NF-κB Regulation of c-FLIP Promotes TNFα-Mediated RAF Inhibitor Resistance in Melanoma

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Targeted inhibitors elicit heterogeneous clinical responses in genetically stratified groups of patients. Although most studies focus on tumor intrinsic properties, factors in the tumor microenvironment were recently found to modulate the response to inhibitors. Here, we show that in cutaneous BRAF V600E melanoma, the cytokine tumor necrosis factor-α (TNFα) blocks RAF inhibitor–induced apoptosis via activation of NF-κB. Several NF-κB-dependent factors are upregulated following TNFα and RAF inhibitor treatment. Of these factors, we show that death receptor inhibitor cellular caspase 8 (FLICE)-like inhibitory protein (c-FLIP) is required for TNFα-induced protection against RAF inhibitor. Overexpression of c-FLIP_S or c-FLIP_L isoform decreased RAF inhibitor–induced apoptosis in the absence of TNFα. Importantly, targeting NF-κB enhances response to RAF inhibitor *in vitro* and *in vivo*. Together, our results show mechanistic evidence for cytokine-mediated resistance to RAF inhibitor and provide a preclinical rationale for the strategy of cotargeting the RAF/MEK/ERK1/2 pathway and the TNFα/NF-κB axis to treat mutant BRAF melanomas.

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INTRODUCTION

Targeted inhibitors, such as vemurafenib and dabrafenib, elicit strong but heterogeneous effects in mutant BRAF V600E cutaneous melanoma patients (Flaherty *et al.*, 2010; Hauschild *et al.*, 2012; Long *et al.*, 2012; Sosman *et al.*, 2012). However, ~50% of patients do not achieve a partial or complete response. Furthermore, many patients who achieve an objective response ultimately develop acquired resistance (Hartsough *et al.*, 2014). Heterogeneous modes of resistance have been identified in patient samples with reactivation of extracellular signal–regulated kinase 1/2 (ERK1/2) signaling being a frequent mechanism. Simultaneously targeting multiple steps in the RAF/MEK/ERK1/2 pathway is more efficacious than single-agent therapy (Flaherty *et al.*, 2012); however, resistant tumors still emerge. These findings indicate that the efficacy of single pathway targeting is limited, and strategies need to be developed to cotarget alternative pathways.

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Abbreviations: c-FLIP, cellular caspase 8 (FLICE)-like inhibitory protein; ERK1/2, extracellular signal–regulated kinase 1/2; IAP, inhibitor of apoptosis; shRNA, short hairpin RNA; siRNA, small interfering RNA; TAM, tumor-associated macrophage; TNFα, tumor necrosis factor-α

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The tumor microenvironment modulates the response to targeted therapies. Growth factors including hepatocyte growth factor and neuregulin 1 provide protective signals counteracting the effects of vemurafenib (Straussman *et al.*, 2012; Wilson *et al.*, 2012; Abel *et al.*, 2013). Accordingly, cotargeting the corresponding receptor tyrosine kinase pathway and the RAF/MEK/ERK pathway elicited better outcome than single pathway targeting (Lito *et al.*, 2012; Straussman *et al.*, 2012; Wilson *et al.*, 2012; Abel *et al.*, 2013). However, because of the complex composition of the tumor microenvironment, additional compensatory pathways will likely modulate the response to RAF inhibitors and will need to be targeted to maximize the effects of RAF and MEK inhibitors in melanoma.

Advanced-stage melanoma cells often have constitutive IκB kinase and NF-κB activity (Meyskens *et al.*, 1999; Yang and Richmond, 2001). NF-κB is a family of protein complexes that controls the transcription of genes involved in inflammatory response and cell survival (Karin and Lin, 2002; Lawrence, 2009). Antiapoptotic targets of NF-κB include Bcl-2 (Catz and Johnson, 2001) and Bcl-xl (Chen *et al.*, 2000), the inhibitor of apoptosis (IAP) family proteins (c-IAP1 (Stehlik *et al.*, 1998a), c-IAP2 (Stehlik *et al.*, 1998a), XIAP (Stehlik *et al.*, 1998b), survivin (Kawakami *et al.*, 2005)), and death receptor inhibitor cellular caspase-8 (FLICE)-like inhibitory protein (c-FLIP) (Kreuz *et al.*, 2001), and therefore NF-κB is implicated in radiation- and chemo-resistance in many cancers (Wang *et al.*, 1999; Magne *et al.*, 2006).

In the tumor microenvironment, tumor-associated macrophages (TAMs) produce cytokines such as tumor necrosis factor-α (TNFα) and IL-1β, which are potent inducers of the NF-κB pathway. Studies have shown that TAM infiltration is associated with melanoma stage and angiogenesis

(Torisu *et al.*, 2000) and may serve as a prognostic marker for AJCC (American Joint Committee on Cancer) stage I/II melanoma (Jensen *et al.*, 2009). Furthermore, targeting the tumor NF-κB pathway or blocking TNFα signaling in TAMs sensitized melanoma cells to chemotherapy or radiation therapy (Amiri *et al.*, 2004; Meng *et al.*, 2010). TNFα also blocks apoptosis induced by MEK inhibitor or BRAF depletion in mutant BRAF melanoma cells, although the underlying mechanism remains unclear (Gray-Schopfer *et al.*, 2007). Here, we tested the role of the NF-κB pathway in the responses of mutant BRAF melanoma cells to RAF inhibition. We show that activation of the NF-κB pathway by TNFα protects melanoma cells against the RAF inhibitor via upregulation of the antiapoptotic protein c-FLIP. Importantly, targeting the NF-κB pathway sensitized melanoma cells to the RAF inhibitor *in vitro* and *in vivo*. Our data provide the rationale for cotargeting the RAF/MEK/ERK and NF-κB pathways to treat mutant BRAF melanoma.

