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Advances in Brief

^{V599E}B-RAF is an Oncogene in Melanocytes

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Abstract

The oncogenic version of B-RAF, ^{V599E}B-RAF, is found in approximately 70% of human melanomas. However, the role that this oncogene plays in melanoma is unclear because ^{V559E}B-RAF is also found in approximately 80% of benign nevi. We have examined the role of oncogenic B-RAF in the early stages of melanoma by expressing ^{V599E}B-RAF in cultured melanocytes. In these cells, ^{V599E}B-RAF induced constitutive mitogen activated ERK-activating kinase (MEK) and extracellular signal-regulated kinase (ERK) signaling, 12-*O*-tetradecanoylphorbol-13-acetate-independent growth, and tumorigenicity in nude mice. Intriguingly, in RAS-transformed melanocytes, B-RAF depletion did not block MEK-ERK signaling or cell cycle progression. Similarly, B-RAF depletion blocked MEK-ERK signaling in human melanoma cells harboring oncogenic B-RAF, but not in melanoma cells harboring oncogenic RAS. Thus, although B-RAF can act as a potent oncogene in the early stages of melanoma by signaling through MEK and ERK, it is not required for this signaling in RAS-transformed melanocytes due to innate redundancy within the pathway. These findings have important implications for future therapeutic strategies.

Introduction

The RAS proteins regulate cell proliferation, survival, and differentiation by activating a number of effector proteins, including the RalGDS exchange factors, the phosphatidylinositol 3′-kinases, and the three RAF protein kinases (A-RAF, B-RAF, and C-RAF). Once activated, RAF proteins stimulate a signaling cascade involving the MEK and extracellular signal-regulated kinase (ERK) protein kinases. For many years, RAS signaling has been implicated in initiation and progression of melanoma. Approximately 15–20% of human melanomas have mutations in RAS genes (1, 2), but RAS activation in melanocytes is not sufficient to induce melanoma in mouse models unless the cell cycle progression inhibitor $p16^{INK4A}$ is also deleted (3). Importantly, the majority of sporadic melanomas are $p16^{INK4A}$ negative (4), and germ-line mutations in $p16^{INK4A}$ lead to a dramatically increased lifetime risk of melanoma (4).

Although RAS clearly plays a role in human melanoma, its importance has recently been superseded by the discovery that the RAS effector B-RAF is mutated in approximately 70% of cutaneous melanomas (5–7). However, like RAS, oncogenic B-RAF is not sufficient to induce melanoma because B-RAF mutations are also found in 80% of nevi, which are benign melanocytic lesions (6, 8). The most frequent mutation in B-RAF (80%) is a glutamic acid for valine substitution at position 599, which produces a protein that has elevated kinase activity *in vitro* and induces constitutive activation of ERK (5). Importantly, ERK activity is elevated in the majority of melanoma cell lines (7), and elevated ERK signaling appears to be required for melanocyte proliferation in culture (9–11). This, together with the observation that B-RAF and RAS mutations are mutually exclusive, suggests that RAS induces ERK activation in a B-RAF-dependent manner and that these oncogenes transform melanocytes through a common mechanism. Oncogenic RAS transforms cultured mouse and human melanocytes, suggesting a role in melanoma initiation (12, 13), but it is not known whether B-RAF can do the same. In view of its high rate of mutation in melanoma, it is essential to investigate whether B-RAF can transform melanocytes to allow us to determine whether it has a role in initiation of melanoma.

Here we show that ^{V599E}B-RAF stimulates constitutive ERK activity and transforms melan-a cells, immortalized melanocytes that possess all of the characteristics of normal melanocytes (14). However, B-RAF is not essential for ERK activation in RAS-transformed melanocytes. Similar results were obtained in human melanoma cell lines expressing oncogenic B-RAF or oncogenic RAS, demonstrating the physiological significance of our results. These data show that ^{V599E}B-RAF is an important melanocyte oncogene but that wild-type B-RAF (^{WT}B-RAF) is not required for ERK signaling in RAS-transformed melanocytes or in melanoma cells harboring oncogenic RAS because there is innate redundancy in this pathway.

