Oncogenic B-RafV600E Inhibits Apoptosis and Promotes ERK-dependent Inactivation of Bad and Bim*

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Recent studies have revealed that B-Raf mutations are very common in malignant melanoma and are required for tumor growth and maintenance. The majority of melanoma-associated B-Raf mutations involve a single point mutation, V600E, which results in greatly elevated B-Raf kinase activity and constitutive activation of MAPK/ERK downstream. Here we show that B-RafV600E increases resistance to apoptosis induced by chemotherapeutic drugs and promotes ERK-dependent phosphorylation of the BH3-only proteins Bim and Bad that are involved in setting thresholds for apoptosis. ERK-dependent phosphorylation of Bim resulted in degradation of this BH3-only protein, whereas phosphorylation of Bad has previously been shown to result in its sequestration by 14-3-3 proteins. Consistent with this, inhibition of ERK activity in a panel of melanoma cell lines resulted in stabilization of Bim and dephosphorylation of Bad. Furthermore, apoptosis induced through overexpression of Bad or Bim was efficiently blocked by coexpression of mutant B-RafV600E. However, small interfering RNA-mediated silencing of Bim and Bad expression conferred only modest protection against cytotoxic drugs, whereas oncogenic B-Raf strongly protected against the same stimuli. These observations suggest that B-Raf-initiated inactivation of Bad and Bim only partly contributes to the anti-apoptotic activities of this oncogene and that other points within the cell death machinery are also targeted by deregulated ERK signaling.

Malignant melanoma is notable for its highly aggressive nature and resistance to conventional chemotherapeutic drugs (1). Consequently, five-year survival rates for melanoma patients are notoriously poor, and little improvement in treatment modalities for this type of cancer has occurred over the past 40 years (1). A significant breakthrough in our understanding of malignant melanoma occurred with the discovery that ~60% of melanomas harbor mutations in the B-Raf kinase (2–4), the majority of which affect valine 600 and result in dramatic elevation of B-Raf kinase activity (2, 5). Mutant B-RafV600E results in elevated activity of its direct downstream target, MEK4 and of the ERK1/2 kinases in turn (2, 5). However, it remains unclear whether elevated B-Raf kinase activity contributes to the enhanced resistance to chemotherapy seen with malignant melanoma.

Previous studies have shown that growth factors such as interleukin 3, epidermal growth factor, and fibroblast growth factor can regulate the phosphorylation status of certain members of the Bcl-2 family through Ras-dependent MAPK/ERK activation (6–11). Bcl-2 family proteins regulate entry into apoptosis through controlling permeability of the mitochondrial outer membrane (12–15). Mitochondrial outer membrane permeabilization facilitates apoptosis through release of cytochrome c, which acts as a trigger for assembly of a caspase-activating complex in the cytosol called the apoptosome (16). BH3-only members of the Bcl-2 family play a pivotal role in the cell death machinery by acting as sensors for stress, cytokine deprivation, radiation, death receptor activation, cytotoxic lymphocyte attack, and several other triggers of apoptosis (15, 17, 18). Upon activation, BH3-only proteins promote apoptosis through neutralizing apoptosis inhibitory proteins such as Bcl-2 and Bcl-xL and also by promoting opening of the mitochondrial pore or channel comprised by the death-promoting members of the Bcl-2 family, Bax and Bak (19, 20). BH3-only proteins can be subdivided into two sub-groups based upon their ability to act as putative direct activators of Bax and/or Bak, or their ability to neutralize anti-apoptotic Bcl-2 family proteins, such as Bcl-2, Bcl-xL, and Mcl-1 (21–23). Recent studies have shown that Bim and Bid may be the most potent direct activators of the Bax/Bak channel, whereas other members of the BH3-only family act primarily to set a threshold for Bax/Bak channel opening through neutralizing Bcl-2 and its relatives (21–23).