RESULTS

The NF-κB agonist, TNFα, protects melanoma cells from RAF inhibitor–induced apoptosis

To test the role of the NF-κB pathway in the response of melanoma cells to RAF inhibitor, we treated three mutant BRAF melanoma cells with TNFα and examined their response to PLX4720, the tool compound for vemurafenib (Tsai *et al.*, 2008). Treatment with TNFα significantly enhanced NF-κB reporter activity in both untreated and RAF inhibitor–treated cells, although activation was slightly lower in the presence of PLX4720 (Figure 1a). Consistent with previous observations (Shao and Aplin, 2010), PLX4720 elicited strong cytotoxic effects in BRAF V600E melanoma cells (Figure 1b and c and Supplementary Figure S1 online). TNFα treatment alone slightly increased the basal level of cell death in A375 but not M238 and M229 cells. Importantly, TNFα strongly protected multiple BRAF V600E melanoma cells against the cytotoxic effect of PLX4720 but had little impact on cell cycle progression (Figure 1b and c and Supplementary Figure S1 online). TNFα was also protective against the RAF plus MEK inhibitor combination but did not reduce death induced by etoposide (Supplementary Figure S1c online). Cleaved caspase 3 was detected 24–48 hours following PLX4720 treatment and was strongly inhibited by TNFα stimulation (Figure 1c). Furthermore, activation of both intrinsic (caspase 9) and extrinsic (caspase 8) apoptosis pathways was detected following PLX4720 treatment and was inhibited by TNFα (Figure 1c). Cleavage of caspases 3 and 9 was first detected at ~18 hours following PLX4720 treatment, whereas caspase 8 cleavage showed a delay of 2–4 hours (Supplementary Figure S2a online), suggesting that caspase 9 activation is an upstream event in RAF inhibitor–induced apoptosis. Interestingly, caspase 8 depletion reduced cleavage of caspases 3 and 9 (Supplementary Figure S2b online), suggesting cross-talk between these caspases. Together, these results show that TNFα protects melanoma cells from RAF inhibitor–induced apoptosis and counteracts the activation of caspases 3, 8, and 9.

NF-κB signaling is required for TNFα-mediated protection from RAF inhibitor–induced apoptosis

Next, we tested the requirement for NF-κB in TNFα-mediated protection. We selectively depleted the NF-κB components, p50 and p65, alone and in combination, as confirmed by western blot (Figure 2a). Depletion of p50 and p65 individually or in combination completely reversed the TNFα-mediated protection against the RAF inhibitor cytotoxicity (Figure 2b–d). Knockdown of NF-κB components also sensitized melanoma cells to TNFα treatment, indicating that NF-κB may confer natural resistance of melanoma cells to TNFα-induced apoptosis. In the case of A375 cells, TNFα treatment in p65 and p65 plus p50 knockdowns further augmented the apoptotic effect of PLX4720 (Figure 2b). These data indicate that TNFα-mediated protection against RAF inhibitor requires NF-κB. In contrast, NF-κB p65 and p50 knockdowns had little impact on the sensitivity of melanoma cells to nontargeted chemotherapeutics such as etoposide and cisplatin (Supplementary Figure S3a and b online).

c-IAP2 and c-FLIP are upregulated upon TNFα and PLX4720 treatment in an NF-κB-dependent manner

To identify the downstream effectors of NF-κB that may account for the protection against PLX4720, we analyzed the expression of NF-κB target proteins known to regulate apoptosis. We focused on differences in target expression in cells treated with PLX4720 alone and with a combination of PLX4720 and TNFα. Upregulation of the BH3-only proteins, Bim-EL and Bmf, and downregulation of the Bcl-2 family protein, Mcl-1, by PLX4720 (Boisvert-Adamo *et al.*, 2009; Shao and Aplin, 2010) were not significantly altered by TNFα (Supplementary Figure S4a online). Furthermore, no apparent TNFα-regulated differences were observed in other Bcl-2 family proteins and most IAP family proteins (XIAP, survivin, livin, and c-IAP1) (Supplementary Figure S4a online). In contrast, two antiapoptotic proteins, c-IAP2 and c-FLIP, were upregulated by TNFα and PLX4720 when compared with PLX4720 alone (Figure 3a).

c-IAP2 was upregulated by TNFα alone in A375 and M238 cells and cotreatment with PLX4720 weakened this upregulation, possibly because of a decreased NF-κB activity upon RAF inhibitor treatment (Figure 3a). The mRNA levels of c-IAP2 were regulated in a similar pattern (Figure 3b). Regulation of c-FLIP was more complex. TNFα alone induced c-FLIP protein expression in M238 cells, whereas PLX4720 treatment alone slightly enhanced c-FLIP expression in A375 cells (Figure 3a and Supplementary Figure S5 online). Multiple forms of c-FLIP provide protection from death receptor–mediated apoptosis (Scaffidi *et al.*, 1999; Krueger *et al.*, 2001; Golks *et al.*, 2005), and we detected upregulation of the long isoform, c-FLIP_L, in our studies. The mRNA levels of c-FLIP were upregulated by TNFα alone or TNFα with PLX4720 in A375 and M238 cells (Figure 3b). Despite this complexity, a consistent observation was that c-FLIP expression was upregulated in the combined TNFα- and PLX4720-treated cells versus PLX4720-alone-treated cells (Figure 3a, and quantitated in Supplementary Figure S5 online). This observation was also confirmed in M229 cells (Supplementary Figure S4b and c online).