Materials and Methods

Cell Culture. The melan-a cells (14) were maintained in RPMI 1640 (Gibco) containing 10% FCS, 200 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma), and 300 pM cholera toxin (Sigma). For generation of stable B-RAF- or ^{V599E}B-RAF-expressing clones, 4×10^4 cells per 24-well plate were transfected with pMCEF-B-RAF or pMCEF-^{V599E}B-RAF using LipofectAMINE (Invitrogen) as described previously (11). TPA (200 nM) was added to the medium for cells transfected with either the empty vector or ^{WT}B-RAF but not to cells transfected with ^{V599E}B-RAF. Individual clones were isolated and subcloned after 3 weeks. Human melanoma cell lines were maintained in DMEM (CHL, WM266-4, and SK-MEL-2) or RPMI 1640 (WM35) containing 10% FCS. Melanocytes transformed by ^{G12V}Ha-RAS (LTRras cells; Ref. 12) and activated MEK (MEKEE cells) were cultured in RPMI 1640/10% FCS.

Thymidine Incorporation and Soft Agar Growth. A total of 4×10^4 melan-a cells were seeded per well on a 24-well plate. When present, 10 μM U0126 was added 24 h before the cells were harvested. After 20 h, [^{3}H]thymidine (0.4 μCi/ml) was added, and after 4 h, incorporated [^{3}H]thymidine was quantified by liquid scintillation counting. For long-term growth experiments, cells were seeded at 1×10^6 cells/10-cm dish in medium containing either 10% FCS or 10% FCS + 100 nM TPA. Cells were counted every 3 days and replated at 1×10^6 cells/10-cm dish. For the anchorage-independent growth assay, 1×10^4 cells were suspended in 0.45% low melting point (LMP)-agarose in RPMI 1640/10% FCS and overlaid onto 0.9% LMP-agarose/RPMI 1640 in a 6-cm dish. Before colonies were stained with crystal violet, 1 ml of fresh medium was added twice a week for 3 weeks.

Cell Lysis, Western Blotting, Antibodies, and Inhibitors. Cells were lysed in 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% NP40, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 10 μg/ml leupeptin, and 10 μg/ml aprotinin for 20 min at 4°C. Proteins were separated by SDS-PAGE, and Western blot

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analysis was performed following standard protocols using the following antibodies: B-RAF (F-7; Santa Cruz Biotechnology); C-RAF (M40091.G; Anogen); phospho-ERK (MAPK-YT; Sigma); 9E10 (15); ERK2 (#122; Ref. 16); and phospho-MEK (New England Biolabs). U0126 was from Promega, and the RAF inhibitor BAY 43-9006 was synthesized as described elsewhere.[3]

RNA Interference (RNAi). Cells were seeded at 2.5×10^3 cells/35-mm well the day before transfection. Cells were transfected in 1 ml of OPTIMEM with 6 μl of 20 μM B-RAF-specific (5′-AAGUGGCAUGGUGAUGUGGCA-3′), C-RAF-specific (5′-AAUAGUUCAGCAGUUUGGCUA-3′), or control scrambled small interfering RNA (siRNA) (5′-AAGUCCAUGGUGACAG-GAGAC-3′) using LipofectAMINE (Gibco). After a 5-h incubation with the RNA-complex, 1 ml of medium containing FCS was added. Cells were harvested at the indicated times after the transfection. For DNA synthesis measurements after siRNA treatment, cells were incubated with [^{3}H]thymidine from 92 to 96 h after the addition of siRNA.

***In Vivo* Studies.** Female CD1 nude mice (Charles River United Kingdom) weighing 19–32 g were used. Cell suspensions were inoculated s.c. in a volume of 0.2 ml into age-matched mice to give groups of seven mice; each mouse received injection with 1×10^7 cells of either WTB-RAF- or V599EB-RAF-expressing melan-a cells (clones B2 and VE11, respectively). Experiments were conducted in accordance with the United Kingdom Home Office regulations and United Kingdom Coordinating Committee on Cancer Research Guidelines (17).

