Tumor Necrosis Factor-α Blocks Apoptosis in Melanoma Cells when BRAF Signaling Is Inhibited

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Abstract

The protein kinase BRAF, a component of the RAS/RAF/mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK signaling pathway, regulates cell fate in response to extracellular signals. Activating mutations in BRAF occur in ~70% of human melanomas. The active proteins stimulate constitutive pathway signaling, proliferation, and survival. Thus, inhibition of BRAF signaling in melanoma cells causes cell cycle arrest and induces cell death through apoptosis, validating BRAF as an important therapeutic target. Here, we show that the apoptosis induced by inhibition of BRAF signaling in melanoma cells can be prevented if the cells are treated with tumor necrosis factor (TNF)-α. This allows the cells to recover from the inhibition of BRAF signaling and reenter the cell cycle. This effect occurs due to a specific TNF-α and BRAF interaction because TNF-α does not prevent cell death in the presence of cisplatin, nitrogen mustard or thapsigargin. Furthermore, the cytokines Fas ligand, TNF-related apoptosis-inducing ligand, interleukin (IL)-1, and IL-6 do not prevent cell death when BRAF signaling is inhibited. The survival mechanism requires nuclear factor-κB (NF-κB) transcription factor activity, which is strongly induced by TNF-α in these cells. These findings suggest that drugs that target the BRAF/MEK pathway could be combined with agents that target TNF-α and/or NF-κB signaling to provide exciting new therapeutic opportunities for the treatment of melanoma. [Cancer Res 2007;67(1):122–9]

Introduction

Melanoma is a form of cancer that arises from melanocytes, the specialized pigmented cells found in the epidermis (1). The only known environmental risk factor is exposure to UV irradiation in the form of natural sunlight or from unnatural sources. The incidence of melanoma is rising at alarming rates in most Western societies, with a doubling of cases within the last 20 years. When treated early, melanoma can be cured by simple surgical resection, but in its metastatic malignant form, it is particularly difficult to treat with a median survival rate of 6 months and a 5-year survival rate of <5%. In part, this is because melanoma is highly resistant to most currently available forms of therapy, leading to extremely high mortality rates.

Programmed cell death (apoptosis) is an essential process in normal tissue development, but it is also essential for normal tissue homeostasis (2). It is induced in response to stress and by various biological effectors, such as some of the hormones and cytokines that are secreted by activated lymphocytes and macrophages. Importantly, apoptosis also plays a role in cancer. Resistance to apoptosis is a well-documented hallmark of cancer (3) and seems to play a critical role in melanoma, where many antiapoptotic pathways provide survival signals. Furthermore, the resistance of melanoma to currently used therapies occurs in part because melanoma cells safeguard their survival through mechanisms that allow them to escape death receptor–mediated apoptosis. Of particular note are cytokines of the tumor necrosis factor (TNF) family, which includes TNF-related apoptosis-inducing ligand (TRAIL), Fas ligand (FasL), and TNF-α (4), the latter two of which are secreted by cells of the immune system. When these ligands bind to their receptors [TRAIL receptor, Fas receptor, and TNF receptor (TNFR) 1/2], they induce receptor trimerization and thereby stimulate recruitment of several proteins which form a complex called the death-inducing signaling complex (5). This complex dissociates from the receptors and binds to the adaptor molecule Fas-associated death domain, leading to activation of a caspase cascade and consequently to cell death.

Another important signaling pathway in melanoma is the RAS/RAF/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK cascade. RAS is a small G protein that is embedded in the plasma membrane and that is activated downstream of growth factor receptor tyrosine kinases (RTKs), cytokines, and hormones (6). RAF is a protein kinase that is activated following its recruitment to the plasma membrane by RAS (7). RAF then phosphorylates and activates the protein kinase MEK, which in turn phosphorylates and activates ERK, the third protein kinase in the cascade. ERK phosphorylates numerous cytosolic and nuclear proteins to regulate gene expression, metabolism, and the cytoskeleton, thereby regulating cell proliferation, differentiation, senescence, and survival. This pathway also seems to play an important role in human cancer. *RAS* genes are mutated in ~15% of human cancers, and recently, it was discovered that one of the three RAF isoforms in humans, *BRAF*, is mutated in ~7% of human cancers (8). This pathway is particularly important in melanoma, with *NRAS* being mutated in 15% to 30% of melanomas and *BRAF* in 50% to 70% of cases (8). Consequently, ERK is hyperactivated in the majority of melanoma cases (1).

More than 70 different cancer-associated mutations have been described in BRAF in human cancer, but the most common substitution is a glutamic acid for valine substitution at position 600 (V600E, formally V599E), which accounts for >90% of all cases.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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^{V600E}BRAF is 500-fold activated and stimulates constitutive MEK-ERK signaling in cancer cells (9). In melanoma, ^{V600E}BRAF provides both proliferation and prosurvival signals (1); thus, when BRAF signaling is inhibited through the use of RNA interference (RNAi) or inhibition of MEK-ERK signaling by small molecular weight inhibitors of MEK, melanoma cell proliferation is inhibited and the cells die through apoptosis (10, 11). Together, these studies have identified oncogenic BRAF and this signaling pathway as an exciting and important new therapeutic target.

Here, we examine the interplay between BRAF and death receptor signaling in melanoma cells. We show that although inhibition of oncogenic BRAF signaling stimulates death of melanoma cells, when the cells are treated with TNF-α, this death is reversed and the cells recover to reenter the cell cycle. These prosurvival effects are due to a specific interaction between BRAF and TNF-α because they are not seen with other cytokines or when death is induced using other cytotoxic drugs. The TNF-α-mediated survival seen when BRAF signaling is inhibited requires the transcription factor nuclear factor-κB (NF-κB) and, in at least one of the lines tested, the cell cycle regulator p21^{Cip1} is required. Taken together, these results suggest that therapies based on inhibition of oncogenic BRAF may be ineffective in melanoma unless they are combined with agents that inhibit TNF-α or NF-κB signaling, providing exciting new strategies that could be used to treat this potentially devastating disease.

