

ORIGINAL ARTICLE

CRAF inhibition induces apoptosis in melanoma cells with non-V600E BRAF mutationsKSM Smalley^{1,7}, M Xiao¹, J Villanueva¹, TK Nguyen¹, KT Flaherty^{2,6}, R Letrero³, P Van Belle⁴, DE Elder⁴, Y Wang⁵, KL Nathanson^{3,6} and M Herlyn¹

¹The Wistar Institute, Philadelphia, PA, USA; ²Division of Hematology–Oncology, University of Pennsylvania School of Medicine, Philadelphia, PA, USA; ³Division of Medical Genetics, University of Pennsylvania School of Medicine, Philadelphia, PA, USA; ⁴Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, USA; ⁵Department of Otorhinolaryngology, University of Pennsylvania School of Medicine, Philadelphia, PA, USA and ⁶The Abramson Cancer Center, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

Here, we identify a panel of melanoma lines with non-V600E mutations in *BRAF*. These G469E- and D594G-mutated melanomas were found to exhibit constitutive levels of phospho-extracellular signal-regulated kinase (pERK) and low levels of phospho-mitogen-activated protein kinase/ERK kinase (pMEK) and were resistant to MEK inhibition. Upon treatment with the CRAF inhibitor sorafenib, these lines underwent apoptosis and associated with mitochondrial depolarization and relocalization of apoptosis-inducing factor, whereas the *BRAF*-V600E-mutated melanomas did not. Studies have shown low-activity mutants of *BRAF* (G469E/D594G) instead signal through CRAF. Unlike BRAF, CRAF directly regulates apoptosis through mitochondrial localization where it binds to Bcl-2 and phosphorylates BAD. The CRAF inhibitor sorafenib was found to induce a time-dependent reduction in both BAD phosphorylation and Bcl-2 expression in the D594G/G469E lines only. Knockdown of CRAF using a lentiviral shRNA suppressed both Bcl-2 expression and induced apoptosis in the D594G melanoma line but not in a V600E-mutated line. Finally, we showed in a series of xenograft studies that sorafenib was more potent at reducing the growth of tumors with the D594G mutation than those with the V600E mutation. In summary, we have identified a group of melanomas with low-activity *BRAF* mutations that are reliant upon CRAF-mediated survival activity.

Oncogene advance online publication, 15 September 2008; doi:10.1038/onc.2008.362

Keywords: melanoma; BRAF; CRAF; targeted therapy; sorafenib

Introduction

The discovery of activating V600E *BRAF* mutations in approximately 50% of melanomas has raised the expectations for targeted therapy (Davies *et al.*, 2002; Houben *et al.*, 2008). The main downstream target of the *BRAF* V600E mutation is the mitogen-activated protein kinase (MAPK) pathway, and it is known that high constitutive MAPK activity accounts for the increased proliferation rates, enhanced cell survival and invasive behavior of melanomas (Smalley, 2003; Gray-Schopfer *et al.*, 2007). As a result, the pharmacological targeting of BRAF/MAPK signaling in melanoma is now being intensively studied in both the clinical and preclinical settings (Eisen *et al.*, 2006).

There is now a growing realization that there are a substantial group of melanomas (>33%) that do not harbor *BRAF* V600E mutations that may require alternate therapeutic strategies. One possible alternate oncogene in melanoma is the closely related serine-threonine kinase CRAF (or Raf-1). Like BRAF, CRAF is also associated with the plasma membrane and can activate MAPK signaling (Kyriakis *et al.*, 1992). However, unlike BRAF, CRAF also has other functions independent of MAPK signaling and is known to regulate downstream effectors such as MST-2 and ASK-1 (Chen *et al.*, 2001; O'Neill *et al.*, 2004). Another intriguing aspect of CRAF signaling is its association with the mitochondria, where it directly regulates apoptosis independently of MAPK signaling (Wang *et al.*, 1996a, b). The anti-apoptotic effects of CRAF arise through its direct binding to Bcl-2 (Wang *et al.*, 1996a), an interaction that leads to the phosphorylation of BAD (von Gise *et al.*, 2001; Jin *et al.*, 2005). Although melanomas are not known to harbor activating *CRAF* mutations, it has been shown that melanomas harboring mutations in *NRAS* may signal through CRAF (Dumaz *et al.*, 2006). Recent studies have also shown that BRAF can activate CRAF, through direct protein–protein interaction and the phosphorylation of CRAF by BRAF (Dhomen and Marais, 2007). Although most work to date has focused upon the *BRAF* V600E mutation, at least 70 other low-frequency

Correspondence: Dr KSM Smalley, Department of Molecular Oncology, The Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, SRB, Tampa, FL 33612, USA.

E-mail: k.smalley@mac.com or M Herlyn, The Wistar Institute, 3601 Spruce Street, Philadelphia, PA, USA.

E-mail: herlynm@wistar.org

⁷Current address: Department of Molecular Oncology, The Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, USA.

Received 13 May 2008; revised 6 August 2008; accepted 18 August 2008

BRAF mutations have been identified (Wan *et al.*, 2004). Unlike the *BRAF* V600E mutation, which can activate MAPK signaling directly, many of the other *BRAF* mutations are of 'low-activity' and are only able to weakly activate MAPK signaling in isolated kinase assays (Wan *et al.*, 2004). However, when these same low-activity *BRAF* mutants are expressed in COS-1 cells, they induce high levels of constitutive MAPK activity: a process driven through the activation of CRAF (Wan *et al.*, 2004).

In this study we identified a panel of melanoma cell lines with three non-V600E *BRAF* mutations (K601E, G469E and D594G). Two of these (G469E and D594G) are low-activity *BRAF* mutants and these cell lines are highly resistant to treatment with a mitogen-activated protein kinase/ERK kinase (MEK) inhibitor but highly sensitive to sorafenib-induced apoptosis. Sorafenib is a kinase inhibitor that has undergone extensive clinical evaluation in melanoma. Although suggested to be a BRAF inhibitor, sorafenib actually has a fourfold higher selectivity for CRAF > BRAF, as well as inhibitory effects against a number of other kinases (Wilhelm *et al.*, 2004). Here, we have shown that CRAF inhibition using either sorafenib or a CRAF shRNA led to a MEK-independent decrease in Bcl-2 expression and apoptosis. It is therefore likely that there exists a group of melanomas with low-activity *BRAF* mutations that may be highly sensitive to sorafenib-induced apoptosis.