Results

We generated melan-a cell lines expressing myc epitope-tagged WTB-RAF or V599EB-RAF, and, as a control, we generated a cell line (pM4) that only expresses the *neo*^{R} gene. Western blotting for the myc tag was used to identify cell lines expressing exogenous B-RAF, and the levels of expression were compared with endogenous protein by blotting for total B-RAF protein. Exogenous WTB-RAF was highly expressed compared with endogenous B-RAF in some but not all of the cell lines (B2, B7, B9, and B10), whereas V599EB-RAF was consistently expressed at low levels that did not significantly alter the total levels of B-RAF in these cells (VE11, VE14, and VE16; Fig. 1*A*). The lack of melanocyte lines expressing high levels of V599EB-RAF protein suggests that these cells do not tolerate high B-RAF activity, presumably because it stimulates excessively high levels of ERK activity (see below). Despite the low levels of expression, all of the lines expressing V599EB-RAF had constitutive MEK and ERK activation as revealed by Western blotting with antibodies that only bind to the dually phosphorylated, active forms of these proteins (Fig. 1*A*). By contrast, even the lines expressing the highest levels of WTB-RAF did not have increased MEK or ERK phosphorylation (Fig. 1*A*). Thus, overexpression of WTB-RAF is not sufficient to trigger constitutive MEK/ERK signaling in melanocytes, whereas even low levels of V599EB-RAF stimulate constitutive signaling through this pathway.

TPA is an essential mitogen for melanocytes *in vitro*, and in the absence of TPA, melanocytes growth arrest (14). However, the mechanism of TPA action is not clear, although it does activate ERK in melanoma cells (18). Because V599EB-RAF also stimulates constitutive ERK activity, we tested whether V599EB-RAF-expressing clones still require TPA for growth. First, we analyzed the effect of TPA on ERK activity. TPA stimulated strong and sustained ERK activation in the parental melan-a cells, the vector control pM4 line, and the cells expressing WTB-RAF (Fig. 1*B*). By contrast, TPA had only a weak effect on ERK phosphorylation in cells expressing V599EB-RAF (Fig. 1*B*). In the presence of TPA, all of the melan-a lines showed a 4–7-fold increase in the cell number over a 3-day period (Fig. 1*C*). In the absence of TPA, the parental cells, the pM4 cells, and the lines expressing WTB-RAF all failed to grow, whereas the lines expressing V599EB-RAF continued to grow at the same rate as in the presence of TPA (Fig. 1*C*).