Materials and Methods

Cell culture and treatments. A375, WM266.4, and Colo829 were cultured and lysates were prepared as described (11). Western blotting was done using standard protocols and the following antibodies: ERK2, p21^{Cip1}, p50, p65 (Santa Cruz Biotechnology, Calne, United Kingdom), phospho-ERK (Sigma, Poole, United Kingdom), poly(ADP-ribose) polymerase (PARP; BD PharMingen, Cowley, United Kingdom), TNFR1, and IL-1 receptor (IL-1R; Abcam, Cambridge, United Kingdom). Cells were treated with recombinant TNF-α, TRAIL (Sigma), FasL, IL-1, or IL-6 (Peprotech, London, United Kingdom) at 50 ng/mL for the times indicated. Enbrel (2.5 μg/mL; Wyeth UK, Maidenhead, United Kingdom) was premixed (37°C, 60 min) with TNF-α in cell culture medium before incubation with cells. As required, TNF-α was added 24 h after small interfering RNA (siRNA) transfection. Cells were incubated with U0126 (10 μmol/L; Promega, Southampton, United Kingdom), PD184352 (1–2 μmol/L for A375 and Colo829 and 5 μmol/L for WM266.4, synthesized as described in ref. 12), cisplatin (5 μmol/L for A375 and Colo829 and 20 μmol/L for WM266.4; Sigma), thapsigargin (10 nmol/L for A375, 0.5 μmol/L for Colo829, and 2 μmol/L for WM266.4; Sigma), nitrogen mustard (10 μmol/L for A375, 2 μmol/L for Colo829, and 20 μmol/L for WM266.4; Sigma) for 48 h before analysis.

Detection of cell death. The cellular sub-G_1 fraction was determined by fluorescence-activated cell sorting (FACS) using propidium iodide staining and standard protocols. Lactate dehydrogenase (LDH) release was measured using a colorimetric assay following the supplier's protocols (Sigma) and caspase-3 activity was determined using an Ac-DEVD-AMC fluorogenic substrate as recommended by the supplier (BD PharMingen). All assays were done in triplicate.

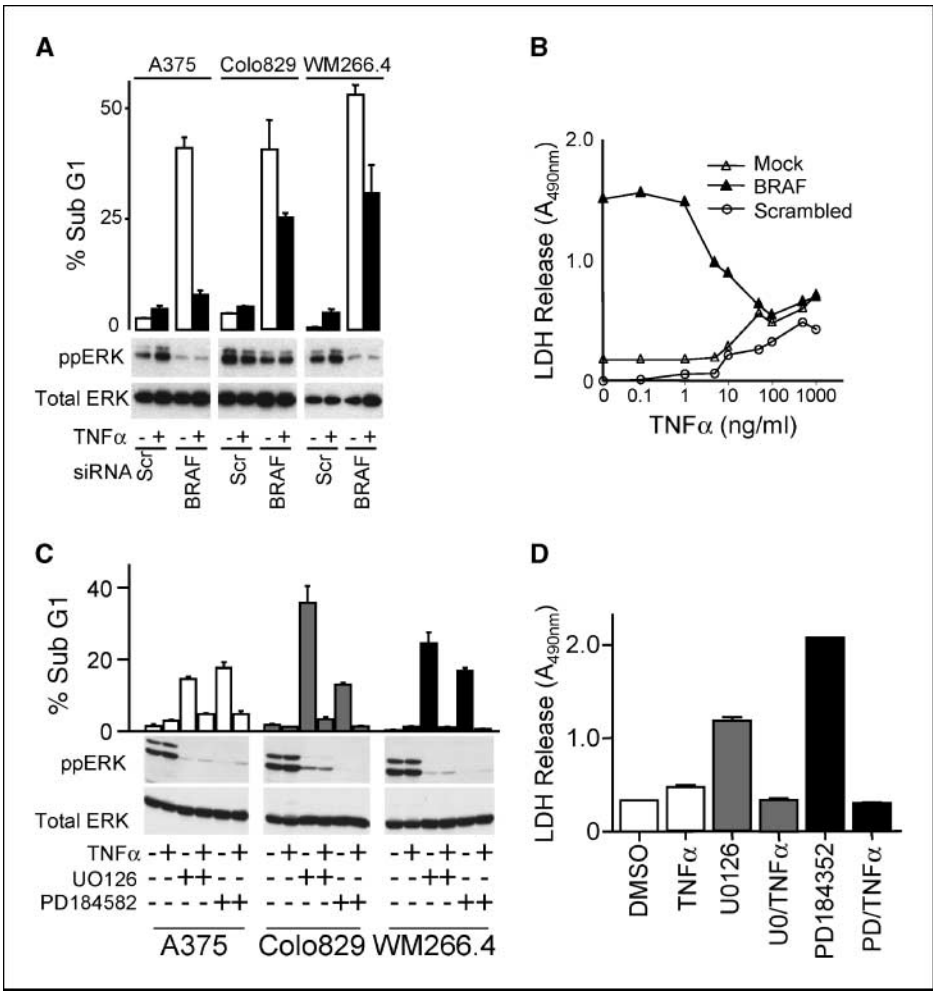

Figure 1. TNF-α rescues melanoma cells from apoptosis induced by inhibition of BRAF signaling. *A,* A375, Colo829, and WM266.4 cells were transfected with a BRAF or a scrambled (*Scr*) control siRNA and, after 24 h, TNF-α was added for an additional 72 h. Proportion of sub-G_1 cells (*% sub-G1*). Data are compiled from three independent experiments. *Columns,* mean; *bars,* SD. Western blot for phospho-ERK (*ppERK*) and total ERK levels. *B,* LDH release from Colo829 cells treated with the indicated siRNA oligonucleotides in the presence of the indicated concentrations of TNF-α. Similar results were obtained in three independent experiments. *C,* A375, Colo829, and WM266.4 cells were treated with U0126 or PD184352 in the presence or absence of TNF-α for 72 h. Sub-G_1 fraction. *Bottom,* Western blots for phospho-ERK and total ERK. *D,* LDH release from Colo829 cells treated with U0126 or PD184352 in the presence or absence of TNF-α for 72 h.