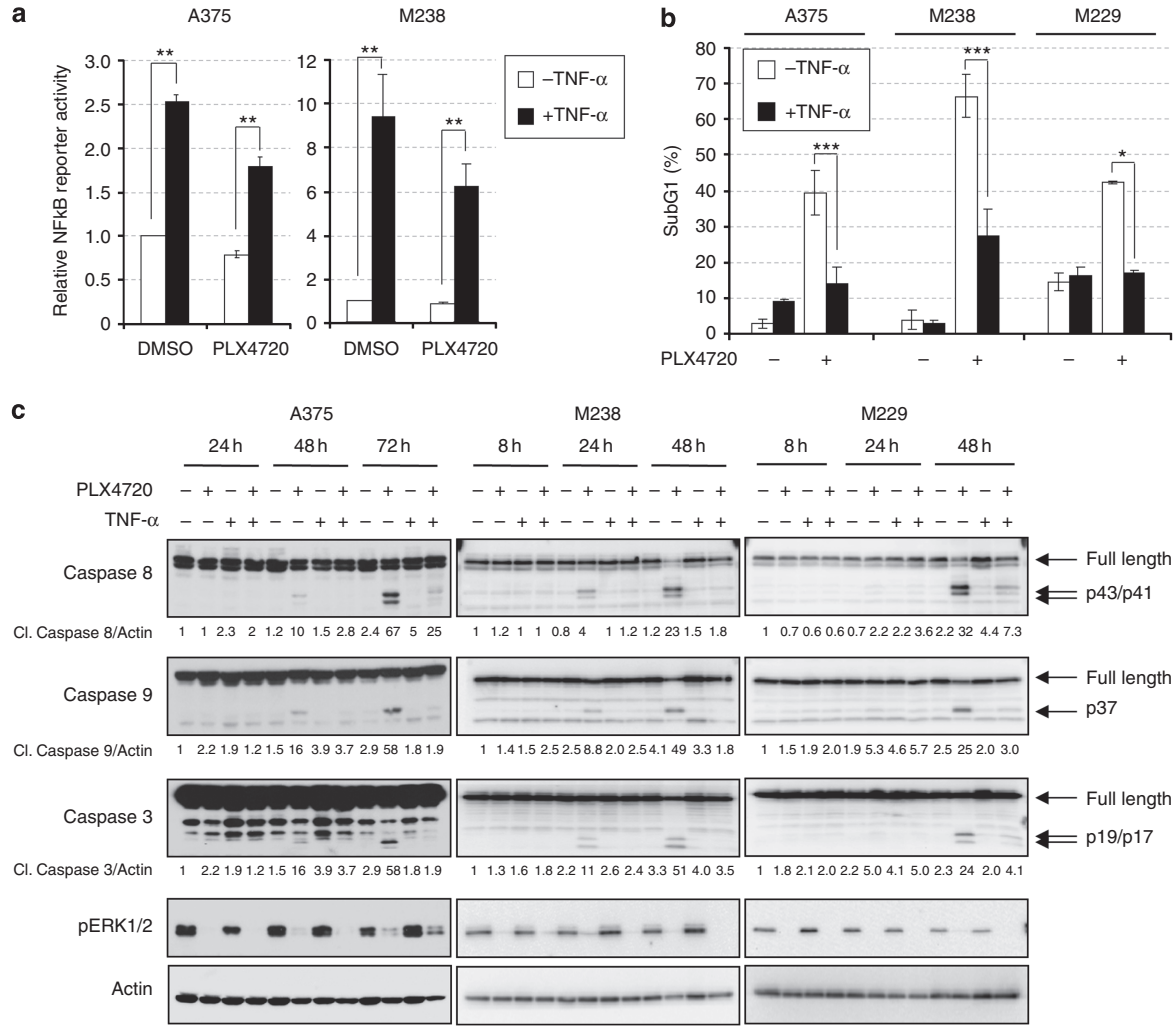

Figure 1. Tumor necrosis factor-α (TNFα) protects melanoma cells from RAF inhibitor–induced apoptosis. (**a**) Luciferase assays were performed as described in the Materials and Methods. Cells were treated with ± 50 ng ml^{-1} TNFα and ± 5 μM PLX4720 for 24 hours. Average relative luciferase activities from three experiments are shown. Error bars represent SD. $^{**}P<0.01$. (**b**) Cells were treated with ± 50 ng ml^{-1} TNFα and either ± 10 μM PLX4720 for 72 hours (A375) or ± 5 μM PLX4720 for 48 hours (M238, M229). Cells stained with propidium iodide (PI) for cell cycle analysis. Average percentages of sub-G1 populations from three experiments are quantified. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. (**c**) A375, M238, and M229 cells were treated with ± 50 ng ml^{-1} TNFα and ± 5 μM PLX4720 for 8–72 hours. Cells lysates were analyzed by western blotting. Arrows indicate full-length or cleaved (Cl.) products of caspases. Relative levels of cleaved caspases are indicated below the corresponding blots. pERK1/2, phosphorylated extracellular signal–regulated kinase 1/2.

To confirm that the regulation of c-IAP2 and c-FLIP by TNFα is dependent on NF-κB, we analyzed their expression in A375 and M238 cells depleted of NF-κB subunits (Figure 3c). Consistent with our data, in control small interfering RNA (siRNA)–treated A375 and M238 cells, PLX4720 and TNFα combination treatment enhanced the expression of c-IAP2 and c-FLIP when compared with PLX4720-alone treatment. However, the upregulation of both proteins was blunted in p50- or p65-depleted cells, indicating that the regulation of c-IAP2 and c-FLIP is NF-κB dependent.

c-FLIP but not c-IAP2 is required for TNFα-induced protection against PLX4720

As both c-IAP2 and c-FLIP have antiapoptotic roles (Liston *et al.*, 1996; Safa and Pollok, 2011), we tested individually the requirement for these two proteins in TNFα-induced protection against PLX4720. Selective siRNAs effectively reduced the induction of c-IAP2 (>90% and 75% downregulation in M238 and M229, respectively) and c-FLIP (>90% and 87% downregulation in M238 and M229, respectively) by TNFα and PLX4720 (Figure 4a). Surprisingly, cells depleted of c-IAP2 were still protected by TNFα against PLX4720-induced apoptosis to a level comparable to control-transfected cells (Figure 4b). In contrast, depletion of c-FLIP completely restored cell death in the presence of TNFα and PLX4720 (Figure 4b and Supplementary Figure S6 online). Similarly, the cleavage of caspases 3, 8, and 9 in the TNFα plus PLX4720 treatment samples was recovered, at least partially, to the level of PLX4720 treatment alone samples in c-FLIP-depleted but not