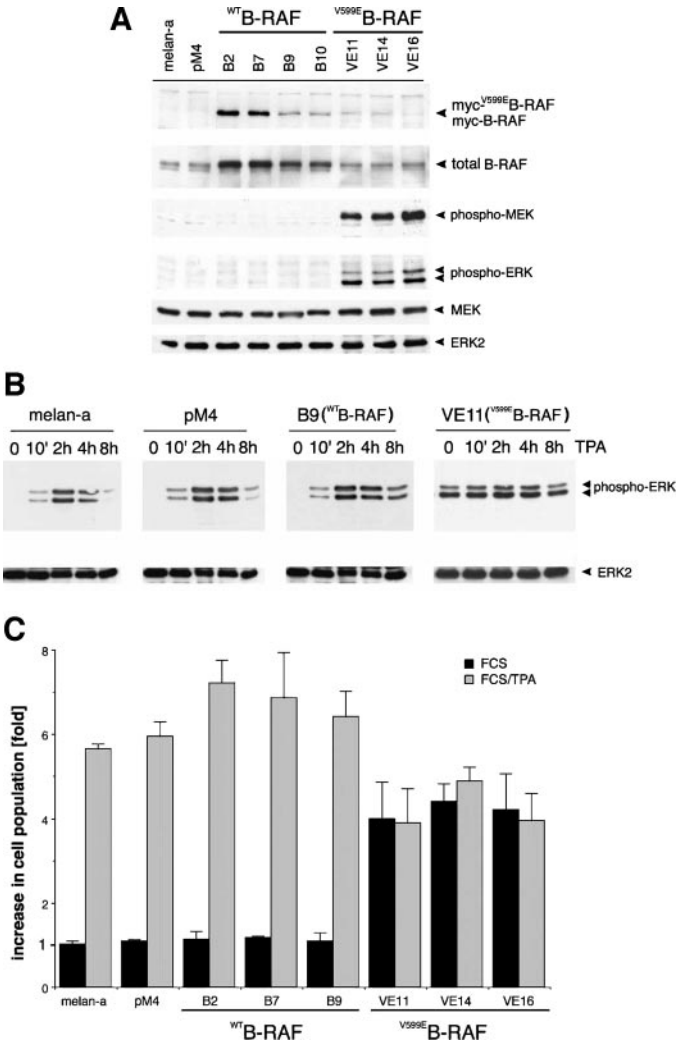

Fig. 1. V599EB-RAF induces constitutive mitogen activated ERK-activating kinase (MEK) and extracellular signal-regulated kinase (ERK) phosphorylation and mediates 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-independent growth in melanocytes. *A,* expression of B-RAF and activation of MEK and ERK. Parental melan-a cells, the control pM4 line, and melan-a clones expressing WTB-RAF (B2, B7, B9, and B10) or V599EB-RAF (VE11, VE14, and VE16) were probed for myc-tagged WTB-RAF, myc-tagged V599EB-RAF, total B-RAF, phospho-MEK, phospho-ERK, total MEK, or total ERK2. *B,* effects of TPA on ERK activity. Melan-a, pM4, B9, and VE11 cells were cultured in medium lacking TPA for 24 h and then stimulated with 200 nM TPA for the indicated times and analyzed for phospho-ERK and total ERK. Data shown are for one clone expressing WTB-RAF and one clone expressing V599EB-RAF, but similar results were obtained with three independent clones in each case. *C,* cell proliferation. The melan-a cells, pM4 cells , and the clones indicated were cultured in the presence or absence of 200 nM TPA, and cells were counted after 3 days. The fold increase in growth is shown.

The parental melan-a cells, the pM4 vector controls, and the clones expressing WTB-RAF did not grow in soft agar. By contrast, melan-a derivatives expressing V599EB-RAF, oncogenic RAS (LTRras cells; Ref. 12), and activated versions of MEK (MEKEE cells) all formed colonies in soft agar (Fig. 2, *A* and *B*). Previous studies have established that melan-a cells do not grow as tumors in nude mice (14), and melan-a cells expressing WTB-RAF also failed to grow in nude mice (Fig. 2*C*). By contrast, V599EB-RAF-expressing melan-a cells did grow as tumors in nude mice (Fig. 2*C*).

Our data show that V599EB-RAF transforms melan-a cells, so we examined whether this required MEK-ERK signaling. Both the MEK

[3] P. T. C. Wan, M. J. Garnett, M. S. Roe, S. Lee, D. Niculescu-Duvaz, V. M. Good, Cancer Genome Project, C. M. Jones, C. J. Marshall, C. J. Springer, D. Barford, R. Marais. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 2004; in press.