Here we have explored whether oncogenic B-Raf can suppress apoptosis, potentially contributing to the chemoresistance frequently seen in malignant melanoma. We have found that the constitutively active B-RafV600E mutant can promote robust ERK-dependent phosphorylation and destabilization of the BH3-only protein, Bim. Furthermore, oncogenic B-Raf also induced phosphorylation of Bad, which previous studies have shown results in sequestration of the latter by 14-3-3 phosphoserine-binding proteins. Consistent with this, B-RafV600E

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The abbreviations used are: MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; GFP, green fluorescent protein; WT, wild-type.

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increased resistance to apoptosis, and this could be reversed through knockdown of B-Raf but not of C-Raf. These observations suggest that melanoma-associated B-Raf mutations may contribute to chemoresistance in part through ERK-mediated inactivation of the BH3-only proteins Bim and Bad and also at other points within the cell death machinery.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies were obtained from the following suppliers. Anti-B-Raf, anti-Bax, and anti-Bik were from Santa Cruz Biotechnology; anti-phospho-ERK, anti-ERK, anti-phospho-Bad, and anti-Bad were from Cell Signaling Technology; anti-Noxa and anti-Bim were from Calbiochem; anti-Bid and anti-C-Raf were from BD Biosciences; and anti-Puma from Prosci and anti-Actin were from MP Biomedicals. Anti-mouse and anti-rabbit secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. Actinomycin D, daunorubicin, cycloheximide, and cisplatin were obtained from Sigma. LY294002 and MG132 were obtained from Calbiochem, and U0126 was obtained from Cell Signaling. Tumor necrosis factor was obtained from Roche Applied Science. siRNAs were purchased from Ambion.

**Cell Lines**—Melanoma cell lines SK-MEL-1, SK-MEL-3, and SK-MEL-30 were purchased from DSMZ and cultured in RPMI medium supplemented with 10% fetal calf serum. SK-MEL-2 and SK-MEL-28 cells were purchased from LGC Promochem and cultured in RPMI medium supplemented with 10% fetal calf serum. SK-MEL-31 cells were purchased from LGC Promochem and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

**Apoptosis Assays**—HeLa cells were plated at 1 × 10^5 cells/well in 6-well plates and were transfected with various plasmid combinations along with a pAAV-GFP-reporter plasmid using GeneJuice (Novagen). After 24 h, GFP-expressing cells were scored for apoptotic morphology, or alternatively, were treated with pro-apoptotic drugs for an additional 12 h prior to enumeration of apoptosis. A minimum of 300 cells was counted in each treatment.

**Transfection and Western Immunoblotting**—To examine the effect of B-Raf^V600E^ on BH3-only proteins, HeLa cells were plated at 2 × 10^5 cells/well in 6-well plates or 2 × 10^6 cells/10-cm dish. After 24 h, cells were transfected with vector, pEFmB-Raf^WT^, or pEFmB-Raf^V600E^ using GeneJuice. Cell lysates were prepared using SDS-PAGE loading buffer and were electrophoresed on 12% SDS-polyacrylamide gels. Protein expression was examined by immunoblotting. HEK293T cells were plated at 2 × 10^6 cells/cm^2^ and transfected with vector, pEFmB-Raf^WT^, or pEFmB-Raf^V600E^ using calcium phosphate precipitation. To inhibit ERK signaling in melanoma cells, cells were plated at 1 × 10^6 cells/10-cm plate and after 24 h were treated with U0126 (20 μM) or LY294002 (20 μM). Cell lysates were prepared after 12 h later.