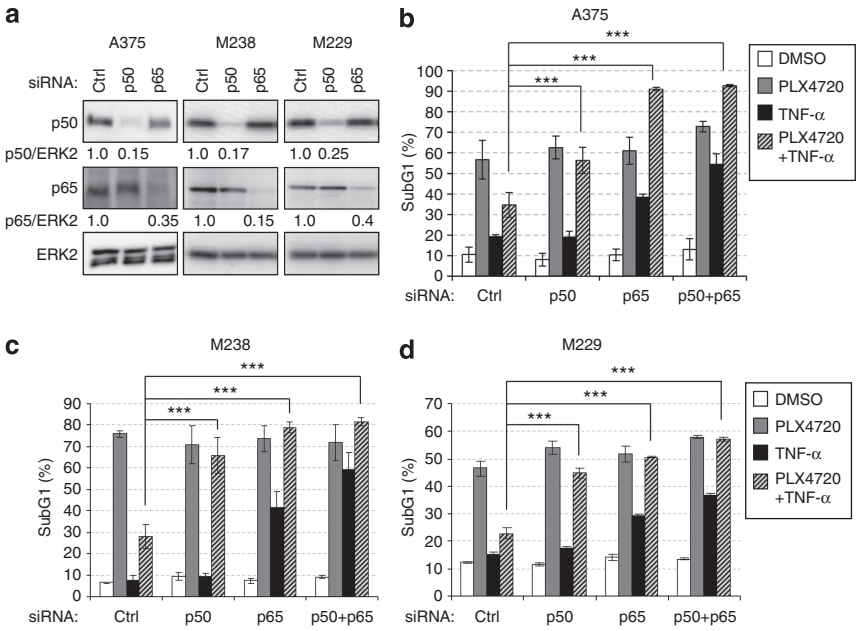

Figure 2. Tumor necrosis factor-α (TNFα)–induced protection from RAF inhibitors is mediated by NF-κB. (**a**) Melanoma cells were transfected with control (Ctrl), p50, or p65 small interfering RNAs (siRNAs) for 72 hours. Cells lysates analyzed by western blotting. Knockdown efficiency was estimated by band intensity quantitation and shown below the blots. (**b**–**d**) Melanoma cells were transfected with indicated siRNAs for 72 hours and treated with ±50 ng ml^{-1} TNFα and ±5 μM PLX4720 for 48 (M229 and M238) or 72 (A375) hours before being harvested for propidium iodide (PI) cell cycle analysis. Average percentage of sub-G1 population from three experiments was plotted for each condition. ERK2, extracellular signal–regulated kinase 2. Error bars represent SD. For statistics, one-way analysis of variance (ANOVA) analyses followed by the Bonferroni test were performed on TNFα+PLX4720 treatment groups. ***$P<0.001$.

c-IAP2-depleted M238 and M229 cells (Figure 4a). c-FLIP depletion had no impact on cell death induced by ERK1/2 pathway-independent drugs such as etoposide and cisplatin (Supplementary Figure S3 online). These results suggest that c-FLIP but not c-IAP2 is required for TNFα-mediated protection against RAF inhibitor.

Overexpression of c-FLIP protects melanoma cells against PLX4720-induced apoptosis

To further test the role of c-FLIP as the prosurvival effector downstream of NF-κB, we examined whether c-FLIP was sufficient to protect against PLX4720-induced apoptosis. We stably expressed either c-FLIP_S (short isoform), c-FLIP_L (long isoform), or LacZ (as a control) in A375 cells, as verified by western blotting (Figure 5a). We then tested for the response to PLX4720. PLX4720 treatment led to decreased expression of transgenes, presumably due to reduced CMV promoter activity upon ERK1/2 signaling inhibition (Chen and Stinski, 2002). Consistent with prior results, treatment of RAF inhibitor induced ~45% cell death in LacZ-expressing A375 cells (Figure 5b). Ectopic expression of c-FLIP_S or c-FLIP_L decreased cell death to 12% or 16%, respectively. The reduction in cell death was associated with lower detectable levels of cleaved caspases 3, 8, and 9 (Figure 5a). To confirm that the protective role of c-FLIP was not cell line dependent, we conducted similar experiments in M229 cells and used another negative control, green fluorescent protein (Figure 5c and d). The green fluorescent protein–expressing M229 cells exhibited 48% cell death after RAF inhibitor treatment, and this was decreased to 34% or 28% following c-FLIP_S or c-FLIP_L expression, respectively. Therefore, c-FLIP isoforms are sufficient to protect mutant BRAF melanoma cells from PLX4720-induced cell death.

Targeting p65 NF-κB enhances the response to PLX4720 *in vitro* and *in vivo*

Prompted by our observations, we hypothesized that targeting the NF-κB pathway may synergize with the RAF inhibitor, PLX4720. p65 is a major component of the NF-κB signaling complex; thus, we generated two 1205Lu cell lines (harboring doxycycline-inducible short hairpin RNAs (shRNAs) targeting p65. In the absence of doxycycline, PLX4720 treatment induced 5–10% cell death after 24 hours and 40–50% after 48 hours in both cell lines *in vitro* (Figure 6b). Treatment of doxycycline led to efficient knockdown of p65 and reduction in c-FLIP expression in both cell lines and significantly increased cell death after 24 and 48 hours of PLX4720 treatment (Figure 6a and b).