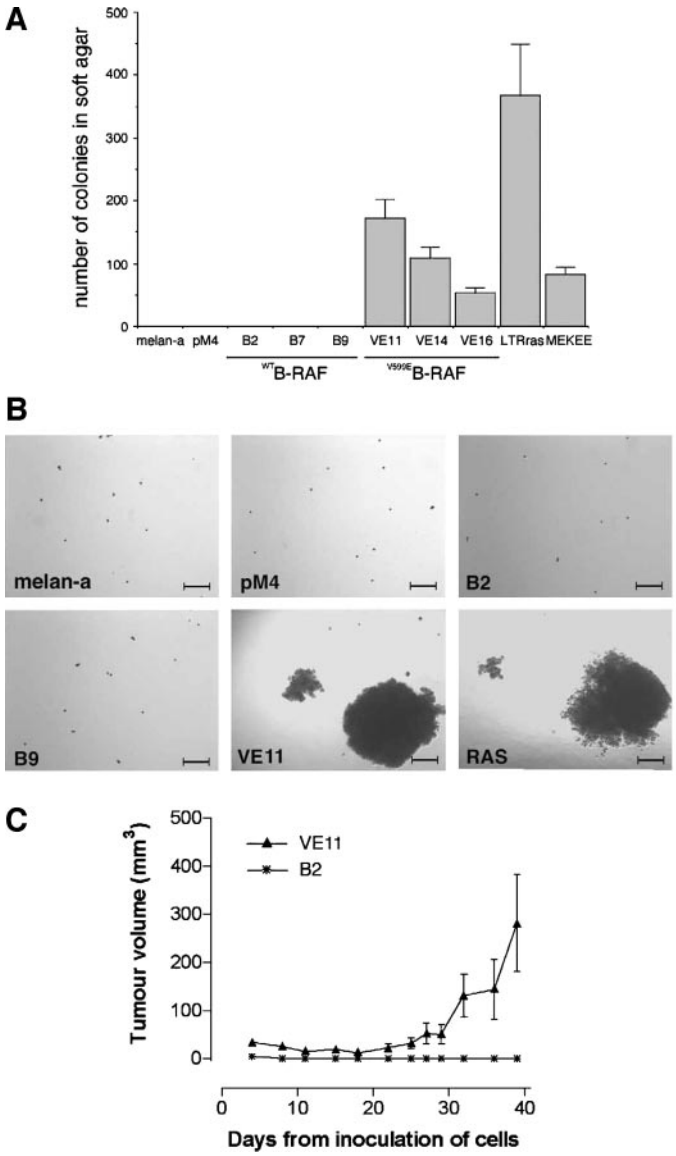

Fig. 2. V599EB-RAF transforms melan-a cells. *A*, colony formation in soft agar. Quantification of colonies formed in soft agar for melan-a cells and the cell lines pM4, B2, B7, B9, VE11, VE14, VE16, LTRras, and MEKEE. *B*, morphology of soft agar colonies. Photographs are presented to show the morphology of cells grown in soft agar for melan-a, pM4, B2, B9, VE11, and LTRras cells. *C*, tumor formation in nude mice. Volumes are shown for tumors formed by inoculation of 1×10^7 VE11 or B2 cells as indicated.

inhibitor U0126 and the RAF inhibitor BAY43-9006 blocked ERK activation and DNA synthesis in V599EB-RAF-transformed melan-a cells (Fig. 3, *A* and *B*). U0126 and BAY43-9006 also blocked constitutive ERK activity and DNA synthesis in LTRras cells (Fig. 3, *A* and *B*). BAY43-9006, previously reported to be a potent inhibitor of C-RAF (19), is also a potent inhibitor of V599EB-RAF (IC_{50} = ~43 nM; data not shown) and is likely to target other cellular protein kinases. However, we have used this compound in conjunction with U0126, a compound from a distinct chemical class that targets another kinase on the same pathway, to demonstrate that signaling through RAF-MEK is essential for ERK activation and proliferation of melanocytes transformed with either V599EB-RAF or G12VRAS.

To determine the contribution made by B-RAF in V599EB-RAF- and G12VRAS-transformed melanocytes, the cells were treated with siRNA oligonucleotides specific for B-RAF or C-RAF. The specificity of these probes is demonstrated by their ability to deplete their target proteins without affecting expression of the other RAF isoform (Fig. 4*A*). A control, scrambled siRNA (Fig. 4A, *sc*) did not affect expression of either protein. B-RAF depletion led to a significant decrease in ERK and MEK activities in V599EB-RAF-transformed melanocytes and strongly suppressed DNA synthesis in these cells (Fig. 4, *A* and *B*; results shown are for the VE11 clone, but identical results were obtained with the VE14 and VE16 clones), whereas C-RAF depletion was without effect. In the LTRras cells, depletion of B-RAF or C-RAF did not reduce MEK and ERK activity or block cell cycle progression, demonstrating that B-RAF is not essential to couple RAS to MEK-ERK activation in G12VRAS-transformed melanocytes.

