveal melanoma.

**DISCUSSION**

ERK1/2 is a key signaling pathway for the acquisition of oncogenic behavior in melanoma cells, but the control of its activation for cell proliferation differs between uveal and cutaneous melanoma cells/melanocytes. The Ras/Raf/MEK/ERK cascade is a major intracellular mediator of mitogenic signaling that regulates numerous biological processes (28, 29). Activating point mutations in \( \text{RAS} \) and \( \text{BRAF} \) are found in 9–15% and 60–66% of cutaneous melanoma, respectively (1). It has been suggested that a gain-of-function mutation in \( \text{BRAF}^{\text{V600E}} \) is primarily responsible for the constitutive activation of ERK1/2 and thus for the acquisition by cutaneous melanoma cells of self-sufficiency in the
growth signal (1). In ocular melanoma, no RAS mutations and rare B-RAF mutations have been detected, although constitutive activation of ERK1/2 has been detected in primary uveal melanoma tumors, suggesting that WT B-Raf may be involved in the activation of the ERK1/2 signaling pathway (10–18).

In this study, we reported that uveal melanoma cells expressing WT B-Raf have similar constitutively high ERK1/2 activation as cells expressing V600E B-Raf, as seen by Western blot analysis and enzyme-linked immunosorbent assay. This is in contrast to the absence of constitutive activation of ERK1/2 in cutaneous melanoma cell lines expressing WT B-Raf and in COS cells ectopically expressing WT B-Raf (8, 9). The blockade of ERK1/2 activation by UO126 greatly inhibited the proliferation of uveal melanoma cells expressing WT B-Raf to a similar extent as cells expressing V600E B-Raf. However, UO126 either weakly inhibited or was unable to inhibit the proliferation of cutaneous melanoma cells expressing WT B-Raf (1, 8). The constitutive activation of ERK1/2 in the absence of RAS and B-RAF activating mutations suggests other mechanisms for activating ERK signaling in uveal melanoma cells. This has also been suggested for only about 20% of cutaneous melanomas that do not carry any mutation in either RAS or B-RAF (1, 5, 31). Although it is well known that the activating V600E mutation of B-Raf is central to the ERK1/2-mediated growth of both cutaneous and uveal melanoma cells, the role of WT B-Raf in the control of ERK1/2 for cell proliferation in uveal melanoma cells remained unclear. Our results show that endogenous WT B-Raf has a growth-promoting potential in uveal melanoma cells without RAS mutations. Unlike cutaneous melanoma cells, this growth-promoting potential is not due to the overexpression of WT B-Raf or to the repression of PTEN, RKIP, or SPRY2 expression in uveal melanoma cells (27–30). Since RAS and B-RAF mutations are mutually exclusive, it has been suggested that these genes are on the same oncogenic signaling pathway in cutaneous melanoma. Therefore, it has been postulated that Ras may also act upstream from WT B-Raf in the control of ERK1/2 in uveal melanoma cells. The present study rules this out by showing that the inhibition of Ras activation did not affect B-Raf-mediated constitutive activation of ERK1/2 in WT B-Raf uveal melanoma cells. This result reveals a large difference between cutaneous and uveal melanoma cells.