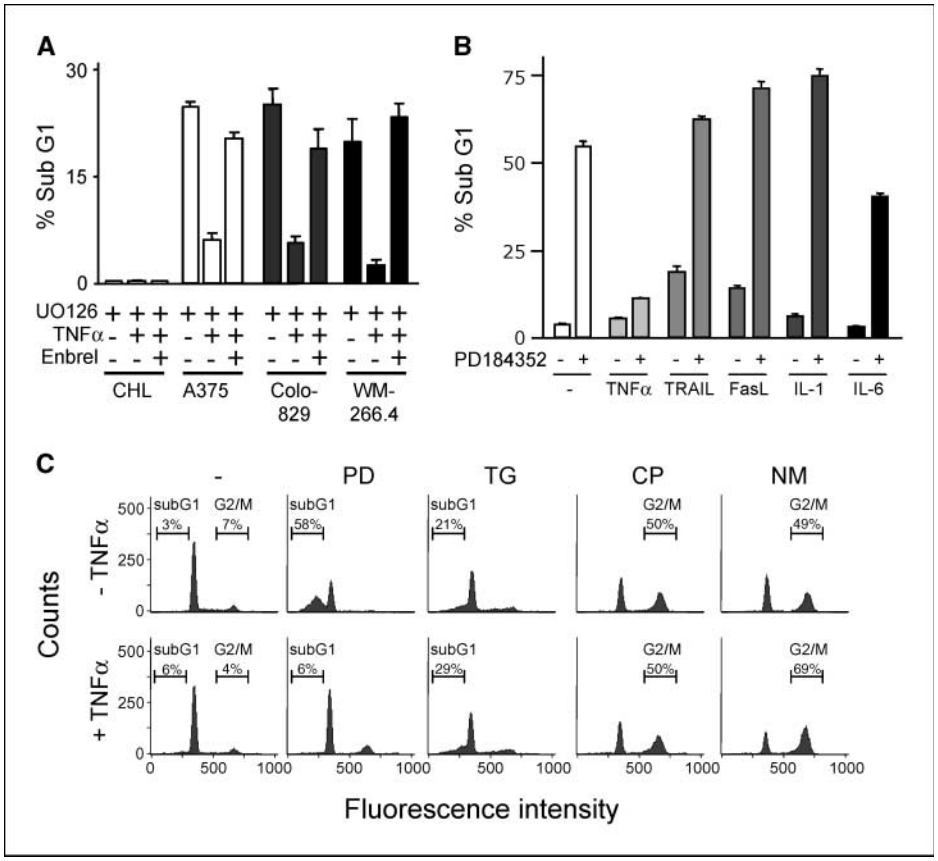

Figure 2. The TNF-α-mediated rescue of melanoma cells, in which BRAF is mutated, is specific. *A,* the sub-G_1 fractions from CHL, A375, Colo829, and WM266.4 cells treated with U0126 in the presence or absence of TNF-α and the TNF-α antagonist Enbrel for 72 h. Data are for one experiment done in triplicate. *Columns,* mean; *bars,* SD. *B,* the sub-G_1 fraction from A375 cells treated with PD184352 for 72 h in the absence or presence of TNF-α, TRAIL, FasL, IL-1, and IL-6. Results are for one experiment done in triplicate. *Columns,* mean; *bars,* SD. *C,* cell cycle profiles for A375 cells treated with PD184352 (*PD*), thapsigargin (*TG*), cisplatin (*CP*), or nitrogen mustard (*NM*) in the presence or absence of TNF-α. Where indicated, the proportion of cells either in the sub-G_1 or in the G_2-M phase of the cell cycle is shown.

Immunofluorescence. TNFR1, IL-1R, and $p21^{Cip1}$ expression was detected using standard protocols with 3.7% formaldehyde (Sigma)–fixed cells. Intact cells were used to detect TNFR1 and IL-1R, but the cells were permeabilized using 0.5% Triton X-100 (Sigma) to reveal $p21^{Cip1}$ expression. The cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain (Sigma) and mounted in 1,4-diazabicyclooctane (Sigma).

RNA interference. RNAi was done as described (11) using the following siRNA oligonucleotides: BRAF, AAAGAATTGGATCTGGATCAT; $p21^{Cip1}$, AATGGCGGGCTGCATCCAGGA; p65, GCCCTATCCCTTTACGTCA; p50, AAGGGGCTATAATCCTGGACT; and scrambled, AAACCGTCGATTT-CACCCGGG.

Clonogenic survival assay. A375 cells were treated with the indicated drugs and TNF-α for the times indicated and then replated at equal densities. After 14 to 21 days, the cells were fixed in 3.7% formaldehyde and stained with 1% crystal violet (Sigma) in 70% ethanol. Cell density was quantified by dissolving the crystal violet bound to the cells in 10% (v/v) acetic acid and by measuring the absorbance of this acetic acid solution at 595 nm.

Luciferase reporter assay. Cells were transfected using LipofectAMINE (Invitrogen, Paisley, Scotland) with 0.8 μg total DNA [0.3 μg NF-κB-responsive luciferase reporter construct (13) and 0.5 μg β-galactosidase control] and extracts were prepared 48 h post-transfection for luciferase activity measurement, using a luciferase assay system (Promega) according to the manufacturer's instructions. Luciferase activity was normalized relative to the β-galactosidase control.

Results

It has been shown recently that cancer cells that harbor mutations in BRAF are more sensitive to MAPK pathway inhibition than cells where BRAF is not mutated (14). In agreement with this, we have reported that inhibition of BRAF signaling in three melanoma cell lines that harbor V600 mutations in BRAF, A375 (^{V600E}BRAF), Colo829 (^{V600E}BRAF), and WM266.4 cells (^{V600D}BRAF) induces cell cycle arrest and apoptosis (11). When BRAF signaling was suppressed through the use of RNAi, cell death became apparent within 48 h and peaked at 72 h. In contrast, death induced by small-molecule inhibitors of MEK, such as PD184352, occurred more rapidly and was evident within 24 h and peaked at 48 h (data not shown). This presumably reflects the fact that MEK inhibitors block ERK activity more rapidly than when BRAF protein is depleted through use of RNAi. The induction of apoptosis in these cells can be measured by the appearance of a sub-G_1 peak in FACS profiles following treatment with siRNA to BRAF (Fig. 1*A*).

Because melanoma cells are resistant to death receptor–mediated apoptosis, we wished to investigate if TNF-α could augment the levels of cell death induced by BRAF siRNA. Unexpectedly however, rather than augmenting cell death, TNF-α attenuated cell killing in BRAF siRNA-treated cells, as shown by the suppression of the sub-G_1 peak in the FACS profiles (Fig. 1*A*). We extended these findings by measuring release of LDH from the cytoplasm of these cells as an independent measure of cell death. BRAF siRNA caused robust LDH release from Colo829 cells, but TNF-α suppressed this in a dose-dependent manner (Fig. 1*B*). Similar results were observed when these cells were treated with U0126 and PD184352, two small-molecule inhibitors of MEK. Both agents induced death in all three lines, as shown by the appearance of a sub-G_1 peak in the FACS profiles (Fig. 1*C*) and by the release of cytosolic LDH (Fig. 1*D*). Importantly, in each case, the induction of death by these MEK inhibitors was attenuated by TNF-α, but note that TNF-α did not activate ERK in these cells (Fig. 1*A* and *C*), showing that it did not reverse death induced by MEK inhibition through cryptic reactivation of ERK.