Results

Identification of human melanomas with low-activity *BRAF* mutations with sensitivity to sorafenib-induced apoptosis

Most studies to date have focused upon the role of the *BRAF* V600E mutation in melanoma. In this study, we profiled a total of 90 melanoma samples that were mutationally screened for mutations in *BRAF* (Exon 11 and 15), *NRAS* and *KIT*, in the order indicated in Supplementary Figure 1. The most prevalent mutation was the Exon 15, *BRAF* V600E mutation (Table 1). A number of other V600 mutations, such as V600K and V600R, were also identified, although at much lower frequency. One patient was identified with a low-activity Exon 11, G469A *BRAF* mutation. The next most significant group of patients harbored mutations in *NRAS*, with a limited number of patients harboring mutations in *KIT* (Table 1). Mutational profiling of our melanoma cell line panel identified three cell lines with non-V600E mutations in *BRAF* (Table 2). Of these cell lines, one (WM3629) had the D594G *BRAF* mutation, another (WM3670) had the G469E *BRAF* mutation and one line (WM3130) had a K601E *BRAF* mutation.

Western blot analysis revealed all three of the non-V600E *BRAF* mutant cell lines to have constitutive levels of phospho-extracellular signal-regulated kinase (pERK) (Figure 1a). Levels of pERK were only serum-dependent in the 1205Lu and WM3629 cell lines

Table 1 Mutational status of human melanoma samples

Mutation	Number of patients
<i>BRAF</i> —Exon 15	67
V600E	60
V600K	5
V600R	1
<i>BRAF</i> —Exon 11	1
G469A	1
<i>NRAS</i> —Exon 3	16
Q61K	5
Q61L	2
Q61R	5
<i>NRAS</i> —Exon 2	4
G13R	2
G13D	2
<i>KIT</i>	2
G565V/N822I	1
P577L	1

A total of 90 melanoma samples were analyzed. The scheme for analysis is shown in Supplemental Figure 1.

Table 2 Mutational status of the melanoma cell lines

Cell line	<i>BRAF</i>	<i>BRAF</i> kinase activity ^a
1205Lu	V600E	478
451Lu	V600E	478
WM3130	K601E	138
WM3629	D594G	0.6
WM3670	G469E	1.3

A total of 85 cell lines were mutationally profiled.

^aKinase activity as determined by Wan *et al.* (2004).

(Supplementary Figure 2). The *BRAF* V600E-mutated and the K601E melanoma cell lines also had constitutive phospho-MEK (pMEK), whereas this was lacking in the cell lines with the D594G (WM3629) and G469E (WM3670) *BRAF* mutation (Figure 1a). All of the cell lines tested had some degree of phospho-AKT activity. Although the low-activity mutant melanoma cell lines retained PTEN expression (Figure 1a), the protein was phosphorylated, indicating its inactivity.

Previous studies have suggested that the presence of the *BRAF* V600E mutation predicts response to MEK inhibition (Solit *et al.*, 2006). Here, we show that the D594G (WM3629) and the G469E (WM3670) melanoma cell lines are nearly completely resistant to the growth inhibitory effects of the MEK inhibitor U0126 (Figure 1b). In contrast, the melanoma cell lines harboring either the *BRAF* V600E or K601E mutation were markedly growth-inhibited following U0126 treatment (Figure 1b). Interestingly, the D594G mutated melanoma line WM3629 was highly sensitive to growth inhibition following treatment with the kinase inhibitor sorafenib (Figure 1c), whereas the cell lines with the V600E, G469E and K601E *BRAF* mutations were growth-inhibited, but not as highly sensitive.

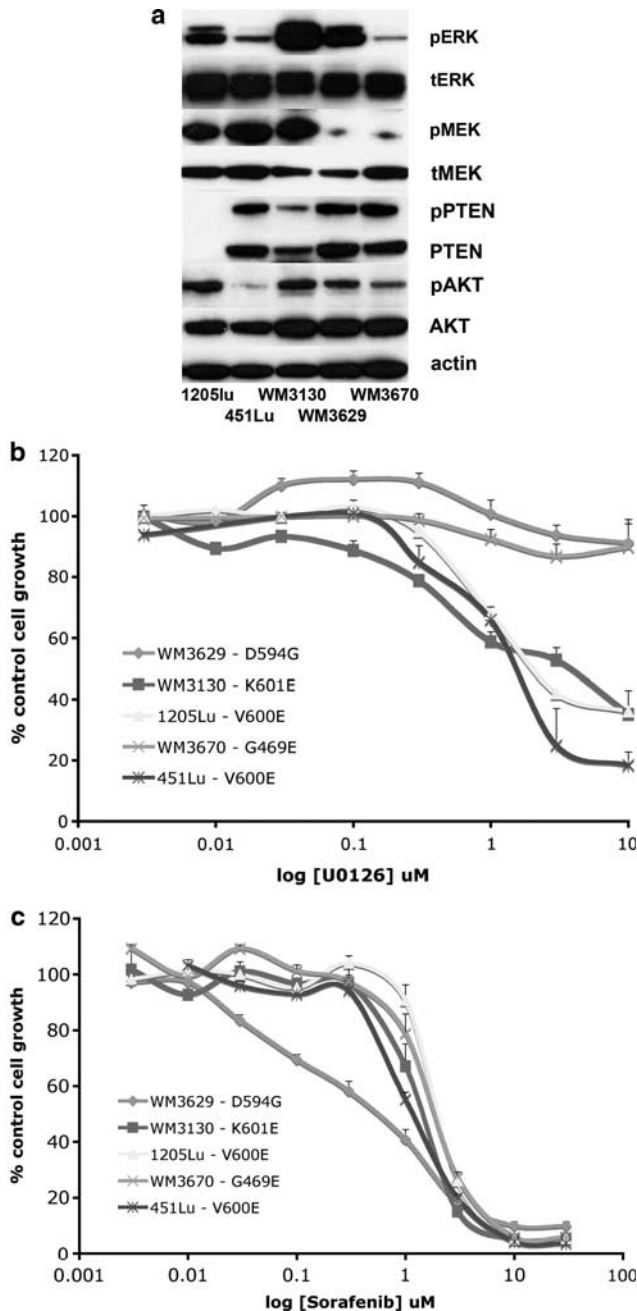


Figure 1 Melanomas with low-activity *BRAF* mutants have low pMEK and are resistant to MEK inhibition. **(a)** Protein expression of phospho-ERK (pERK), total ERK (tERK), phospho-MEK (pMEK), total MEK (tMEK), phospho-PTEN (pPTEN), total PTEN (PTEN), phospho-AKT (pAKT) and total AKT (AKT) in melanoma cell lines with the *BRAF* V600E mutation (1205Lu, 451Lu), the K601E mutation (WM3130), the D594G mutation (WM3629) and the G469E mutation (WM3670). **(b)** The G469E- and D594G-mutated melanoma cells are resistant to MEK inhibition. Cells were treated with increasing concentrations of a MEK inhibitor (U0126) (3 nM–10 μ M) for 72 h and cell proliferation was measured by the MTT assay. **(c)** D594G-mutated melanoma cells are highly sensitive to the CRAF inhibitor sorafenib. Cells were treated with increasing concentrations of sorafenib for 72 h; cell proliferation was measured as in **(b)**. Data shows the mean of three independent experiments \pm s.e.m.