**RNA Interference**—To ablate B-Raf^V600E^ expression in SK-MEL-1 melanoma cells, 1.5 × 10^6 cells were transfected with 2 μM siRNA duplex using Amaxa cell line solution V and program T-020 as described by the manufacturer’s protocol. siRNAs were as follows: B-Raf sense, 5′-GGGCAUCGUAG-UUGCA-3′; C-Raf sense, 5′-UAGUUCAGCGUUGCCUA-3′; and control sense, 5′-GAUCGCCAGUGGAAGCUGAC-3′. To ablate B-Raf expression in SK-MEL-2 cells, 2 × 10^5 cells were transfected with 200 nm siRNA duplexes using Oligofectamine (Invitrogen). To ablate Bad, Bim, and Noxa expression in HeLa cells, 2 × 10^6 cells were transfected with 100 nm siRNA using Oligofectamine. Cells were incubated with siRNA for 48 h followed by treatment with cytotoxic drugs for 12 h. siRNAs were as follows: Bad sense, 5′-GUACUUCCUCUCAG-GCCUAU-3′; Bim sense, 5′-GCACCCAUAGAUGUUGA-C-3′; and Noxa sense, 5′-GGUGCAGCUUUCUAUCAAU-3′.

**Image Acquisition and Analysis**—The images presented in Figs. 3B and 7A were taken on an inverted microscope (Olympus IX71) with a ×20 objective. Images were acquired using Analysis image acquisition software and processed using Adobe Indesign. For confocal microscopy presented in Fig. 7C, HeLa cells were grown on coverslips for 24 h, transfected with various plasmid combinations, and treated with the caspase inhibitor z-VAD-fmk (50 μM) to prevent loss of cells from apoptosis. After 18 h, cells were incubated in MitoTracker-CMXRos (Molecular Probes) for 1 h. Cells were then washed in phosphate-buffered saline, pH 7.2, fixed with 3% paraformaldehyde in phosphate-buffered saline for 10 min, and incubated in 20 μM Hoechst (Sigma) for 10 min. Cells were mounted with Slow Fade (Invitrogen) and observed on a laser scanning microscope (Olympus FV1000) using a 488-nm argon laser (green fluorescence), a 543-nm HeNe laser (red fluorescence), and a 405-nm LD laser (Hoechst). Confocal images were acquired with Fluoview 1000 version 1 application software, and images were processed with Adobe Indesign.

**RESULTS**

Previous studies have found that a mutant form of B-Raf, B-Raf^V600E^, is frequently expressed in melanomas and is required for survival of such tumors (2, 3). B-Raf^V600E^ exhibits elevated kinase activity that has been estimated to be 50–100-fold more potent than wild-type B-Raf (5); however, it remains unknown how B-Raf^V600E^ contributes to the increased chemoresistance seen in melanoma. One way in which deregulated oncogene expression may contribute to resistance toward chemotherapy drugs is through increasing the threshold for apoptosis in cells expressing such oncogenes. However, it is unclear whether oncogenic B-Raf can act as an apoptosis-inhibitory kinase.

**Overexpression of B-Raf^V600E^ Blocks Apoptosis**—To address this question, we transiently expressed wild-type B-Raf and the oncogenic B-Raf^V600E^ mutant in HeLa cells to explore whether either of these kinases could increase resistance to pro-apoptotic stimuli. As shown in Fig. 1A, transient overexpression of either wild-type B-Raf or oncogenic B-Raf^V600E^ resulted in activation of the downstream kinase, ERK, with oncogenic B-Raf^V600E^ being much more effective in this regard, despite similar levels of expression of both proteins. Strikingly, both forms of B-Raf blocked apoptosis in response to actinomycin D, daunorubicin, and cisplatin, with B-Raf^V600E^ being considerably more potent than wild-type B-Raf in this regard (Fig. 1B). Thus, deregulated B-Raf activity is capable of raising the threshold for apoptosis, and this correlated with the degree of ERK
activation seen upon overexpression of oncogenic versus wild-type B-Raf.