We next wished to examine if the responses to TNF-α were specific. First, we showed that all three cell lines express TNFR1 to similar levels (Supplementary Fig. S1*A*). As important controls, we show that the prosurvival activity of TNF-α was blocked by Enbrel, a specific antagonist of TNF-α and also that U0126 did not induce apoptosis in CHL cells, a melanoma line, in which BRAF is not mutated (Fig. 2*A*; ref. 8). We also show that TRAIL and FasL, two cytokines related to TNF-α, did not rescue PD184352-induced cell death (Fig. 2*B*). Furthermore, interleukin (IL)-1 and IL-6, two other cytokines that are secreted by macrophages, also failed to block PD184352-induced cell death (Fig. 2*B*). We also wished to examine if TNF-α was prosurvival under all conditions that induce death in these cells. A375 cells were treated with the DNA alkylating agents cisplatin or nitrogen mustard or with the Ca^{2+} pump inhibitor thapsigargin and the cell cycle profiles were examined. As expected, PD184352 induced a G_1 cell cycle arrest and the appearance of a sub-G_1 peak that was reversed by TNF-α (Fig. 2*C*). Thapsigargin also induced a G_1 cell cycle arrest and sub-G_1 peak, but TNF-α augmented, rather than suppressed, the effects of thapsigargin (Fig. 2*C*). Cisplatin and nitrogen mustard induce cell death by cross-linking DNA and this is apparent by the appearance of a G_2-M cell cycle arrest in the FACS profiles (Fig. 2*C*). TNF-α did not suppress the induction of the G_2-M cell cycle arrest induced by cisplatin and actually enhanced the G_2-M cell cycle arrest induced by nitrogen mustard (Fig. 2*C*). Similar results were observed in WM266.4 and Colo829 cells (data not shown).

We next examined the long-term consequences of MEK inhibition. A375 cells were treated with PD184352 in the presence or absence of TNF-α for various times and then assessed for their long-term survival by clonogenic assay. PD184352 caused a profound reduction in the long-term survival of A375 cells, particularly at later time points, but the induction of death was significantly reduced by TNF-α (Fig. 3*A*). In contrast, TNF-α was unable to rescue the cells treated with cisplatin and it actually augmented the killing observed with thapsigargin (Fig. 3*B*).

Together, these results show that the prosurvival activity of BRAF in melanoma is specific to the oncogenic form and that the ability of cytokines to rescue cells when this signaling is inhibited is specific to TNF-α. Furthermore, TNF-α does not provide prosurvival signals to cells under other conditions that induce death in melanoma cells. Importantly, one consequence of TNF-α treatment when BRAF signaling is inhibited is that melanoma cells recover and reenter the cell cycle.

We next investigated the mechanism(s) underlying the prosurvival activity of TNF-α in melanoma cells when BRAF signaling is suppressed, examining the role of NF-κB, because this transcription factor is constitutively activated in melanoma, is downstream of TNF-α receptors, and has been implicated in many aspects of melanoma initiation and progression (15). First, we examined NF-κB activity in these melanoma cells, using a luciferase reporter gene whose activity depends on a κB element (13). All three cell lines displayed basal levels of luciferase reporter activity and this was suppressed when the p65 subunit of NF-κB was depleted (Supplementary Fig. S1*B*), showing that the reporter construct activity is dependent on NF-κB signaling. Importantly, TNF-α induced a 140-fold increase in luciferase expression from this reporter in A375 cells (Fig. 4*A*). In the presence of the MEK inhibitor, TNF-α was still able to stimulate luciferase activity albeit to a lower level (Fig. 4*A*), but importantly, this luciferase activity was suppressed when the p65 subunit of NF-κB was depleted (Fig. 4*B*), again confirming that the reporter activity is dependent on NF-κB activity. Notably, NF-κB activation by IL-1 and IL-6 was moderate compared with TNF-α-induced activity and FasL did not activate NF-κB in A375 cells (Fig. 4*C*). Similar results were observed in Colo829 and WM266.4 cells (data not shown), suggesting common responses in all three cell lines. For IL-1, the lack of