We further tested this hypothesis in a mouse xenograft model using A375 cells expressing either control nontargeting or p65-specific shRNAs controlled by doxycycline. A375TR cells were used for their reproducible tumor-forming ability in nude mice and efficient doxycycline inducible knockdowns (Abel *et al.*, 2013). Doxycycline treatment significantly decreased p65 expression in A375TR p65 shRNA 2 and 3 cells (Figure 6c). *In vivo*, p65 shRNA induction had marginal impact on tumor growth in the absence of the RAF inhibitor. Treatment with the RAF inhibitor alone partially inhibited tumor growth, consistent with partial resistance of this cell line to RAF inhibitor–induced cell death

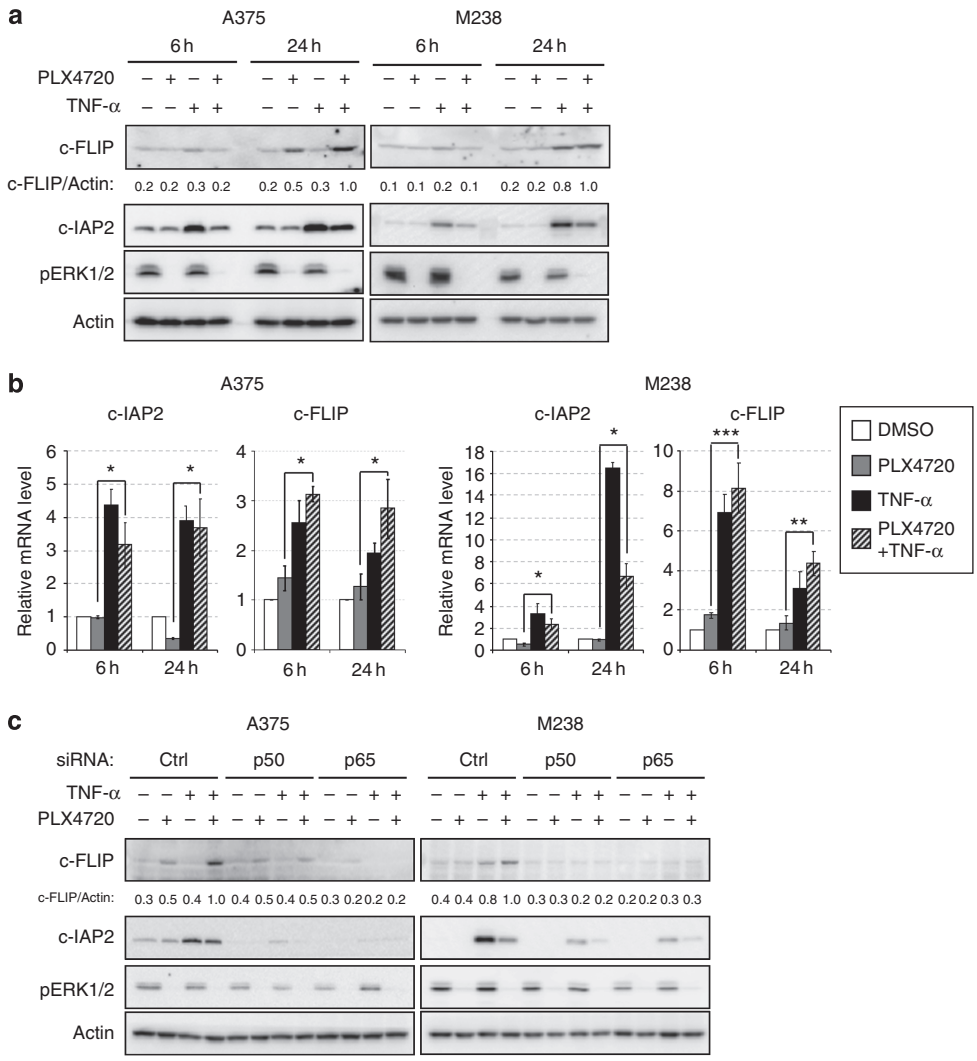

Figure 3. c-IAP2 (cellular inhibitor of apoptosis 2) and c-FLIP (cellular caspase 8 (FLICE)-like inhibitory protein) are upregulated by tumor necrosis factor-α (TNFα) and PLX4720 cotreatment. (**a**) A375 and M238 cells were treated with ± 50 ng ml^{-1} TNFα and ± 5 μM PLX4720 for 6 or 24 hours before analysis by western blotting. Normalized c-FLIP/actin band intensity ratios are shown. pERK1/2, phosphorylated extracellular signal–regulated kinase 1/2. (**b**) A375 and M238 cells were treated as in **a** and total RNA isolated and quantitative reverse transcriptase–PCR (qRT-PCR) performed on c-IAP2 and c-FLIP genes. Housekeeping genes, actin or EEFA1, were internal controls. Average results from three independent experiments are shown. Error bars represent SD. *$P<0.05$; **$P<0.01$; ***$P<0.001$. (**c**) A375 and M238 cells were transfected with indicated small interfering RNAs (siRNAs) for 72 hours and then treated with ± 50 ng ml^{-1} TNFα and ± 5 μM PLX4720 for an additional 24 hours. Ctrl, control. Cells were lysed for western blotting analysis on indicated proteins. Normalized c-FLIP/actin band intensity ratios are indicated.

(Shao and Aplin, 2010). Remarkably, tumor growth was further inhibited when RAF inhibitor was combined with p65 depletion (Figure 6d). Analysis of harvested tumors at the end of the xenograft experiment showed a reduction in p65 with shRNAs (Supplementary Figure S7 online), although levels were variable because of the late time point of harvesting (day 28) and the presence of stromal cells. In summary, targeting the NF-κB pathway enhances the response to RAF inhibitor *in vitro* and *in vivo*.

DISCUSSION

The response in mutant BRAF melanoma patients to RAF inhibitors is heterogeneous (Hartsough *et al.*, 2014). Although many forms of acquired resistance involve changes in the allelic frequency of mutations that reactivate the RAF/MEK/ERK1/2 pathway, factors in the tumor microenvironment are important in modulating the primary response to RAF inhibitors (Straussman *et al.*, 2012; Wilson *et al.*, 2012; Abel *et al.*, 2013). In this study, we showed that TNFα modulates the apoptotic response of mutant BRAF melanoma cells to the RAF inhibitor, PLX4720, in an NF-κB-dependent manner. This work builds on previous studies showing that TNFα blocks apoptosis in melanoma cells treated with MEK inhibitors depleted for BRAF (Gray-Schopfer *et al.*, 2007) and adds TNFα to the list of factors that provide protection against RAF inhibitor monotherapy and the RAF/MEK inhibitor combination.