B-Raf Promotes Phosphorylation of the BH3-only Proteins Bad and Bim—Because accumulating evidence indicates that BH3-only proteins act as key upstream integrators of diverse forms of cellular stress or damage, we wondered whether one or more of these proteins may be targeted for inactivation by oncogenic B-RafV600E. To address this question, we transiently expressed wild-type B-Raf and B-RafV600E in HEK293T and HeLa cells and examined the effects of this on the stability and SDS-PAGE mobility of multiple BH3-only proteins, including Bim, Bad, Bid, Puma, Bik, and Noxa (Fig. 1, C and D). Notably, we observed marked phosphorylation and degradation of Bim and increased phosphorylation of Bad in cells expressing B-RafV600E (Fig. 1, C and D). In contrast, the stability and SDS-PAGE mobility of the other BH3-only proteins examined was not affected under the same conditions, with the exception of Noxa, where increases in expression were seen in the presence of oncogenic B-Raf (Fig. 1, C and D). Titration of B-Raf and B-RafV600E into HeLa cells demonstrated that the effects seen on Bim and Bad were robust and broadly correlated with ERK activation status (Fig. 2A).

Inhibition of ERK Activity in Melanoma Cells Results in Dephosphorylation of Bim and Bad—The preceding experiments suggested that deregulated activation of B-Raf is associated with increased phosphorylation of Bim and Bad and that this correlated with deregulated ERK activation status. To ask whether ERK activity was required for the effects seen, we repeated these experiments in the presence of the ERK pathway inhibitor U0126. As Fig. 2B illustrates, U0126 treatment almost...
completely suppressed B-Raf-induced ERK activation and phosphorylation of Bim and Bad.

We also used a panel of melanoma cell lines, several of which carried B-RafV600E mutations. As Fig. 2C shows, all of the melanoma cell lines displayed constitutively high levels of phosphorylated ERK, and this was efficiently suppressed using the MEK/ERK inhibitor U0126, but not the PI3 kinase inhibitor LY294002. Strikingly, inhibition of ERK activity led to a dramatic accumulation and dephosphorylation of Bim and an almost complete inhibition of Bad phosphorylation in all six of the melanoma lines examined. These data strongly suggest that constitutive ERK activation, as a result of Ras/Raf/ERK pathway mutations, directly impacts on the cell death machinery through phosphorylation of Bim and Bad. Furthermore, Noxa levels were also clearly decreased in response to MEK/ERK inhibition (Fig. 2C), in line with the observation that Noxa expression is also influenced by ERK activation status (Fig. 1, C and D).

**Ablation of B-Raf Results in Spontaneous Apoptosis Associated with Dephosphorylation of Bad and Bim**—To confirm that B-Raf was required for the constitutive ERK activation and Bim and Bad phosphorylation seen in the melanoma lines, we used B-Raf-specific siRNA to ablate expression of this protein in the B-RafV600E-positive cell line, SK-MEL-1 (Fig. 3A). As a control, siRNA directed against C-Raf was used. Knockdown of B-Raf expression dramatically reduced the levels of active phospho-ERK in these cells, whereas ablation of C-Raf expression had little effect (Fig. 3, A and D). Furthermore, ablation of B-Raf resulted in dephosphorylation of Bad and Bim, with the latter event being especially noticeable at 72 h post siRNA treatment (Fig. 3, A and D). In contrast, ablation of C-Raf had little effect on Bad and Bim phosphorylation status. Importantly, SK-MEL-1 cells treated with B-Raf-specific siRNA underwent spontaneous apoptosis and were also sensitized toward a range of chemotherapeutic drugs (Fig. 3, B and C). These experiments provided further evidence that oncogenic B-Raf exerts suppressive effects upon the cell death machinery and that this is correlated with the phosphorylation and inactivation of the BH3-only proteins Bad and Bim. Of note, whereas B-Raf knockdown in SK-MEL-1 cells abolished much of the phospho-ERK seen in these cells (Fig. 3, A and D), this was not the case in Ras mutant SK-MEL-2 cells, where B-Raf knockdown had little effect on phospho-ERK levels (Fig. 3E). Instead, silencing of C-Raf in SK-MEL-2 cells led to reduction in phospho-ERK levels in this context (Fig. 3E), highlighting the increased dependence of the B-RafV600E-positive SK-MEL-1 cells on B-Raf for ERK activation.