Sorafenib preferentially induces apoptosis in melanoma cell lines with low-activity mutations in BRAF

Sorafenib is a small-molecule kinase inhibitor with selectivity for CRAF over BRAF. Treatment of the melanoma cell line panel with sorafenib (3 μ M, 24 h) led to a marked apoptosis in the cell lines with the D549G and G469E *BRAF* mutations (Figure 2a). The two cell lines with the *BRAF* V600E mutation (1205Lu and 451Lu) were found to undergo only a G1-phase cell cycle arrest (Figure 2a) and the cell line with the K601E *BRAF* mutation underwent limited apoptosis (<15%). Treatment of three NRAS-mutated melanoma cell lines (WM1361A, WM1366, WM1346) with sorafenib (3 μ M, 24 h) showed a G1-phase cell cycle arrest but no apoptosis induction (not shown). Treatment of the V600E-, D594G-, G469E- and K601E-mutated melanoma lines with U0126 (30 μ M, 24 h) did not lead to apoptosis induction, demonstrating that the sorafenib-induced apoptosis was MEK independent (not shown). As CRAF is known to regulate apoptosis at the level of the mitochondria, we next looked at the ability of sorafenib in reducing mitochondrial membrane potential using tetramethyl rhodamine methylester (TMRM). Treatment of the D594G- and G469E-mutated melanoma cell lines, with sorafenib (3 μ M, 8 h) led to 31 and 36% loss of TMRM, respectively, compared with a TMRM loss of 10% in the *BRAF* V600E-mutated 1205Lu cell line (Figure 2b). Consistent with the increased TMRM loss seen in the low-activity *BRAF*-mutated melanoma cell lines, it was also shown that sorafenib (3 μ M, 0–24 h) induced a selective cleavage of caspase-3 in the WM3629 and WM3670 cell lines (Figure 2c and not shown) and not the 1205Lu cell line. Pretreatment of the WM3629 cells with z-vad-FMK (15–50 μ M) was not found to significantly reduce the extent of apoptosis induction, indicating that caspase cleavage may have been a secondary effect of apoptosis induction. Although sorafenib was not pro-apoptotic in the 1205Lu cell line, it induced p53 expression in a concentration-dependent manner, whereas treatment of the WM3629 cell line with sorafenib did not alter the expression of p53 (Supplementary Figure 3A). Interestingly, the upregulated p53 in the 1205Lu cell line was not associated with increased p21 expression, demonstrating a lack of transcriptional activity.

Apoptosis arising following mitochondrial depolarization is also known to involve the release of apoptosis-inducing factor (AIF) from the mitochondria and its relocalization to the nucleus. Initial studies, costaining the WM3629 cells with both mitotracker orange and AIF, showed that AIF associated with the mitochondria (Supplementary Figure 3B). It was further found that treatment of the WM3629 and WM3670 cells with sorafenib (3 and 10 μ M, 24 h) but not U0126 (30 μ M, 24 h) led to the nuclear localization of AIF (Figure 3). At the same time, sorafenib treatment also led to the loss of mitotracker orange staining, demonstrating loss of mitochondrial integrity.

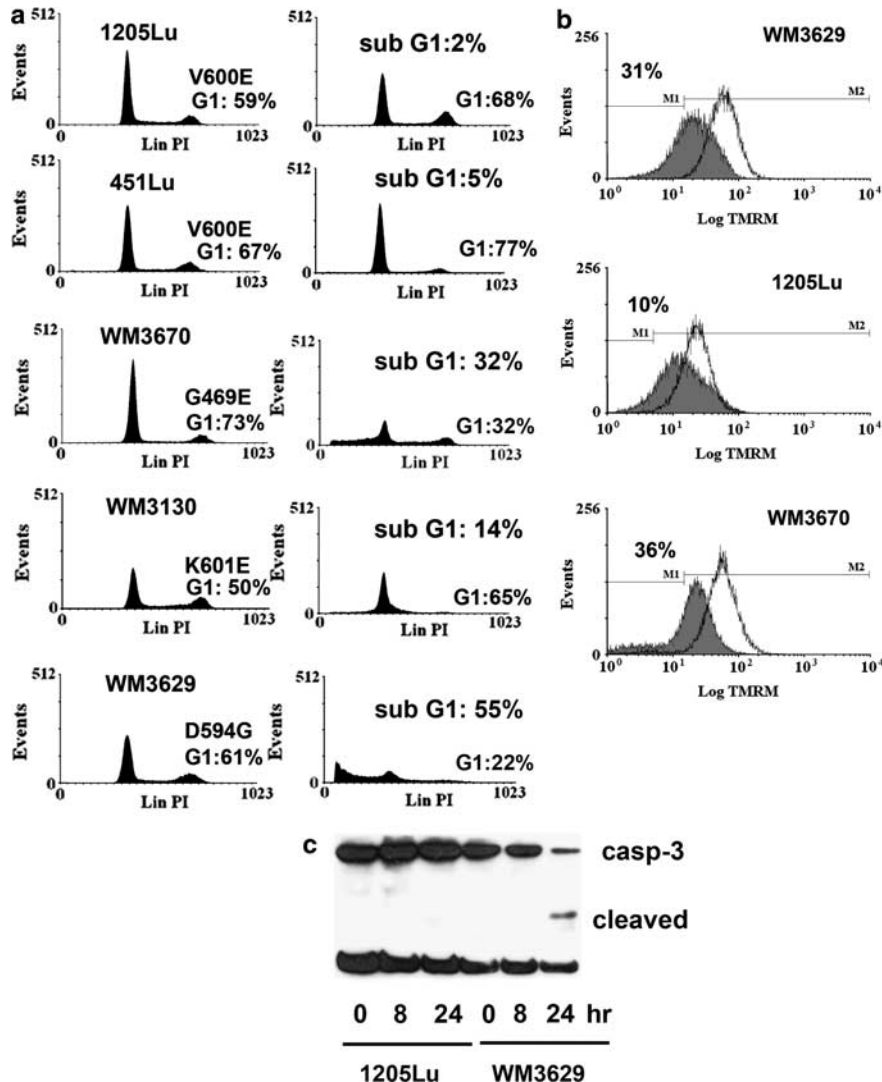


Figure 2 Low-activity *BRAF* mutant melanoma cells are sensitive to sorafenib-induced apoptosis. **(a)** Selective sorafenib-induced apoptosis in non-V600E-mutated melanoma cell lines. Cells were treated with sorafenib ($3 \mu\text{M}$, 24 h), before being harvested, fixed and stained with propidium iodide. Figure shows sample cell cycle profiles. Extent of apoptosis induced is indicated by the sub-G1 peak. **(b)** Sorafenib treatment enhances the loss of mitochondrial membrane potential in melanoma cells with D594G/G469E mutations. Cells were treated with sorafenib ($3 \mu\text{M}$ 8 h), before being stained with TMRM and analysed by flow cytometry. **(c)** Sorafenib selectively induces caspase-3 cleavage in D594G-mutated melanoma cells. Melanoma cells were treated with sorafenib ($3 \mu\text{M}$, 0–24 h), followed by protein extraction and probing for cleavage of caspase-3. Equal protein loading is shown by stripping of the blot and probing for actin expression.