Our results showed that TNFα protects melanoma cells against the RAF inhibitor–induced apoptosis by upregulation

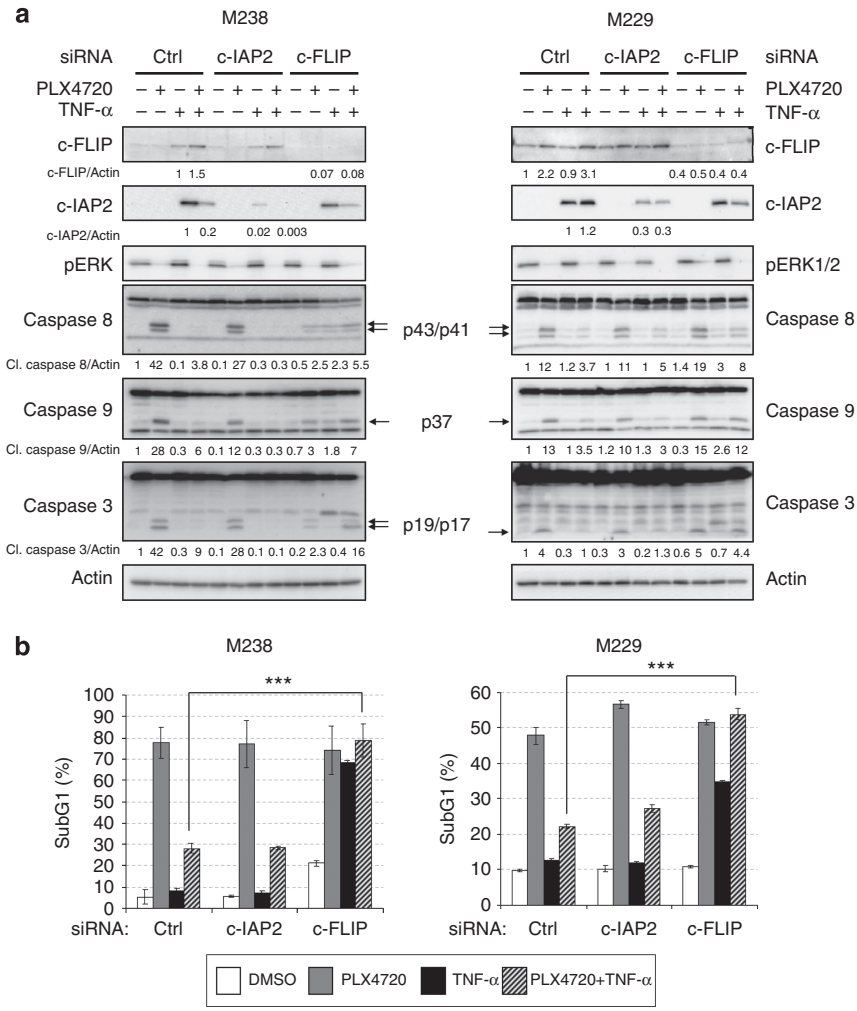

Figure 4. c-FLIP (cellular caspase 8 (FLICE)-like inhibitory protein) but not cIAP2 (cellular inhibitor of apoptosis 2) is required for tumor necrosis factor-α (TNFα)–induced protection against PLX4720. (**a**) M238 or M229 cells were transfected with control (Ctrl), c-IAP2, or c-FLIP-specific small interfering RNAs (siRNAs) for 72 hours. Cells were then treated with ± 50 ng ml^{-1} TNFα and ± 5 μM PLX4720 for an additional 24 hours before lysis for western blot analysis on indicated proteins. Arrows indicate the cleaved (Cl.) forms of the respective caspases. c-IAP2 and c-FLIP knockdown efficiency was determined by quantitating the band intensity. (**b**) M238 and M229 cells were treated with ± 50 ng ml^{-1} TNFα and ± 5 μM PLX4720 for an additional 48 hours after siRNA transfection as in **a**. Cells were then harvested for propidium iodide (PI) cell cycle analysis. Average percentages of sub-G1 cells from three independent experiments are shown for each knockdown condition. Error bars represent SD. ***$P<0.001$.

of c-FLIP. Furthermore, ectopic expression of c-FLIP is sufficient to decrease RAF inhibitor–induced apoptosis in the absence of exogenous TNFα. The molecular details of c-FLIP-mediated protection remain unclear. c-FLIP is generally implicated in protection against extrinsic apoptosis, although the long isoform, c-FLIP_L, may either promote or inhibit apoptosis depending on its expression level (Chang *et al.*, 2002). In the extrinsic apoptosis pathway, activated death receptors induce the assembly of death-inducing signaling complex. The death-inducing signaling complex recruits the FADD (Fas-associated death domain-containing protein) that in turn recruits procaspase 8 via its death-effector domain for self-cleavage and activation. All three c-FLIP isoforms (c-FLIP_L, c-FLIP_S, and c-FLIP_R) carry two death-effector domains at the N terminus that enable them to compete with procaspase 8 for binding to FADD and prevent apoptosis (Irmler *et al.*, 1997; Rasper *et al.*, 1998). c-FLIP may contribute to RAF inhibitor resistance by preventing caspase 8 activation induced by RAF inhibitor. Indeed, overexpression of c-FLIP rescued PLX4720-induced apoptosis with a concurrent reduction in caspase 8 cleavage, as well as cleavage of caspases 3 and 9 (Figure 5a); knockdown of c-FLIP led to the recovery of caspase 8 cleavage that was inhibited by TNFα plus PLX4720 treatment (Figure 4a). In addition, caspase 8 interference protected melanoma cells against PLX4720 but not etoposide treatment (Supplementary Figure S8 online). Although our data implicate the c-FLIP/caspase 8 axis in the protective role of TNFα against the RAF inhibitor, it is still possible that c-FLIP protects melanoma cells against PLX4720 through other downstream targets. For example, c-FLIP may counteract the activation of caspase 10, another caspase activated upon death-inducing signaling complex