**B-Raf-dependent Phosphorylation of Bim Occurs at Ser55 and Ser65**—ERK has previously been implicated in the phosphorylation of Bim and Bad in the context of growth factor-mediated suppression of apoptosis (6–11). To further explore the role of ERK in B-RafV600E-mediated Bim phosphorylation, we used a panel of Bim mutants where individual ERK consensus phosphorylation sites at Ser55, Ser65, and Ser100 were mutated to Ala residues by site-directed mutagenesis (Fig. 4A). Using this panel of mutants, as well as a triple mutant where all putative ERK phosphorylation sites were replaced, we found that the S55A and S65A mutants were both resistant to B-Raf-initiated phosphorylation and degradation (Fig. 4B). Importantly, the proteasome inhibitor, MG132, blocked B-Raf-induced degradation of Bim, confirming that the decreased levels of the latter seen in response to elevated ERK signaling resulted from enhanced proteasome-mediated degradation of Bim under these conditions (Fig. 4C).

**Oncogenic B-Raf Blocks Bad and Bim-dependent Apoptosis**—Because Bim and Bad were phosphorylated as a consequence of B-RafV600E expression, this suggested that this oncoprotein may repress apoptosis initiated by one or both of these BH3-only proteins. To explore this possibility, we transiently overexpressed Bad or Bim in HeLa cells, in the presence or absence of wild-type or oncogenic B-Raf, and assessed apoptosis under
these conditions. Strikingly, oncogenic B-RafV600E afforded substantial protection against Bim-associated apoptosis (Fig. 5A), which is in agreement with its ability to target this protein for phosphorylation and degradation (Fig. 2A). Furthermore, B-Raf also protected against apoptosis initiated through overexpression of Bad (Fig. 5B). In contrast, apoptosis initiated by overexpression of Noxa or Bid was poorly inhibited by deregulated B-Raf activity, suggesting that the effects of B-Raf on apoptosis initiated via BH3-only proteins may be relatively specific (Fig. 5, C and D). In line with this, oncogenic B-Raf failed to block apoptosis induced by tumor necrosis factor receptor ligands (Fig. 5E), which is known to be highly Bid-dependent in HeLa cells (24). Recent studies have also shown that apoptosis resulting from inhibition of the proteasome is Noxa-dependent (25, 26), and B-Raf also failed to protect against cell death induced by the proteasome inhibitor, MG132 (Fig. 3B).

**B-Raf-mediated Targeting of Bad and Bim Is Not Sufficient to Explain B-Raf-mediated Resistance to Apoptosis**—Although Bad and Bim are clearly phosphorylated in response to oncogenic B-Raf, it is not clear whether inactivation of these BH3-only proteins is sufficient to explain the protection seen against diverse pro-apoptotic stimuli (Fig. 1B). To explore this issue, we knocked down expression of Bad and Bim in HeLa cells using specific siRNAs, either individually or in combination, and asked whether this resulted in robust protection from pro-apoptotic stimuli. However, as Fig. 6A illustrates, the protection seen when Bad or Bim were knocked down individually was only minimal, even though expression of both BH3-only proteins was very substantially reduced. Simultaneous knockdown of both Bad and Bim resulted in better protection against actinomycin D and daunorubicin, whereas this had no effect on cisplatin-induced apoptosis (Fig. 6A). Because Noxa was upregulated in response to deregulated B-Raf expression (Fig. 1, C and D), we also explored the effects of Noxa ablation, either alone or in combination with Bad and Bim knockdown (Fig. 6B). Triple knockdown of Bad, Bim, and Noxa resulted in greater protection from actinomycin D- and daunorubicin-induced apoptosis (Fig. 6B) but was still not as effective as B-Raf overexpression (Fig. 6C). Furthermore, expression of B-RafV600E further augmented apoptosis resistance even in cells where Bad, Bim, and Noxa were knocked down (Fig. 6C), suggesting that oncogenic B-Raf targets multiple points within the cell death machinery to suppress apoptosis.
Oncogenic B-Raf Targets Bim and Bad for Inactivation