Sorafenib induces apoptosis by modulating *Bcl-2* expression in a *MEK*-independent manner

Having shown that sorafenib-induced apoptosis was independent of *MEK* inhibition, we next turned our attention to the mechanism of sorafenib-induced apoptosis in the D594G- and G469E-mutated melanoma cell lines. We first examined the relative selectivity of sorafenib to block pERK activity in the V600E- and D594G-mutated melanoma cell lines. Sorafenib completely blocked pERK activity of the D594G-mutated cell line WM3629 at $< 100 \text{ nM}$, whereas little effect was seen in the 1205Lu cell line until $3 \mu\text{M}$ of sorafenib (Figure 4a). As this possibly indicated that sorafenib was having a CRAF-specific effect in the WM3629 cell line, we next tested the ability of the *MEK* inhibitor

U0126 to inhibit pERK in the two cell lines and found that U0126 had equivalent activity in inhibiting pERK in the WM3629 and 1205Lu cell lines (Figure 4b), suggesting that the selective effects of sorafenib upon the WM3629 cell line were likely to be CRAF mediated.

CRAF is known to suppress apoptosis through a direct association with *Bcl-2*. Cellular fractionation studies showed CRAF to be associated with the mitochondria in both the WM3670 and WM3629 cell lines (Supplementary Figure 4). Sorafenib treatment led to a time-dependent ($< 8 \text{ h}$) downregulation in both *Bcl-2* and phospho-BAD expression in the two cell lines with the D594G and G469E *BRAF* mutations, but not those with either the V600E or K601E *BRAF* mutation (Figures 4b and c and data not shown). In contrast,

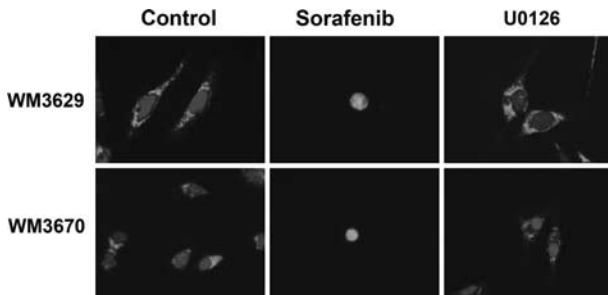


Figure 3 Sorafenib but not U0126 leads to the nuclear relocalization of apoptosis-inducing factor (AIF). WM3629 and WM3670 cells were treated with either vehicle (control), sorafenib (3 μ M, 24 h) or U0126 (30 μ M, 24 h) before being fixed, permeabilized and stained for AIF (green) and DAPI (blue). Original magnification $\times 60$. See online version for colour figure.

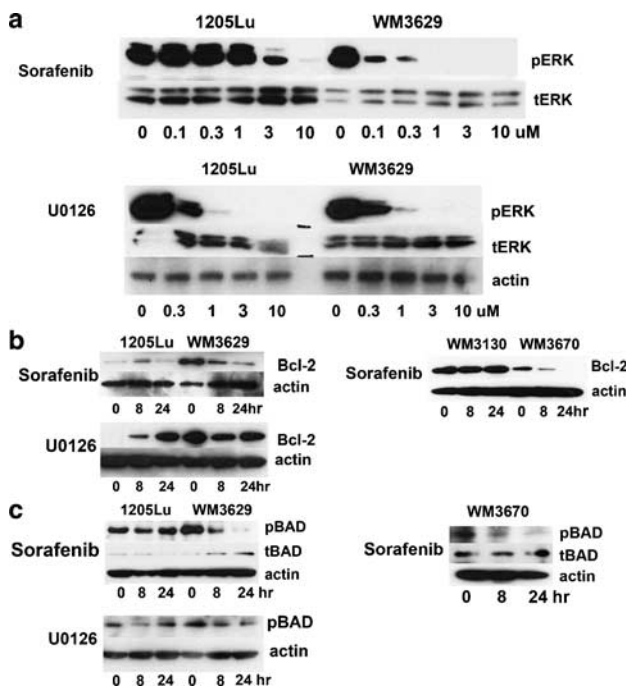


Figure 4 Sorafenib decreases Bcl-2 and pBAD expression in low-activity *BRAF* mutant melanoma cells in a MEK-independent manner. (a) Sorafenib potently inhibits pERK activity in the D594G *BRAF*-mutated cells. 1205Lu (V600E)- and WM3629 (D594G)-mutated melanoma cells were treated with either sorafenib (0.1–3 μ M, 1 h) or U0126 (0.3–10 μ M, 1 h) before being extracted, resolved and probed for either pERK (pERK) or total ERK (tERK). (b) Sorafenib reduces Bcl-2 expression in the D549G- and G469E-mutated melanoma lines in a MEK-independent manner. Cells were treated with either sorafenib (3 μ M, 0–24 h) or U0126 (30 μ M, 0–24 h) after which time protein was extracted, resolved and probed for expression of Bcl-2. Blots were stripped and probed for actin to demonstrate equal protein loading. (c) Sorafenib selectively downregulates BAD activity in the D594G- and G469E-mutated melanoma cell line in a MEK-independent manner. Cells were treated with drug as in (b), and resulting protein extracts were probed for expression of Ser75 pBAD (pBAD) and total BAD (tBAD). Blots were stripped and probed for actin to demonstrate equal protein loading.

treatment of the cells with U0126 (30 μ M) was not found to reduce Bcl-2 or phospho-BAD expression in the WM3629 and WM3670 cell lines, again suggesting that

the effects of sorafenib were MEK independent (Figures 4b and c). Sorafenib treatment was also found to downregulate expression of Mcl-1 (Supplementary Figure 5). The effects of sorafenib upon Mcl-1 expression were likely to be independent of *BRAF* mutational status, as similar effects were observed in both the 1205Lu and WM3629 cell lines.

CRAF shRNA knockdown reduces MAPK signaling and induces apoptosis in a low-activity BRAF-mutated melanoma cell line

We next looked at the prosurvival role of CRAF signaling in the WM3629 cell line. Infection with an shRNA targeted against CRAF led to protein knockdown (Figure 5a). The shRNA construct that gave the best level of CRAF knockdown (clone no. 3) was found to inhibit pERK in the WM3629 cell line, but not the 1205Lu cells, suggesting that ERK activation is mediated, in part, through CRAF. Knockdown of CRAF using shRNA clone 3 was also shown to reduce the expression of Bcl-2 and pBAD in the WM3629 cell lines, but not the 1205Lu cell line (Figure 5a). The downregulation of CRAF expression was also found to induce apoptosis in the WM3629 cell lines, but not the 1205Lu cell line, as characterized by the appearance of a sub-G1 peak (24% apoptotic cells) (Figure 5b) and increased TdT-mediated dNTP nick end labeling staining (Figure 5c). As further demonstration of the essential role of CRAF in the survival of this subgroup of melanoma cells, we showed that shRNA knockdown of CRAF led to a marked suppression of tumor-forming ability *in vivo* (Figure 5d). In a final series of studies, we demonstrated that the shRNA knockdown of Bcl-2 increased apoptosis in the WM3629 cell line (Supplementary Figure 6A–C), demonstrating the importance of Bcl-2 expression in maintaining the survival of melanoma cell lines with the D549G *BRAF* mutation.