Finally, we wished to demonstrate that our findings in mouse melanocytes were relevant to human melanoma. First, we examined whether oncogenic B-RAF could stimulate constitutive ERK signaling in melanoma cell lines. We used CHL and WM35 cells because both lines are wild type for B-RAF and wild type for all three RAS proteins (5, 7). In these cells, expression of WTB-RAF did not elevate the FCS-induced basal ERK activity, whereas V599EB-RAF stimulated strong ERK activation (Fig. 4*C*). Thus, in common with the mouse melanocytes, V599EB-RAF stimulated constitutive ERK activity in melanoma cells that do not harbor oncogenic B-RAF or oncogenic RAS. Next we used siRNA to investigate ERK signaling in melanoma lines that harbor either oncogenic B-RAF (WM266-4 cells) or oncogenic RAS (SK-MEL-2 cells; Ref. 5). B-RAF ablation blocked constitutive ERK activity in WM266-4 cells, whereas it only caused a small reduction in ERK activity in SK-MEL-2 cells (Fig. 4*D*). Thus, the data obtained with the mouse melanocytes reflect mechanisms found in human melanoma. V599EB-RAF can stimulate constitutive ERK activity in both cell types, and whereas B-RAF is required for constitutive ERK signaling in cells harboring oncogenic B-RAF, it is not required in cells harboring oncogenic RAS.

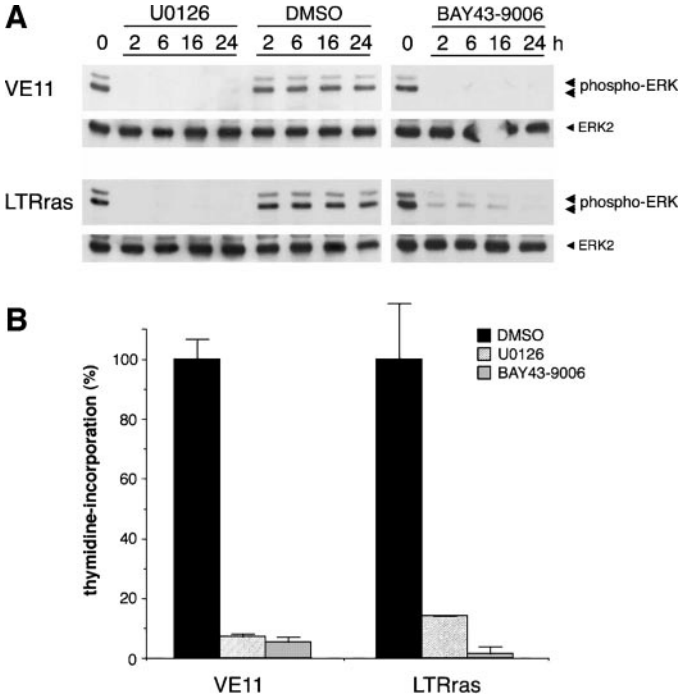

Fig. 3. V599EB-RAF-induced proliferation is dependent on MEK-extracellular signal-regulated kinase (ERK) signaling. *A*, ERK activity. Western blot of phospho-ERK or total ERK from VE11 or LTRras cells treated with U0126 (10 μM), BAY43-9006 (10 μM), or DMSO vehicle for the times indicated. Similar results were obtained with all three V599EB-RAF-expressing clones. *B*, DNA proliferation. [^{3}H]Thymidine incorporation in VE11 and LTRras cells treated with U0126 (10 μM) or BAY43-9006 (10 μM) compared with the DMSO vehicle control, which was set 100%. Results obtained are the average of three independent determinations (*error bars* represent SDs from the mean), and similar results were obtained with all three V599EB-RAF-expressing clones. *P* for each value is $\leq$0.0002.