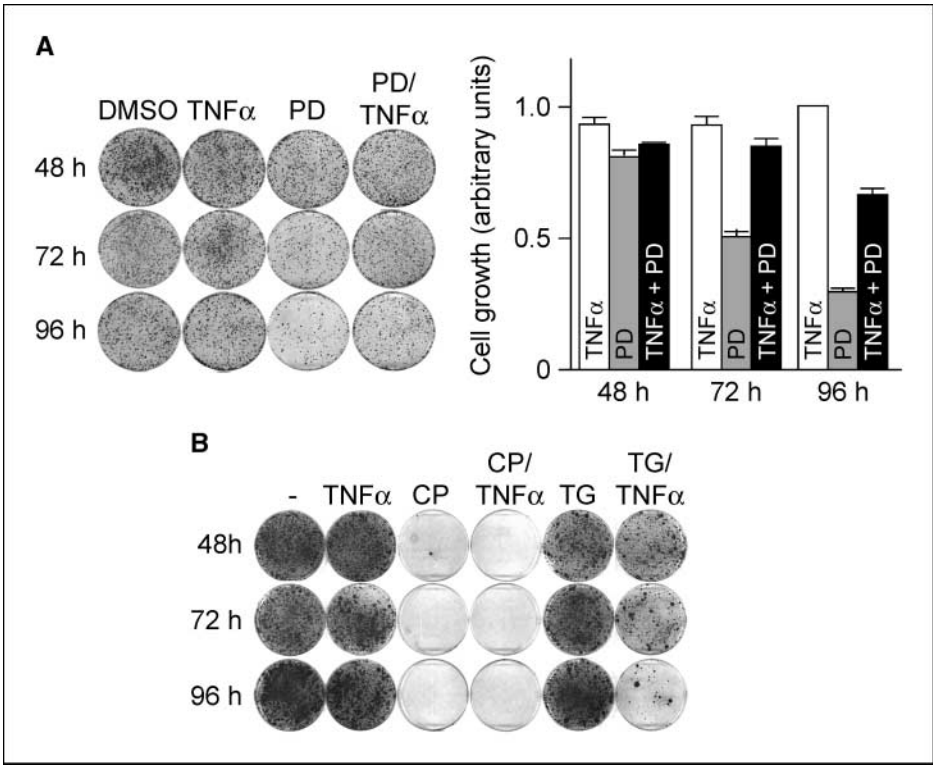

Figure 3. TNF-α restores the long-term survival of melanoma cells treated with MEK inhibitors. *A,* clonogenic survival in A375 cells treated with DMSO, TNF-α, and PD184352 for 48, 72, or 96 h. Representative images of the crystal violet–stained cells are shown together with quantitation of cell density relative to DMSO-treated controls. The quantitative data are compiled from three independent experiments. *Columns,* mean; *bars,* SD. *B,* clonogenic survival assays of A375 cells treated with TNF-α, cisplatin, or thapsigargin for 48, 72, or 96 h. This experiment was done in triplicate.

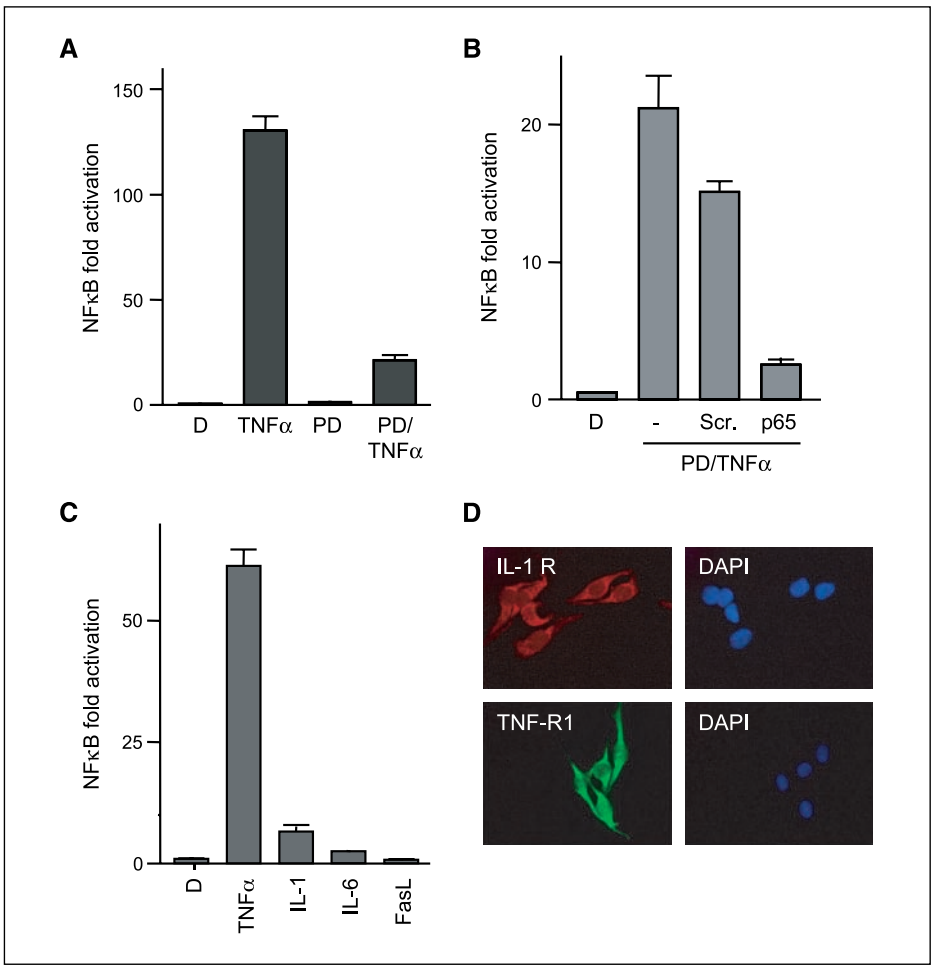

Figure 4. TNF-α activates NF-κB in melanoma cells. *A,* luciferase reporter assay in A375 cells treated with TNF-α and PD184352. Results are from one assay measured in triplicate. *Columns,* mean; *bars,* SD. *B,* luciferase reporter assay in A375 cells treated with DMSO (*D*), PD184352, and TNF-α in the absence or presence of a scrambled or a p65 subunit of NF-κB (p65)–specific siRNA. Results are from one experiment in triplicate. *Columns,* mean; *bars,* SD. *C,* luciferase reporter assay in A375 cells treated with DMSO (*D*), TNF-α, IL-1, IL-6, and FasL. Results are for one experiment. *Columns,* mean; *bars,* SD. *D,* immunostaining analysis of A375 cells stained for the IL-1R (*red*) and TNFR1 (*green*). The cell nuclei were counterstained with DAPI (*blue*).

response does not seem to be due to absence of the receptor because we were able to detect the presence of IL-1R and TNF-α receptor 1 by immunohistochemistry in all three cell lines (Fig. 4*D*; Supplementary Fig. S2).

Depletion of either the p50 or the p65/RelA subunits of NF-κB by RNAi reversed the prosurvival activity of TNF-α in the presence of MEK inhibitors (Fig. 5*A*). These effects on cell survival are specific to NF-κB subunit depletion, as shown by the lack of effect observed with the scrambled control (Fig. 5*B*). To verify these findings, we also examined caspase-3 activation and PARP cleavage, two other markers of apoptosis. Caspase-3 was activated (Fig. 5*C*) and PARP was cleaved (Fig. 5*D*) in PD184352-treated cells and both events were blocked by TNF-α. Notably, depletion of the p65 subunit of NF-κB reversed the ability of TNF-α to block caspase-3 activation and induce PARP cleavage in PD184352-treated cells (Fig. 5*C* and *D*).