One of the major downstream consequences of BH3-only protein activation is the triggering of Bax and/or Bak oligomerization within mitochondrial outer membranes (20). Bax/Bak oligomers form a pore or channel that permits the efflux of mitochondrial membrane lipids and proteins, thereby initiating apoptosis through cytochrome c-dependent apoptosome activation. Whereas Bak is constitutively present within the mitochondrial outer membrane, Bax is normally present in the cytoplasm of healthy cells but translocates to mitochondria upon activation. Because the preceding experiments suggested that oncogenic B-Raf could block apoptosis through targeting certain BH3-only proteins for modification in an ERK-dependent manner, we wished to confirm that this oncogene could block Bax activation and translocation to mitochondria. To explore this issue we used a GFP-labeled Bax and expressed this protein in the presence or absence of wild-type or oncogenic B-Raf. As Fig. 7, A and B illustrate, cells transfected with GFP-Bax alone typically underwent apoptosis, but this was significantly inhibited by oncogenic B-RafV600E and to a much lesser degree by wild-type B-Raf. Furthermore, examination of GFP-Bax expression pattern revealed that Bax was localized to mitochondria in a punctate pattern in the majority of cells where Bax was transfected alone (Fig. 7, C and D). However, in sharp contrast, upon cotransfection with B-RafV600E, Bax was localized to the cytoplasm in the majority of cells (Fig. 7, C and D).

Collectively, these data suggest that oncogenic B-Raf can suppress apoptosis upstream of Bax/Bak-dependent mitochondrial permeabilization through targeting the BH3-only proteins Bim and Bad, and most likely additional proteins that operate upstream of Bax and Bim, for ERK-dependent phosphorylation and inactivation.

DISCUSSION

Here we have shown that oncogenic B-Raf can exert inhibitory effects on the cell death machinery through deregulated ERK activation. Specifically, B-Raf was found to promote ERK-dependent phosphorylation and degradation of the BH3-only protein Bim, as well as phosphorylation of another member of the BH3-only family, Bad. Ablation of B-Raf expression in the B-RafV600E-positive cell line SK-MEL-1 increased the susceptibility of these cells to chemotherapeutic drugs such as cisplatin and daunorubicin and also resulted in spontaneous apoptosis. In contrast, ablation of the related C-Raf kinase did not affect the threshold for apoptosis in the same cell line. Furthermore, ablation of B-Raf expression in SK-MEL-1 cells also led to the dephosphorylation of Bad and Bim and increased stability of the latter. Conversely, transient expression of oncogenic B-Raf suppressed Bad- and Bim-initiated apoptosis. Collectively, these data suggest that oncogenic B-Raf can target BH3-only proteins within the cell death machinery for inactivation, in an ERK-dependent manner, and can increase resistance to apoptosis initiated through pathways dependent on Bad and Bim. However, knockout studies involving Bad and Bim demonstrated that targeting of these proteins for inactivation by oncogenic B-Raf was not sufficient to explain the totality of the resistance to apoptosis seen with this oncogene. Thus, in addition to ERK-dependent phosphorylation of Bad and Bim, B-Raf is also very likely to modulate apoptosis at additional points within the cell death machinery.