Sorafenib induces some regression of D594G BRAF-mutated melanoma xenografts, but only slowing of BRAF V600E-mutated melanoma xenografts

We next grew both the WM3629 and 1205Lu cells as tumor xenografts in severe combined immunodeficient (SCID) mice, to assess whether sorafenib had any selective effects upon the D594G-mutated WM3629 cell line. After tumor establishment (5 \times 5 mm), mice were dosed three times per week with either vehicle or sorafenib tosylate (60 mg/kg) by oral gavage. After 14 days, it was found that sorafenib treatment had suppressed growth of the WM3629 cell line (Figure 6a), leading to some degree of regression. In contrast, although sorafenib treatment reduced the growth of the V600E-mutated 1205Lu melanoma xenograft, these tumors continued to grow in the presence of drug (Figure 6b).

Discussion

Although the most prevalent activating oncogene in melanoma is the V600E-mutated form of *BRAF*, there

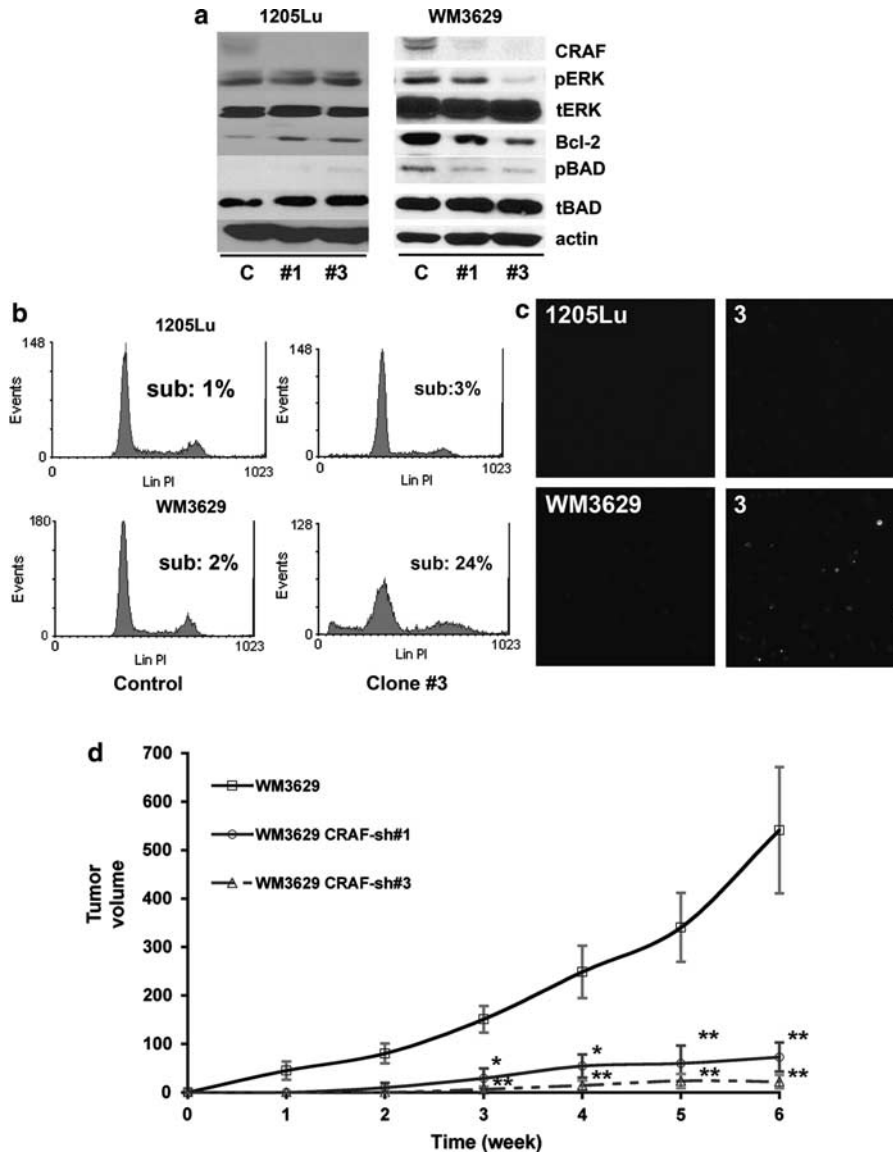


Figure 5 Low-activity *BRAF* mutant melanoma cells depend upon CRAF for MEK activity and Bcl-2-mediated cell survival. (a) Knockdown of CRAF reduces the pERK activity in D594G- and G469E *BRAF*-mutated melanoma cells. Cells with the *BRAF* V600E (1205Lu) or D594G (WM3629) mutation were infected with either a control lentiviral shRNA (c) or one of two clones directed against CRAF (no. 1 or no. 3). Following drug selection, cells were harvested, and proteins were extracted and probed for expression of pERK (pERK), total ERK (ERK), Bcl-2 (Bcl-2) and phospho-BAD (pBAD). Even protein loading was confirmed following the stripping of the blots and probing for expression of actin. (b) After lentiviral infection and drug selection, cells were harvested, stained with propidium iodide and analysed by flow cytometry. The number of sub-G1 cells is indicative of apoptosis. (c) TdT-mediated dNTP nick end labeling staining of melanoma cells infected with either shRNA control (left panel) or clone no. 3 of the CRAF shRNA. (d) CRAF knockdown reduces the tumorigenicity of WM3629 cells. WM3629 cells were infected with shRNA control, clone no. 1, clone no. 3 of the CRAF shRNA. A total of 2 million cells were then injected into the lower back of severe combined immunodeficient mice. Tumor volumes were measured every week over a 6-week period. **Statistically significantly different from tumor volume in control WM3629 animals ($P < 0.001$). * $P < 0.05$.

remains a very large group of melanomas (>33%) of which very little is known. This study has identified a group of melanomas with low-activity non-V600E mutations in *BRAF*, a reliance on CRAF signaling and sensitivity to the pro-apoptotic effects of sorafenib/CRAF knockdown. In agreement with all published studies to date, most of the mutations in *BRAF* identified in our patient population (>95%) were Exon 15 mutations at the V600E position (Davies *et al.*, 2002).