We next examined genes that are downstream of NF-κB to further characterize the pathway that regulates these effects. We examined expression of XIAP, Mcl-1, and Bcl_{XL}, proteins that are known to play a role in regulation of apoptosis (16), but we did not find any preliminary evidence to suggest that these proteins play a role in mediating the survival of melanoma cells treated with TNF-α in the presence of MEK inhibitors (data not shown). However, we found that TNF-α, U0126, and PD184352 all suppressed expression of the small molecular weight cyclin-dependent kinase inhibitory protein $p21^{Cip1}$ in WM266.4 cells (Fig. 6*A*, compare *lanes 1, 2, 5*, and *9*; Fig. 4*B*), a protein that is implicated in chemoresistance in other cancers (17). Surprisingly, however, when the cells were treated with TNF-α and the MEK inhibitors together, rather than augmenting each other as expected, these agents antagonized each other and $p21^{Cip1}$ expression was restored (Fig. 6*A* and *B*). The reduction of $p21^{Cip1}$ expression in the presence of the MEK inhibitors correlated with induction of apoptosis as shown by induction of PARP cleavage in these samples. $p21^{Cip1}$ expression was reduced in the presence of PD184352 and U0126, conditions that lead to PARP cleavage (Fig. 6*A*). However, when the cells were treated with PD184352 or U0126 in the presence of TNF-α, $p21^{Cip1}$ expression was restored and PARP cleavage was blocked (Fig. 6*A*). Note however, that a reduction in $p21^{Cip1}$ protein levels in the absence of MEK inhibition was not sufficient to induce apoptosis; $p21^{Cip1}$ expression was reduced in cells treated with TNF-α, but PARP cleavage was not observed (Fig. 6*A*) and TNF-α did not induce apoptosis in these cells (Figs. 1 and 3).

We therefore examined if the recovery in $p21^{Cip1}$ expression was necessary to prevent apoptosis. WM266.4 cells were treated with siRNA to $p21^{Cip1}$, conditions that lead to a profound reduction in protein levels (Fig. 6*C*). Importantly, when $p21^{Cip1}$ protein levels were suppressed, the combined treatment of PD184352 and TNF-α failed to prevent PARP cleavage (Fig. 6*C*). Similar results were observed with NF-κB. Ablation of the p65 subunit blocked $p21^{Cip1}$ reexpression in the presence of PD184352 and TNF-α and also blocked the inhibition of PARP cleavage. Thus, in WM266.4 cells,

$p21^{Cip1}$ is a critical TNF-α/NF-κB target gene, but the regulation of this gene is complex. Its expression is suppressed when MEK is inhibited or the cells are treated with TNF-α but recovers when MEK is inhibited in the presence of TNF-α and this recovery is dependent on NF-κB function. Importantly, the reexpression of $p21^{Cip1}$ is essential to block induction of apoptosis in these cells. However, this role of $p21^{Cip1}$ is cell type specific because we did not observe these effects in Colo829 or A375 cells (data not shown).

Discussion

In this work, we have examined the interaction between death receptor and BRAF signaling in melanoma. Both pathways play a key role in the biology and progression of this disease and we describe an intriguing interaction between these signaling cascades. We have shown that inhibition of BRAF/MEK/ERK signaling in melanoma cells that harbor mutations in BRAF induces cell death, whereas this does not occur when BRAF and RAS are not mutated. This is consistent with recent findings showing that cells that harbor BRAF mutations are significantly more dependent on BRAF signaling than cells where BRAF is not mutated (10, 11, 14). Importantly, we go on to show that the induction of cell death that occurs when oncogenic BRAF signaling is blocked can be reversed by the cytokine TNF-α. This is a specific response because it is blocked by Enbrel, a specific TNF-α antagonist and it is not seen when the cells are treated with TRAIL, FasL, IL-1, or IL-6. Furthermore, TNF-α did not suppress the death induced by the cytotoxic drugs cisplatin, nitrogen mustard, and thapsigargin. Thus, although melanoma cells can be induced to die when oncogenic BRAF signaling is inhibited, TNF-α attenuates the induction of death. Critically, this allows the cells not only to survive but also to recover and reenter the cell cycle, and consequently, in melanoma patients, inhibition of BRAF signaling could be ineffective if cells of the immune system have infiltrated the tumor and are secreting TNF-α.

The mechanisms underlying TNF-α-mediated recovery seem to be regulated by NF-κB, a heterodimeric transcription factor mainly composed of a p50 and a p65 subunit (15). NF-κB is maintained in an inactive state in cells through its binding to an inhibitory cytosolic anchor protein called inhibitor of NF-κB (IκB). On activation, IκB is degraded through the proteasome, releasing active NF-κB into the nucleus where it regulates gene expression. NF-κB is implicated in a wide variety of cell responses, which in part depend on cell type, context, and other signaling events. In melanoma, constitutive NF-κB signaling is associated with survival, angiogenesis, and metastasis and it is therefore implicated in tumor maintenance and progression. We find that melanoma cells have elevated basal NF-κB signaling, but this is insufficient to reverse the death induced by inhibition of BRAF signaling, and survival only occurs when NF-κB signaling is further enhanced by TNF-α. We show that TNF-α activates NF-κB (Fig. 4) and the importance of NF-κB is established by our RNAi depletion of the p50 or p65 subunits, which block the ability of TNF-α to rescue cells when BRAF signaling is inhibited. Furthermore, we show that TNF-α stimulates increased p50 and p65 subunit levels in melanoma cells, whereas PD184352 suppresses expression of these