Previous studies have linked the Ras/Raf/ERK pathway with regulation of BH3-only protein stability. Cook and co-workers (6, 7) have shown that serum withdrawal leads to decreases in ERK activation and consequent dephosphorylation and accumulation of Bim. Conversely, ectopic expression of a constitutively active Raf-1 led to phosphorylation and inactivation of Bim (6). Phosphorylation of Bim appears to target this protein for polyubiquitination and subsequent degradation via the ubiquitin-proteasome pathway (6, 7), which is consistent with the decreased expression of phosphorylated...
Bim consistently seen in the present study. ERK-dependent phosphorylation of Bad has previously been linked with sequestration of the latter by the phosphoserine-binding 14-3-3 proteins (27, 28) rather than proteasome-mediated destruction of this protein. Upon withdrawal of interleukin 3 from responsive cell lines, Bad becomes dephosphorylated and is available for displacing anti-apoptotic Bcl-2 family members from Bax and/or Bak, thereby lowering the threshold for apoptosis (27).

Several studies have demonstrated the oncogenic potential of B-RafV600E and its involvement in melanoma progression, whereas other studies have implicated this oncogene in various tumor-promoting functions such as vascular development and melanoma immune evasion (29–32). Interestingly, cell death induction following inhibition of B-RafV600E in melanoma using siRNA or the Raf inhibitor BAY 43-9006 has also been described, suggesting a dual role for this oncogene in melanoma cell growth and survival (29, 33). Here we demonstrate that oncogenic B-RafV600E targets the BH3-only proteins Bim and Bad for inactivation in melanoma cells, suggesting that B-Raf enhances melanoma survival by raising the apoptotic threshold in melanoma cells through constitutive ERK-dependent inhibition of these BH3-only proteins. As Bim and Bad have been implicated in apoptosis induction in response to growth factor withdrawal and anoikis, inactivation of these BH3-only proteins by B-RafV600E may aid the development and progression of melanoma (27, 34, 35).

Treatment of melanoma cells with B-Raf siRNA sensitized these cells to a number of chemotherapeutic drugs, demonstrating the potential of targeting B-RafV600E in melanoma treatment. Furthermore, ablation of B-RafV600E but not C-Raf in SK-MEL-1 cells resulted in spontaneous apoptosis, suggesting that these cells become highly dependent on the B-Raf oncogene. Previous observations have similarly described enhanced sensitivity of melanoma cells harboring B-RafV600E to MEK inhibition, suggesting that therapy directed against B-RafV600E may specifically reduce melanoma tumors with few adverse effects on normal melanocytes (35). In addition, preliminary studies using mice models of melanoma demonstrated reduced tumor development and regression of previously existing tumors treated with the MEK inhibitor C1 1040 or short hairpin RNA targeted against B-RafV600E, indicating that B-RafV600E is a promising target for melanoma treatment (30, 36). Thus targeting the MAPK pathway in melanoma cells containing the B-RafV600E mutation, in conjunction with conventional chemotherapy, may be an important

![FIGURE 7. B-RafV600E suppresses Bax translocation to mitochondria. A and B, HeLa cells were transfected with 500 ng of either pCDNA3 vector or expression plasmids encoding B-RafWT or B-RafV600E, along with 30 ng of a GFP-tagged Bax plasmid. After 18 h, apoptosis was analyzed by phase contrast microscopy (A) or by quantification of cell death in GFP-positive cells (B). Results represent triplicate counts of 100 cells/treatment and are representative of three independent experiments. C, HeLa cells were transfected with 500 ng of either pCDNA3 vector or expression plasmids encoding B-RafWT or B-RafV600E, along with 30 ng of a GFP-tagged Bax plasmid. All treatments were carried out in the presence of z-VAD-fmk (50 μM) to block caspase-dependent cell detachment. After 18 h, cells were stained with MitoTracker dye followed by fixation, and cells were subsequently stained with Hoechst dye and analyzed by confocal microscopy. D, cells treated as described in C were scored for Bax localization to mitochondria (punctate) or the cytosol (diffuse). Results represent triplicate counts of 100 cells/treatment and are representative of three independent experiments.]

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means of increasing the efficacy of chemotherapeutic drugs and improving survival rates of patients with this form of cancer.

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