It is well known that cAMP can inhibit cell growth by blocking growth factor activation of ERK1/2 in many cell types. cAMP uses multiple mechanisms to inhibit ERK1/2, including the activation of Rap1 by PKA to disrupt Ras/Raf-1 signaling (24, 25). By contrast, the proliferation of uveal melanocytes required a combined stimulation of cAMP-elevating agents and growth factors, these latter exerting their mitogenic activity, at least in part, through the activation of ERK1/2 (22, 23). In cells that do not express B-Raf, the transfection of B-Raf converts cAMP from an inhibitor to an activator of ERK1/2, suggesting that the expression of B-Raf in uveal melanocytes and melanoma cells makes cAMP a stimulator of cell proliferation (39, 40). Although the requirement of cAMP and ERK1/2 activation for uveal melanocytes greatly suggests that proliferation occurs through the cross-talk between cAMP and ERK1/2, the exact mechanisms by which ERK1/2 and cAMP cross-talk remain unknown in uveal melanocytes. Our study showed that cAMP activation of ERK1/2 requires both PKA and B-Raf, but not Rap1 or Ras, for uveal melanoma cell proliferation. By contrast, cAMP activation of ERK1/2 requires both Ras and B-Raf, but not Rap1 or PKA, for cutaneous melanocyte proliferation (33). This confirms that the signaling pathways involved in cell proliferation differ greatly between cutaneous and uveal cells. Uveal melanoma cells are not the only cell type requiring coupling of cAMP/PKA to B-Raf for activation of ERK1/2. In response to neural growth factor, cAMP stimulates a sustained B-Raf and ERK1/2 signaling that leads to PC12 differentiation (36). However, it has been shown that PKA is not involved in cAMP coupling to B-Raf and that Rap1 is necessary for coupling cAMP to B-Raf. Therefore, the cAMP downstream signaling for ERK1/2 activation greatly differs between proliferating uveal melanoma cells and differentiating PC12 cells. However, the direct activation of B-Raf by Rap1 is still doubtful, leaving the question of how B-Raf is activated downstream from neural growth factor and cAMP in PC12 cells. It has been suggested that Src is needed for ERK activation by cAMP/PKA in B-Raf-positive cells (20, 41, 42). Src inhibition with PP1 does not affect uveal melanoma cell proliferation, thus ruling out a role of Src for mediating PKA-induced ERK1/2 activation.4 TSH-stimulated human thyroid cell proliferation has also been shown to be PKA-dependent, and both TSH and cAMP activate ERK1/2 in rat thyroid cells (43, 44). ERK1/2 activation by cAMP is independent of PKA and is linked to Rap1 activation through Epac. However, TSH-stimulated Rap1 activation does not activate ERK1/2, although it enhances the growth-promoting effects of growth factors (45, 46). These data show that the signaling pathways for ERK1/2 activation downstream from cAMP in thyroid cells also differ from those observed in uveal melanoma. Nevertheless, the central role of PKA in activating B-Raf/ERK signaling for WT B-Raf cell proliferation suggested that uveal melanoma growth may be inhibited by targeting PKA.

B-Raf as a Therapeutic Target for Uveal Melanoma—B-Raf plays a key role in uveal melanoma cell proliferation and cell cycle regulation. Therefore, the use of Raf kinase inhibitors is of great interest in uveal melanoma treatments. BAY43-9006 has recently been tested on cutaneous melanoma cells expressing oncogenic B-Raf mutants and has been shown to induce a substantial growth delay in melanoma xenografts (8, 9). Consistent with this, our study showed that BAY43-9006 is a potent inhibitor of cell growth of uveal melanoma cells expressing V600E B-Raf. Analysis of BAY43-9006 interactions with purified B-Raf showed that the Raf inhibitor interacts with an inactive conformation of B-Raf. This suggested that activating oncogenic mutants of B-Raf would be less sensitive to the inhibitor than WT B-Raf (32) and that BAY43-9006 would be more potent on cells expressing WT B-Raf than V600E B-Raf. Surprisingly, in uveal melanoma cells, inhibition by BAY43-9006 was as efficient in cells expressing WT B-Raf as in those expressing V600E B-Raf. Since BAY43-9006 was designed to inhibit Raf-1-dependent MEK phosphorylation, it was important to exclude a role of Raf-1 in uveal melanoma due to the high sensitivity of Raf-1 toward BAY43-9006 (31, 47). We demonstrated that siRNA-mediated Raf-1 depletion does not affect cell proliferation and ERK1/2 signaling in both WT B-Raf and V600E B-Raf uveal melanoma cells. This ruled out a role of Raf-1 in the inhibitory effect of BAY43-9006 on the proliferation of uveal melanoma cells, confirming previous data showing that Raf-1 depletion did not affect cell proliferation and ERK signaling in ectopically expressing V600E B-Raf cutaneous melanoma cell lines (3, 4, 9), whereas it does affect cell proliferation in cutaneous melanoma cell lines naturally expressing V600E B-Raf (8). However, further experiments are needed to determine why the inhibition of cell proliferation by BAY43-9006 was as efficient in cells expressing WT B-Raf as those expressing V600E B-Raf in uveal melanoma cells. Unlike that observed with siRNA-mediated Raf-1 depletion, we observed a decrease in cell proliferation in uveal melanoma cells treated with phosphorothioate-modified antisense (AS) oligonucleotide (ODN) specifically targeted against Raf-1 mRNA (48). Raf-1 AS ODNs have been shown to induce nonspecific effects on nontargeted proteins, including Bcl-2 (49). Treatment with Raf-1 AS ODN also reduces Bcl-2 expression in uveal melanoma cells.4 Therefore, the decrease in cell

4 F. Mascarelli, personal communication.
proliferation observed in Raf-1 AS ODN-treated uveal melanoma cells may be due to a nonspecific effect of a Raf-1 AS ODN on Bcl-2 expression. The decrease in cell proliferation by Bcl-2 AS ODN in uveal melanoma may be due to a nonspecific effect of Raf-1 AS ODN on Bcl-2 expression.

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