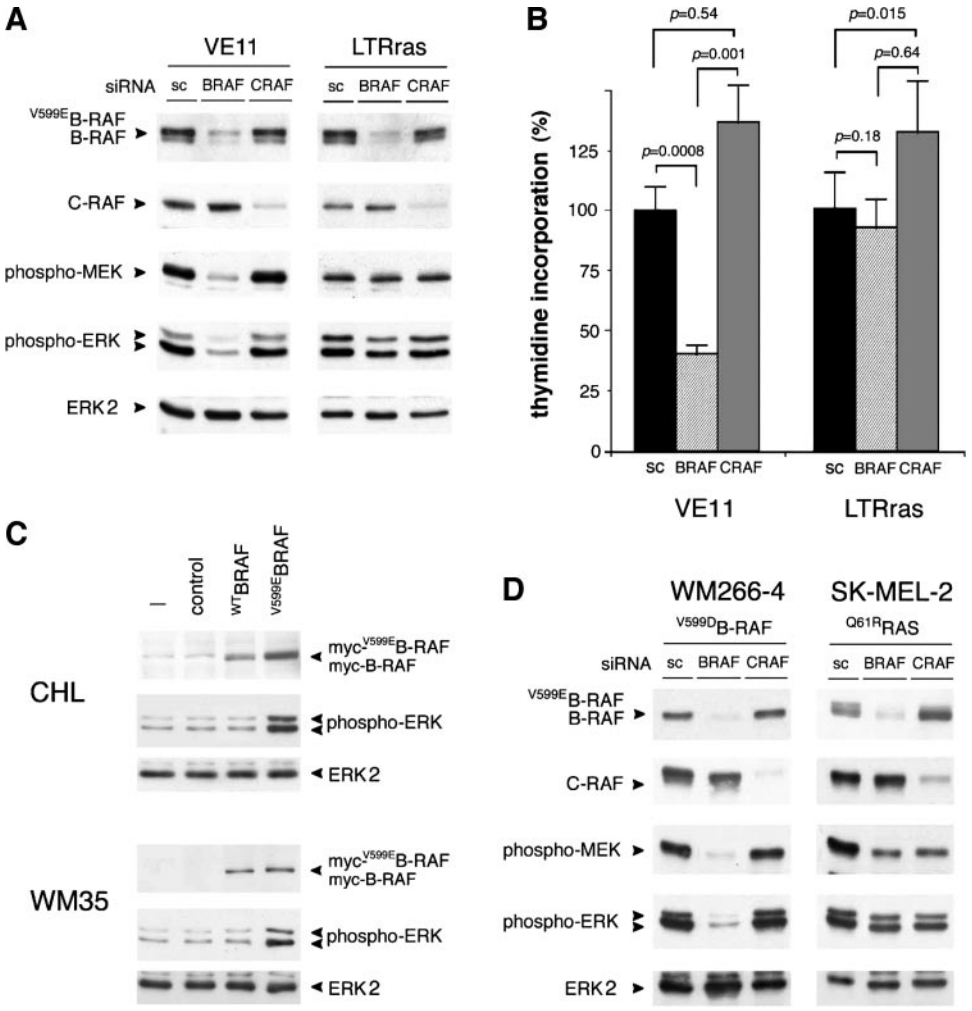

Fig. 4. B-RAF is not required for oncogenic RAS-induced melanocyte proliferation. *A,* MEK and extracellular signal-regulated kinase (ERK) activation. Western blot showing levels of B-RAF, C-RAF, phospho-MEK, phospho-ERK, and total ERK in VE11 and LTRras cells treated with a siRNA to B-RAF, C-RAF, or a scrambled control (*sc*) for 72 h as indicated. Similar results were obtained with all three ^{V599E}B-RAF-expressing clones. *B,* DNA synthesis. [^{3}H]Thymidine incorporation into VE11 or LTRras cells treated with siRNA to B-RAF or C-RAF compared with the scrambled control, which was set 100%. Results shown are the average of three independent determinations (*error bars* represent SDs from the mean); *P*s are indicated. Similar results were obtained with all three ^{V599E}B-RAF-expressing clones. *C* and *D,* ERK activation in melanoma cell lines. *C,* CHL or WM35 cells were transfected with ^{WT}B-RAF or ^{V599E}B-RAF expression constructs or with vector control and Western blotted for the myc-tagged B-RAF proteins, phospho-ERK, or total ERK2 as indicated. *D,* WM266-4 or SK-MEL-2 cells were treated with a B-RAF- or C-RAF-specific siRNA or with a scrambled control (*sc*) as indicated. The cell extracts were blotted for total B-RAF, total C-RAF, total MEK, total ERK, phospho-MEK, and phospho-ERK as indicated.

Discussion

We have shown that ^{V599E}B-RAF is an important oncogene that can transform melan-a cells. Clearly, ^{V599E}B-RAF is not sufficient to induce melanoma because B-RAF is mutated in a high proportion of nevi (6, 8). It has been suggested that the melanocytes in nevi are senescent (4), and it is interesting to note that melan-a cells do not appear to tolerate a high level of B-RAF signaling because all of our ^{V599E}B-RAF-expressing clones expressed the exogenous gene at very low levels compared with ^{WT}B-RAF. Nevertheless, even low levels of ^{V599E}B-RAF transform melan-a cells, possibly because these cells do not express either p16^{INK4A} or p19^{ARF} (20) and thus presumably are unable to enter ERK-induced senescence. Reintroduction of p16^{INK4A} into ^{V599E}B-RAF-transformed melanocytes did not revert the transformed phenotype because the p16^{INK4A} did not enter the nucleus and accumulated in the cytosol (data not shown). Additional experiments are currently underway to address how p16^{INK4A} and ^{V599E}B-RAF cooperate to induce melanocyte transformation and why p16^{INK4A} accumulates in the cytosol.