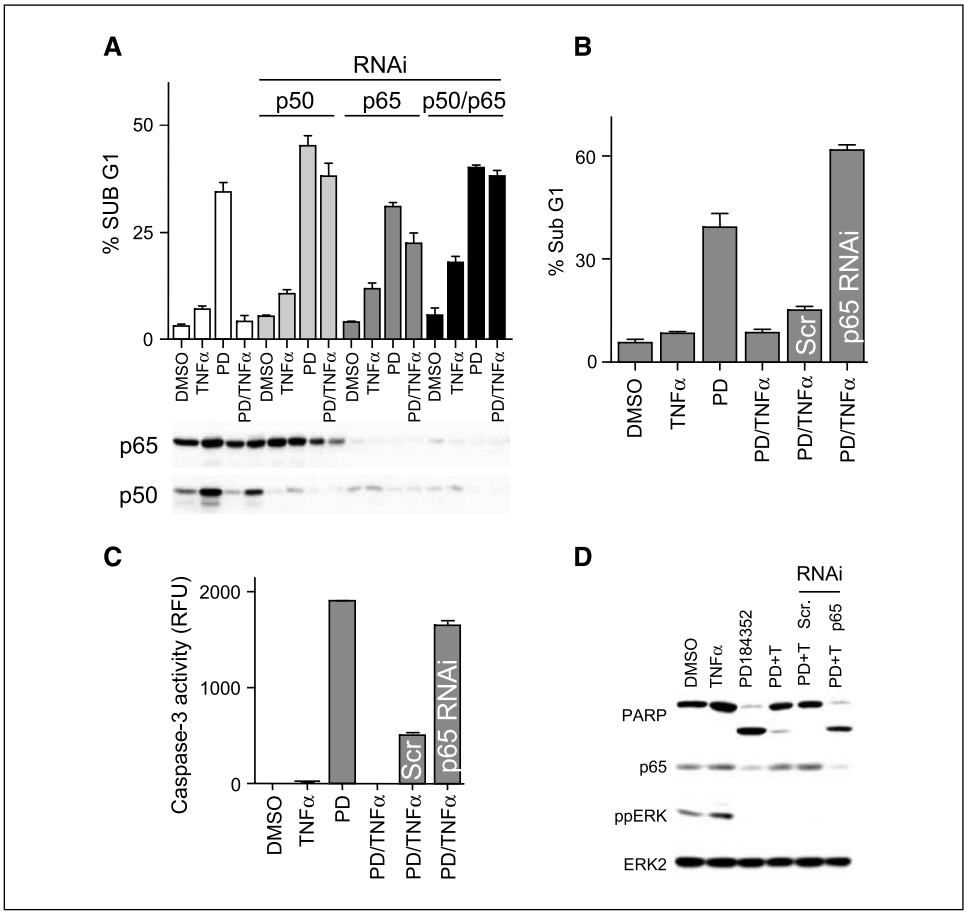

Figure 5. NF-κB is required for TNF-α-mediated reversal of death induced by MEK inhibition. In each experiment, cells were treated with DMSO, PD184352, and TNF-α for 72 h in the presence of various siRNA oligonucleotide probes. *A,* sub-G_1 fraction measurements in Colo829 cells, in which the p50 and/or p65 subunits of NF-κB were depleted. Western blots for p50 and p65 (*bottom*). *B,* sub-G_1 fraction measurements in A375 cells, in which the p65 subunit of NF-κB was depleted compared with cells transfected with a scrambled control. *C,* caspase-3 activity in Colo829 cells, in which the p65 subunit of NF-κB was deleted compared with a scrambled control. *D,* Western blot for PARP, p65, phospho-ERK, and total ERK2 in A375 cells, in which the p65 subunit of NF-κB was depleted.

subunits (Fig. 5*A*) and we find that TNF-α induces IκB degradation (data not shown). We also find that BRAF seems to enhance the NF-κB activity in these cells because inhibition of the pathway using PD184352 caused a reduction in NF-κB-dependent luciferase activity (Fig. 4*A*). Thus, the interplay between these pathways is complex and may be regulated at many levels. Nevertheless, our data show that the interaction between these pathways provides a specific rescue that allows TNF-α to overcome the consequences of BRAF signaling inhibition.

We have also shown that $p21^{Cip1}$ is a critical NF-κB target that mediates the effects of TNF-α in WM266.4 cell, but this is not a universal function of $p21^{Cip1}$ as it did not seem to play a role in Colo829 or A375 cells (data not shown). The regulation of $p21^{Cip1}$ expression in WM266.4 is complex. Its expression is blocked when MEK is inhibited or when the cells are treated with TNF-α and yet together these agents antagonize each other allowing $p21^{Cip1}$ expression to recover in a NF-κB-dependent manner, as shown by fact that p65 subunit prevents $p21^{Cip1}$ reexpression. Furthermore, our data show that depletion of $p21^{Cip1}$ is not sufficient to induce apoptosis because although TNF-α suppresses $p21^{Cip1}$ expression, it does not induce apoptosis. Thus, it seems that a reduction in $p21^{Cip1}$ protein levels is not sufficient to induce apoptosis in WM266.4 cells but that $p21^{Cip1}$ expression is essential to prevent the induction of apoptosis when oncogenic BRAF-MEK signaling is inhibited.

We posit that NF-κB is the critical transcription factor that allows TNF-α to block apoptosis when BRAF signaling is inhibited in melanoma cells. In WM266.4 cells, a key downstream target of NF-κB is $p21^{Cip1}$, but in the other cell lines, other targets presumably mediate the effects of signaling through this pathway. Our preliminary studies suggest that the prosurvival proteins Bcl_{XL}, Mcl-1, and XIAP do not play a role in mediating the survival of melanoma cells, in which BRAF signaling is suppressed. However, many of the other factors within these protein families are NF-κB targets and are implicated in melanoma cell survival (15, 18). For example, ML-IAP is thought to cause resistance to apoptosis and chemotherapy in some melanomas (19) and we are currently attempting to identify the key NF-κB targets in Colo829 and A375 cells.

Our findings have important clinical implications because of the crucial role that inflammation plays in the tumor microenvironment of melanoma. Several studies have shown that there is significant infiltration of cells of the immune system into melanomas, particularly macrophages and mast cells, both of which are known to secrete TNF-α, IL-1, and IL-6 (20–22). TNF-α plays a critical role in various cancers as shown, for example, by the observation that TNF-α-null mice are resistant to murine models of skin carcinogenesis (23). Furthermore, polymorphisms that elevate TNF-α expression are associated with poor prognosis and increased disease severity in cancers, such as non-Hodgkin's lymphoma (24). The intrinsic basal activity of NF-κB in melanoma cells together with the high levels of invasive macrophages seen in melanoma could account for the fact that NF-κB is constitutively activated in most human melanomas (15), and importantly, the invasion of cells of the immune system is associated with a poor prognosis in invasive melanoma (22).