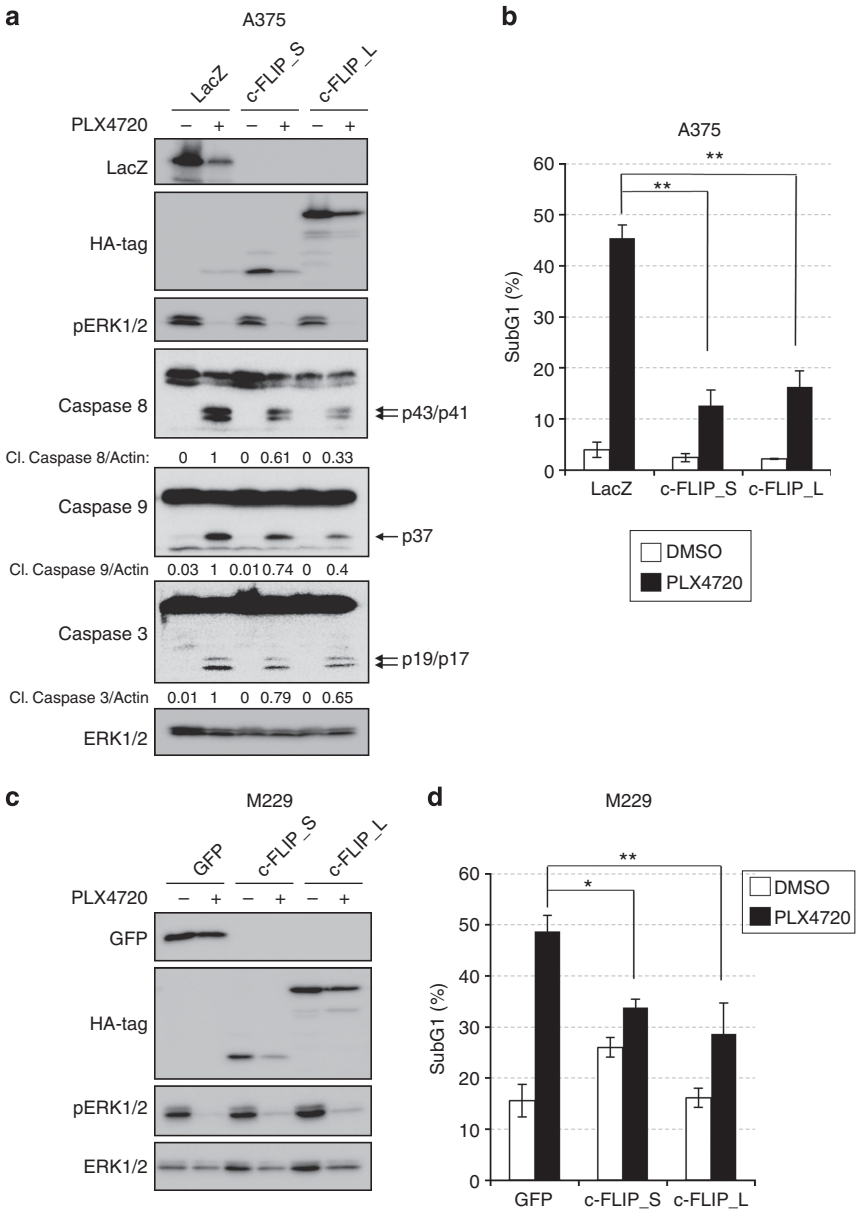

Figure 5. c-FLIP (cellular caspase 8 (FLICE)-like inhibitory protein) overexpression rescues melanoma cells from PLX4720-induced apoptosis. (**a**) A375 cells expressing the indicated transgenes were treated with ± 10 μM PLX4720 for 72 hours. Cells were harvested for western blotting. Cl., cleaved; ERK1/2, extracellular signal–regulated kinase 1/2; HA, hemagglutinin; pERK1/2, phosphorylated ERK 1/2. (**b**) A375 cells were treated as in **a** and harvested for propidium iodide (PI) cell cycle analysis. The average percentage of sub-G1 cells from three independent experiments is shown. Error bars represent SD. $^{**}P<0.01$. (**c**) M229 cells expressing the indicated transgenes were treated with ± 5 μM PLX4720 for 48 hours. Cells were harvested for western blotting. (**d**) M229 cells were treated as in **c** and harvested for PI cell cycle analysis. Average percentage of sub-G1 cells from three independent experiments is shown. Error bars represent SD. $^{*}P<0.05$; $^{**}P<0.01$.

engagement (Safa and Pollok, 2011). Previous studies have shown that c-FLIP expression is elevated in melanoma compared with benign melanocytic lesions (Bullani *et al.*, 2001), and suppression of c-FLIP expression sensitizes melanoma cells to death ligand–induced apoptosis (Geserick *et al.*, 2008). Thus, strategies to decrease c-FLIP expression may prove efficacious for melanoma.

Consistent with previous studies (Kreuz *et al.*, 2001), we found in melanoma cells that TNFα upregulated c-FLIP at the transcriptional level in an NF-κB-dependent manner. The regulation of c-FLIP may be complex as it is regulated by several transcription factors (Safa *et al.*, 2008), and posttranslational regulation may also occur (Chang *et al.*, 2006). Nevertheless, the c-FLIP protein level was consistently elevated in PLX4720 and TNFα cotreated cells compared with cells treated with PLX4720 alone, supporting its protective role against PLX4720.