Despite these limitations, the demonstration that melan-a cells can be transformed by ^{V599E}B-RAF has enabled us to monitor the early changes that lead to pigment cell transformation. ^{V599E}B-RAF induced constitutive ERK activation that is essential for proliferation, as demonstrated by the use of the MEK inhibitor U0126. ^{V599E}B-RAF also overcame the need for TPA, an essential melanocyte mitogen (14) that induces strong and sustained ERK activity in melan-a cells (Fig. 1). These data suggest that ^{V599E}B-RAF is able to replace TPA in the growth medium because of its ability to stimulate constitutive ERK signaling. Importantly, our data show that C-RAF is not required for MEK/ERK activation or proliferation of ^{V599E}B-RAF-transformed melanocytes or in melanoma cells harboring oncogenic B-RAF. This is in agreement with the recent finding that C-RAF is not required for MEK activation or transformation of WM793 cells, another melanoma line that expresses ^{V599E}B-RAF (21). Despite its important role as an oncogene in melanocytes and melanoma, B-RAF is not required for signaling to ERK in RAS-transformed melanocytes or melanoma cells harboring oncogenic RAS. Similarly, C-RAF depletion failed to block MEK/ERK activity and cell cycle progression in oncogenic RAS-expressing melanocytes. However, RAF-MEK-ERK signaling is clearly required for proliferation of both the RAS and the RAF-transformed melanocytes because the RAF inhibitor BAY43-9006 and the MEK inhibitor U0126 blocked the elevated ERK activity in these cells and also blocked their cell cycle progression. Thus, cells that express oncogenic RAS do not require B-RAF to couple signals to MEK because they can route these signals through the other RAF isoforms.

In summary, our results show that B-RAF can act as a potent oncogene in early stages of melanoma development, whereas ^{WT}B-RAF is not required for RAS-induced pigment cell transformation, findings that have important implications for future therapeutic strategies.

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