Only one Exon 11 mutation, the G469A, was identified in our clinical specimens, suggesting that these mutations are relatively rare. In contrast, analysis of our cell lines identified two Exon 11 mutations, the D594G and G469E. The apparent lack of D594G mutations in our clinical population is likely a result of the relatively small sample size in this study.

Previous biochemical analysis of the *BRAF* D594G- and G469E-isolated kinase assays showed them to be

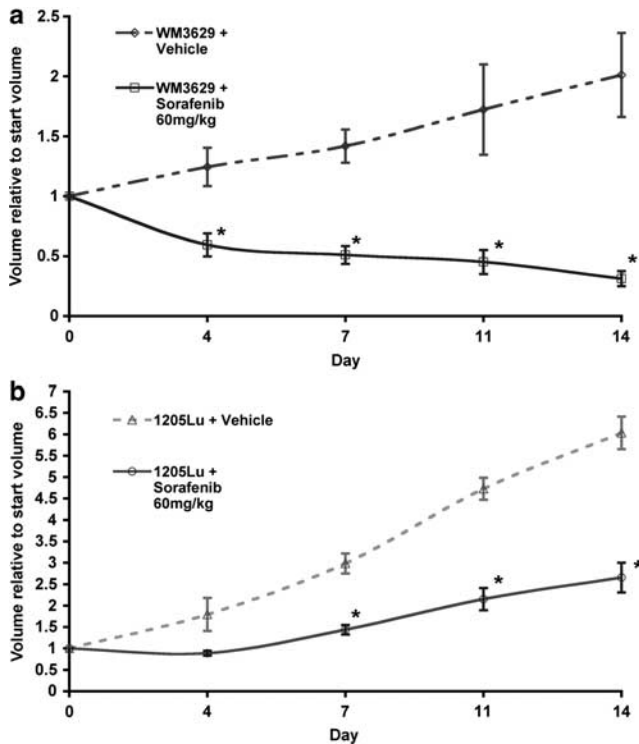


Figure 6 Sorafenib treatment induces some regression of established D594G-BRAF mutated melanoma xenografts. 1205Lu (V600E) and WM3629 (D594G) cells were grown as tumor xenografts in severe combined immunodeficient mice. After tumor establishment, mice were dosed three times per week with either vehicle or sorafenib tosylate (60 mg/kg) by oral gavage for 14 days. Growth curves were normalized to the start volumes. (a) Sorafenib treatment led to some regression of the established D594G mutated (WM3629) melanoma xenografts. (b) Sorafenib treatment led to slowing of the growth of established BRAF V600E-mutated (1205Lu) melanoma xenografts. *Statistically different from vehicle-treated animals ($P < 0.05$).

‘low-activity mutants’ with impaired ERK activation (Wan *et al.*, 2004; Garnett *et al.*, 2005). Relative to BRAF V600E, which was assigned a kinase activity score of 478, the D594G and G469E mutants were found to have kinase activities of 0.6 and 1.8, respectively (Wan *et al.*, 2004). However, when these mutants were expressed in COS-1 cells, they were found to activate ERK, through a mechanism involving the transactivation of CRAF (Wan *et al.*, 2004). The other BRAF mutant identified, K601E, is suggested to be an intermediate activity mutant with a kinase activity score of 138 (Wan *et al.*, 2004); unlike the low-activity mutants, the K601E is not thought to require CRAF for its MEK activity.

The two cell lines with low-activity BRAF mutations were found to have constitutive pERK activity, but very low pMEK activity. Although the presence of constitutive pERK in the low-activity BRAF mutant melanoma cell lines was predicted by previous studies using COS-1 cells, the relative lack of pMEK activity is unexpected (Wan *et al.*, 2004). In addition to having very low MEK activity, the D594G- and G469E-mutated melanoma lines were also highly resistant to the MEK inhibitor

U0126. These results agree with previous studies suggesting that the presence of the BRAF V600E mutation is predictive of response to MEK inhibition (Solit *et al.*, 2006; Haass *et al.*, 2008). Interestingly, it was found that the lowest activity BRAF mutant (D594G) was sensitive to the multi-kinase inhibitor sorafenib (BAY 43-9006, Nexxavar). Treatment of the low-activity mutant cell lines with sorafenib led to profound levels of apoptosis. In contrast, no apoptosis was seen in either of the BRAF V600E-mutated cell lines, and only limited apoptosis was seen in the K601E BRAF-mutated cell line. Although sorafenib can inhibit MEK activity, the pro-apoptotic activity observed was found to be MEK independent, as U0126 treatment did not induce apoptosis in any of the cell lines tested. These findings agree with our previous studies, also showing very limited MEK inhibitor-induced apoptosis across a large panel of melanoma cell lines (Smalley *et al.*, 2006; Haass *et al.*, 2008). One unexpected finding was the ability of sorafenib to induce p53 expression in the 1205Lu cell line in the absence of any apoptosis induction or upregulation of p21 expression. This is likely to be a consequence of the high expression of murine double minute-2 (MDM2), a key negative regulator of p53 activity, in these melanoma cell lines. Previous work from our group has shown that p53-dependent apoptosis proceeds efficiently only when MDM2 expression is suppressed (Smalley *et al.*, 2007a).

Although sorafenib inhibits the activity of a number of kinases, its highest affinity is actually against CRAF (Wilhelm *et al.*, 2004). CRAF is known to suppress apoptosis through a direct association with the mitochondria. In agreement with the idea of the sorafenib effects being CRAF mediated, we found that there was a preferential loss of mitochondrial membrane potential only in the melanoma cells with low-activity BRAF mutations. Sorafenib-induced apoptosis has also been associated with the loss of mitochondrial AIF and its relocalization to the nucleus (Panka *et al.*, 2006). The finding that sorafenib, but not U0126 treatment, led to the loss of mitochondrial AIF expression provides further evidence that the effects of this inhibitor are MEK independent. Although the low-activity mutant melanoma cell lines have low MEK activity, both sorafenib and U0126 were able to block pERK activity in these cells. The fact that sorafenib blocked all pERK activity in the D594G cell line at a 10-fold lower concentration than in the V600E-mutated melanoma cell line again suggested that the preferential effects of sorafenib in the low-activity mutants was a consequence of CRAF inhibition. This idea was confirmed by shRNA knockdown of CRAF, which led to an inhibition of pERK activity in the D594G/G469E lines and not in the V600E-mutated cells. Again, like sorafenib, CRAF knockdown also selectively induced apoptosis in the low-activity mutant melanoma cells, confirming the role of CRAF in the survival of melanomas with low-activity BRAF mutations.

CRAF is known to associate with both the plasma membrane and the mitochondrial membrane (Rapp *et al.*, 2004). Only the plasma membrane-associated

CRAF activates the MAP kinase pathway with the mitochondria-associated CRAF instead regulating apoptosis through direct association with the anti-apoptotic protein Bcl-2 (Wang *et al.*, 1996a). The lack of pro-apoptotic activity in our melanoma cell lines following MEK inhibition suggests that the pro-apoptotic activity seen in the low-activity *BRAF* mutant following sorafenib treatment is likely to be MEK independent.