Several small molecular weight inhibitory drugs that target either BRAF or its downstream effector MEK are in preclinical or clinical development. The most advanced of these is sorafenib, a broad specificity kinase inhibitor that targets BRAF, CRAF, and RTKs, such as the vascular endothelial growth factor receptor (VEGFR) and c-kit (25). However, as a monotherapy, sorafenib provides only a marginal clinical benefit in patients with melanoma and its activity does not track with BRAF mutation status (26). The reason for its lack of activity is unclear. Apparently, sorafenib is not potent enough to target BRAF in melanoma patients and its clinical activity is due to its anti-RTK activity. Indeed, the efficacy of sorafenib in renal cell carcinoma patients is thought to arise from its anti-VEGFR activity (25). Importantly, our data now suggest an intriguing alternative interpretation. We suggest that

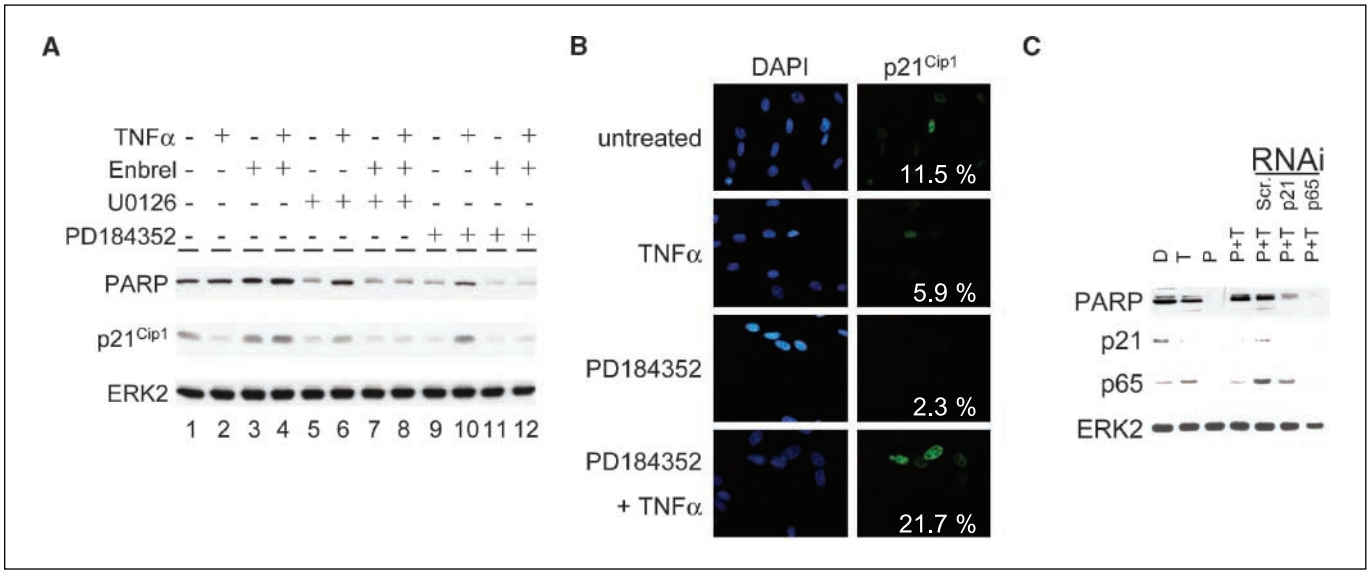

Figure 6. NF-κB-dependent expression of $p21^{Cip1}$ is required for TNF-α-mediated reversal of death in WM266.4 cells treated with MEK inhibitors. *A,* immunoblotting for PARP, $p21^{Cip1}$, and ERK2 (loading control) in WM266.4 cells treated (96 h) with TNF-α, Enbrel, U0126, and PD184352. *B,* immunofluorescence for $p21^{Cip1}$ expression in WM266.4 cells treated (96 h) with TNF-α with or without PD184352. Nuclei were counterstained with DAPI. *Numbers,* the percentage of $p21^{Cip1}$-positive nuclei counted in six independent fields. *C,* Western blotting for PARP, $p21^{Cip1}$, p65, and ERK2 in WM266.4 cells treated with DMSO (*D*), TNF-α (*T*), and PD184352 (*P*). Where indicated, the cells were also transfected with RNAi oligonucleotides against $p21^{Cip1}$, the p65 subunit of NF-κB, or a scrambled control.

TNF-α may present a significant barrier to the successful use of anti-BRAF or anti-MEK therapies in melanoma. Although these drugs will predispose melanoma cells to death, the prosurvival activity of TNF-α could overcome this, causing these approaches to fail.

We therefore propose that strategies that combine drugs that target BRAF or MEK with agents that antagonize TNF-α could provide an effective approach to treating melanoma because they will both induce cell death and remove the rescue pathway provided by TNF-α. Several agents that inhibit TNF-α are available. Biological agents, such as Enbrel and infliximab, antagonize TNF-α by binding to TNF-α or its receptors and are already approved for clinical use in psoriasis, rheumatoid arthritis, and Crohn's disease (27, 28). Alternatively, several widely used anti-inflammatory drugs that target NF-κB, such as aspirin, sodium salicylate, or sulindac (18), could also be tested. Finally, NF-κB could also be inhibited indirectly by proteasome inhibitors, such as Valcade, *Vinca* alkaloids, such as vinblastine, and alkylating agents, such as temozolomide, agents that all work through distinct mechanisms (18). Melanoma cells are particularly resistant to most current forms of treatment and their innate resistance to apoptosis is an enormous barrier to successful treatment. We propose that the agents described above hold enormous promise if combined with BRAF/MEK inhibitors to prime melanoma cells for apoptosis. Further studies are warranted to test the potential of these approaches.

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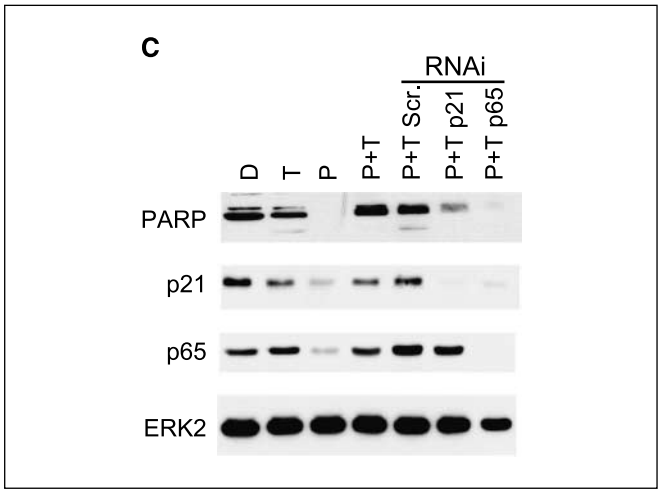
Correction: TNF-α Prevents Melanoma Cell Death

In the article on how TNF-α prevents melanoma cell death in the January 1, 2007 issue of *Cancer Research* (1), there is data missing from Fig. 6*C*. The corrected figure appears below.



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Tumor Necrosis Factor-α Blocks Apoptosis in Melanoma Cells when BRAF Signaling Is Inhibited

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Cancer Res 2007;67:122-129.