Bcl-2 is a BH3 family domain protein that occupies a convergent site in the extrinsic and intrinsic apoptosis pathways. It protects cells by maintaining mitochondrial integrity, thereby suppressing the release of the pro-apoptotic proteins cytochrome-C and AIF. The activity of Bcl-2 is regulated through a dynamic balance between the number of pro- and anti-apoptotic complexes formed, with CRAF forming an anti-apoptotic complex with Bcl-2 and BAD forming a pro-apoptotic complex with Bcl-2 (Zha *et al.*, 1996; Wang *et al.*, 1996a). CRAF is targeted to the mitochondria following its Ser 338 and 339 phosphorylation by p21-activated kinase-1 (Jin *et al.*, 2005). Once associated with the mitochondria, CRAF binds directly to Bcl-2 and displaces BAD by phosphorylating it at Ser75 (Jin *et al.*, 2005). The replacement of BAD with CRAF within the Bcl-2 complex serves to alter the life/death balance of the cell and survival ensues. This study shows that melanoma cell lines with D594G and G469E *BRAF* mutants depend on CRAF for their survival. Inhibition of CRAF activity through either sorafenib or CRAF shRNA treatment leads to a rapid decrease in both Bcl-2 expression and BAD phosphorylation, leading to apoptosis. The wealth of evidence presented in this study suggests that the anti-apoptotic activity of CRAF in these cell lines is independent of MEK activity and is instead mostly a Bcl-2-mediated effect upon cell survival. Treatment of D594G/G469E-mutated cells with sorafenib leads to both loss of mitochondrial integrity (as shown by decreased TMRM staining) and the mitochondrial release of AIF. Furthermore, sorafenib decreases only Bcl-2 and BAD phosphorylation in the D594G/G469E-mutated cell lines, which are known to signal through CRAF, and not those with either the K601E or the V600E *BRAF* mutations. Although *NRAS*-mutated melanomas are also known to signal through CRAF (Dumaz *et al.*, 2006), we did not observe similar sorafenib-mediated effects in cell lines with *NRAS* mutations, suggesting there may be other survival mechanisms within this particular genetic subgroup.

In summary, we have identified for the first time a subgroup of melanomas with low-activity mutants in *BRAF* that instead rely on CRAF signaling for their survival. Striking responses were seen in these melanoma lines to sorafenib, now an FDA-approved drug for renal cell and hepatocellular carcinoma. Previously conducted single-agent and chemotherapy-combination trials with sorafenib in melanoma have included patients regardless of the *BRAF* mutation status of their tumors. Among the subset of patients who received benefit from sorafenib, our data support analysis of these patients'

tumors for the presence of low-activity *BRAF* mutations. We believe that there is a clear rationale for the further clinical testing of this compound in melanoma with a new focus on those melanomas with CRAF dependency.

Materials and methods

Cell culture

Human melanoma cells were isolated and cultured as described by Smalley *et al.* (2005). The lentiviral vector shRNA constructs for Bcl-2, CRAF and scrambled controls were from Sigma (St Louis, MO, USA). Lentiviral infections were performed as described previously (Smalley *et al.*, 2005). TdT-mediated dNTP nick end labeling staining was performed as described previously (Smalley *et al.*, 2007a).

Adherent cell proliferation analysis

Cells were plated into a 96-well plate at a density of 2.5×10^4 cells per ml and left to grow overnight. Cells were treated with increasing concentrations of U0126 (Calbiochem, CA, USA) or sorafenib (Bayer Corporation, Wayne, NJ, USA) in triplicate; after 72 h, the levels of growth inhibition were examined using the MTT assay (Smalley *et al.*, 2007b). Data show the mean of at least three independent experiments \pm the s.e.m.

Western blot analysis

Proteins were extracted and blotted as described by Smalley *et al.* (2005). In some studies, mitochondrial and cytoplasmic cellular fractions were prepared using a fractionation kit (Calbiochem). After analysis, western blots were stripped once and reprobed for β -actin to demonstrate even protein loading. Antibodies to pERK, total ERK, pMEK, phospho-PTEN, total PTEN, caspase-3, phospho-BAD, phospho-AKT and total AKT were obtained from Cell Signaling Technology (Beverly, MA, USA), and the antibodies to AIF, CRAF and Bcl-2 were obtained from Santa Cruz (Santa Cruz, CA, USA).

In vivo melanoma xenograft studies

The study protocol was approved by the Wistar Institute Animal Care and Use Committee. Each group consisted of five SCID CB-17 mice (Charles River Laboratories, Wilmington, MA, USA). Ten mice were injected subcutaneously with either WM3629 or 1205Lu cells (2×10^6) in Matrigel into the lower back. When animals had developed melanoma nodules of about 5 mm in diameter, the study drug administration was initiated (day 1): the SCID mice were randomly assigned to the two experimental groups of five animals each—(1) 200 μ l vehicle (Cremophor EL (12.5%)/ethyl alcohol (12.5%)/distilled water (75%)), (2) 60 mg/kg sorafenib tosylate (in 200 μ l vehicle) three times per week by oral gavage over a period of 14 days. Tumors were measured twice a week using digital calipers. Tumor volume was calculated as a product of the three dimensions. Tumor shrinkage was calculated as a fold-change relative to the starting volume. At treatment day 14, one hour after the final drug application, all animals were euthanized. In other studies, mice (6 per group) were injected with WM3629 cells (2×10^6) in Matrigel into the lower back infected with either scrambled shRNA control or clone1/3 of the CRAF shRNA. Animals were then monitored for melanoma growth over a 6-week period with tumor measurements being made once per week.

Cell cycle analysis

Cells were plated into 10-cm dishes at 60% confluency and left to grow overnight before being treated with sorafenib (3 or 10 μM) or U0126 (10 or 30 μM) for 24 h. Cells were fixed in 70% ethanol overnight, labeled with propidium iodide and analysed by flow cytometry.

Flow cytometric analysis of mitochondrial membrane potential ($\Delta\psi_m$)

After the treatment of the cells with sorafenib (3 μM , 24 h), cells were washed once with binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl and 5 mM CaCl_2) and resuspended in 100 μl binding buffer containing 25 nM TMRM perchlorate (Molecular Probes, Eugene, OR, USA) and incubated at 37 $^\circ\text{C}$ for 15 min. Cells were then analysed for TMRM retention (red fluorescence) using flow cytometry.

Immunofluorescence microscopy

Cells were seeded thinly onto ethanol-sterilized glass coverslips in 6-well plates and left to grow overnight, after which they were treated with either sorafenib (3 μM) or U0126 (30 μM) for 24 h before being fixed in 4% paraformaldehyde and permeabilized with 0.2% v/v Triton X-100. One hour before fixation, cells were treated with mitotracker orange (Molecular Probes) for 30 min. Samples were then blocked in PBS containing 1% bovine serum albumin. Primary antibody incubations to AIF, (Santa Cruz) were performed at 37 $^\circ\text{C}$

References

Chen J, Fujii K, Zhang L, Roberts T, Fu H. (2001). Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK-ERK independent mechanism. *Proc Natl Acad Sci USA* **98**: 7783–7788.

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S *et al.* (2002). Mutations of the BRAF gene in human cancer. *Nature* **417**: 949–954.

Dhomen N, Marais R. (2007). New insight into BRAF mutations in cancer. *Curr Opin Genet Dev* **17**: 31–39.

Dumaz N, Hayward R, Martin J, Ogilvie L, Hedley D, Curtin JA *et al.* (2006). In melanoma, RAS mutations are accompanied by switching signaling from BRAF to CRAF and disrupted cyclic AMP signaling. *Cancer Res* **66**: 9483–9491.

Eisen T, Ahmad T, Flaherty KT, Gore M, Kaye S, Marais R *et al.* (2006). Sorafenib in advanced melanoma: a Phase II randomised discontinuation trial analysis. *Br J Cancer* **95**: 581–586.

Garnett MJ, Rana S, Paterson H, Barford D, Marais R. (2005). Wild-type and mutant B-RAF activate C-RAF through distinct mechanisms involving heterodimerization. *Mol Cell* **20**: 963–969.

Gray-Schopfer V, Wellbrock C, Marais R. (2007). Melanoma biology and new targeted therapy. *Nature* **445**: 851–857.

Haass NK, Sproesser K, Nguyen TK, Contractor R, Medina CA, Nathanson KL *et al.* (2008). The mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitor AZD6244 (ARRY-142886) induces growth arrest in melanoma cells and tumor regression when combined with docetaxel. *Clin Cancer Res* **14**: 230–239.

Houben R, Vetter-Kauczok CS, Ortmann S, Rapp UR, Broecker EB, Becker JC. (2008). Phospho-ERK staining is a poor indicator of the mutational status of BRAF and NRAS in human melanoma. *J Invest Dermatol* **128**: 2003–2012.

Jin S, Zhuo Y, Guo W, Field J. (2005). p21-activated Kinase 1 (Pak1)-dependent phosphorylation of Raf-1 regulates its mitochondrial localization, phosphorylation of BAD, and Bcl-2 association. *J Biol Chem* **280**: 24698–24705.

Kyriakis JM, App H, Zhang XF, Banerjee P, Brautigam DL, Rapp UR *et al.* (1992). Raf-1 activates MAP kinase-kinase. *Nature* **358**: 417–421.

for 1 h under humidified conditions. Slides were prepared as described by Smalley *et al.* (2005).

Mutational testing

The algorithm for genotyping for FFPE melanoma specimens is shown in Supplementary Figure 1. Exons 15 of *BRAF* and 3 of *NRAS* were typed concurrently using either pyrosequencing or direct sequencing as described previously (Spittle *et al.*, 2007). If these exons were wild type, the following exons are typed in order, until a mutation is identified or all are wild type—exon 11 of *BRAF*, exon 2 of *NRAS* and *KIT* (exons 9, 11, 13, 17). Samples were collected from 160 unselected melanomas from patients on clinical trials in full accordance with the Institutional Review Board of the University of Pennsylvania in compliance with HIPPA protocols. All melanomas were superficial spreading or nodular types.

Statistical analysis

Unless otherwise stated, all experiments show the mean \pm s.e.m. of at least three independent experiments. Statistical significance was measured using the Student's *t*-test, where $P < 0.05$ was judged to be significant.

Acknowledgements

We thank the Bayer Corporation for providing the BAY 43-9006 (sorafenib).

O'Neill E, Rushworth L, Baccharini M, Kolch W. (2004). Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. *Science* **306**: 2267–2270.

Panka DJ, Wang W, Atkins MB, Mier JW. (2006). The Raf inhibitor BAY 43-9006 (Sorafenib) induces caspase-independent apoptosis in melanoma cells. *Cancer Res* **66**: 1611–1619.

Rapp UR, Rennefahrt U, Troppmair J. (2004). Bcl-2 proteins: master switches at the intersection of death signaling and the survival control by Raf kinases. *Biochim Biophys Acta* **1644**: 149–158.

Smalley KS, Brafford P, Haass NK, Brandner JM, Brown E, Herlyn M. (2005). Up-regulated expression of zonula occludens protein-1 in human melanoma associates with N-cadherin and contributes to invasion and adhesion. *Am J Pathol* **166**: 1541–1554.

Smalley KS, Contractor R, Haass NK, Kulp AN, Atilla-Gokcumen GE, Williams DS *et al.* (2007a). An organometallic protein kinase inhibitor pharmacologically activates p53 and induces apoptosis in human melanoma cells. *Cancer Res* **67**: 209–217.

Smalley KS, Contractor R, Haass NK, Lee JT, Nathanson KL, Medina CA *et al.* (2007b). Ki67 expression levels are a better marker of reduced melanoma growth following MEK inhibitor treatment than phospho-ERK levels. *Br J Cancer* **96**: 445–449.

Smalley KS, Haass NK, Brafford PA, Lioni M, Flaherty KT, Herlyn M. (2006). Multiple signaling pathways must be targeted to overcome drug resistance in cell lines derived from melanoma metastases. *Mol Cancer Ther* **5**: 1136–1144.

Smalley KSM. (2003). A pivotal role for ERK in the oncogenic behaviour of malignant melanoma? *Int J Cancer* **104**: 527–532.

Solit DB, Garraway LA, Pratilas CA, Sawai A, Getz G, Basso A *et al.* (2006). BRAF mutation predicts sensitivity to MEK inhibition. *Nature* **439**: 358–362.

Spittle C, Ward MR, Nathanson KL, Gimotty PA, Rappaport E, Brose MS *et al.* (2007). Application of a BRAF pyrosequencing assay for mutation detection and copy number analysis in malignant melanoma. *J Mol Diagn* **9**: 464–471.

von Gise A, Lorenz P, Wellbrock C, Hemmings B, Berberich-Siebelt F, Rapp UR *et al.* (2001). Apoptosis suppression by Raf-1 and MEK1 requires MEK- and phosphatidylinositol 3-kinase-dependent signals. *Mol Cell Biol* **21**: 2324–2336.

- Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM *et al.* (2004). Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* **116**: 855–867.
- Wang HG, Rapp UR, Reed JC. (1996a). Bcl-2 targets the protein kinase Raf-1 to mitochondria. *Cell* **87**: 629–638.
- Wang HG, Takayama S, Rapp UR, Reed JC. (1996b). Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1. *Proc Natl Acad Sci USA* **93**: 7063–7068.
- Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H *et al.* (2004). BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* **64**: 7099–7109.
- Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* **87**: 619–628.

Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)