Studies on resistance to RAF inhibitors have mainly focused on the RAF/MEK/ERK and phosphatidylinositol 3 kinase/AKT pathways. We present a mechanism of resistance and

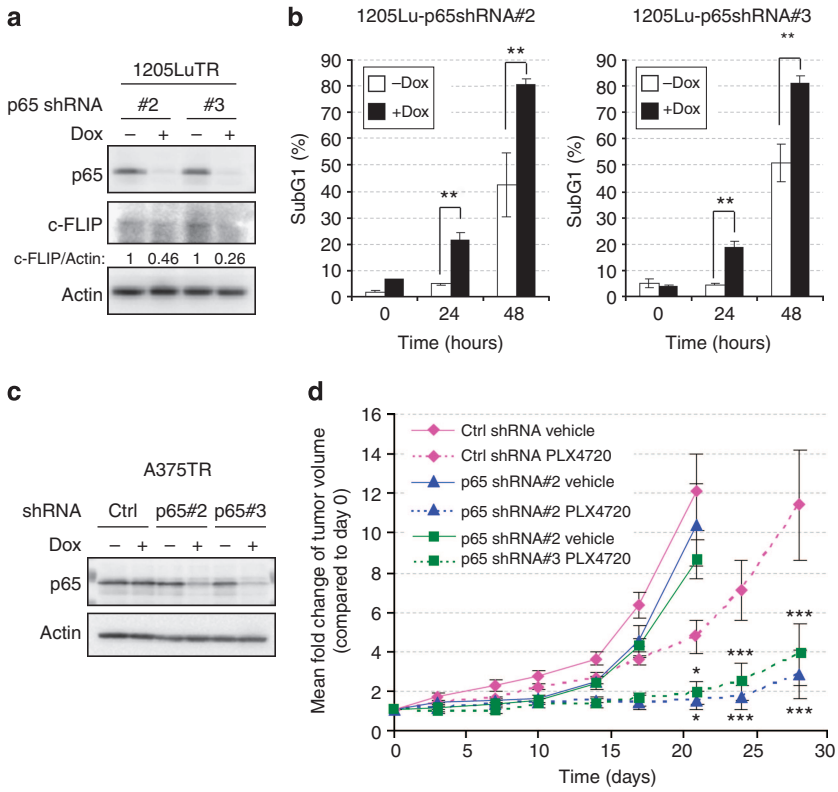

Figure 6. NF-κB targeting enhances responses to PLX4720 *in vitro* and *in vivo*. (**a**) 1205LuTR p65 short hairpin RNA (shRNA) 2 or 3 cells were grown in ± 100 ng ml^{-1} doxycycline (Dox) for 3 days and then lysed for western blotting on p65, c-FLIP (cellular caspase 8 (FLICE)-like inhibitory protein), and actin. Quantitation of the c-FLIP levels is shown. (**b**) As in **a**, except that cells were treated after 3 days with 5 μM PLX4720 for a further 0, 24, or 48 hours and stained with propidium iodide (PI). Error bar represents SD. $^{**}P<0.01$. (**c**) A375TR Ctrl shRNA or A375TR p65 shRNA 2 or 3 cells were cultured in ± 100 ng ml^{-1} doxycycline for 72 hours and lysed for western blotting. (**d**) Growth curves of tumors formed by A375TR cells harboring control or p65 shRNAs in nude mice fed with either PLX4720 or vehicle-laced chow and doxycycline-containing water ($n=6$ per condition). Statistical analysis was performed on relative tumor size differences between PLX4720-treated control shRNA group and PLX4720-treated p65 shRNA 2 or 3 group. Error bars represent SE. $^{*}P<0.05$; $^{***}P<0.001$.

possible therapeutic strategies. Importantly, we identified the NF-κB pathway as an anti–apoptotic signal in RAF inhibitor–treated melanoma cells and cotargeting mutant BRAF and the NF-κB pathway had enhanced antitumor effects *in vitro* and *in vivo* compared with targeting either pathway alone. The NF-κB pathway is implicated in chemo-/radio-therapy resistance of many cancers and is readily targetable by various compounds that target this pathway at different levels. Bortezomib, a proteasome inhibitor, inhibits the NF-κB pathway by preventing the degradation of the NF-κB inhibitor, IκB. The small molecule, BMS-345541, specifically inhibits the NF-κB upstream activating kinase, IκB kinase-β (Burke *et al.*, 2003). Furthermore, NEMO-binding domain peptides block the interaction between NEMO and the IκB kinase complex (May *et al.*, 2000). TAMs are a major source of TNFα in the tumor microenvironment, and TAM infiltration into tumors is enhanced by RAF and MEK inhibitor treatment (Smith *et al.*, 2014). Targeting TAMs may be a viable alternative to direct NF-κB intervention, and the clinical efficacy of combined treatment of macrophage inhibitor PLX3397 and vemurafenib on BRAF V600E melanoma patients is under investigation.

In summary, our findings underline the importance of the tumor microenvironment component, TNFα, to the responsiveness of BRAF V600E melanoma cells to RAF inhibitors and provide a strong rationale for the strategy of cotargeting the RAF/MEK/ERK1/2 pathway and the TNFα/NF-κB axis to treat this subset of melanomas.

MATERIALS AND METHODS

Reagents

PLX4720 was provided by Dr Gideon Bollag and Plexxikon (Berkeley, CA). TNFα was purchased from Sigma-Aldrich (St Louis, MO). Doxycycline was obtained from Thermo Scientific (Rockford, IL).

Cell culture

Melanoma cells were cultured as previously described (Shao and Aplin, 2012; Abel *et al.*, 2013). Cell lines were sequenced at multiple loci. 1205LuTR and A375TR are sublines with high Tet repressor expression (Abel *et al.*, 2013).

Western blotting

Western blotting was performed, as previously described (Shao and Aplin, 2010).

Antibodies against Bcl-xL, phospho-ERK1/2 (Thr202/Tyr204), caspase 8, caspase 9, caspase 3, c-IAP1, cIAP2, XIAP, livin, survivin, p50, and hemagglutinin (HA) tag were purchased from Cell Signaling Technology (Beverley, MA). ERK2 and p65 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). In addition,

antibodies toward Bcl-2 and Mcl-1 (BD Biosciences, San Jose, CA), anti-actin (Sigma-Aldrich), Bim (Stressgen, San Diego, CA), Bmf (ENZO Life Science, Farmingdale, NY), and c-FLIP (Adipogen, San Diego, CA) were utilized.

Luciferase assay

Melanoma cells were transfected with pNF-κB-luc plasmid (2 μg per 33 mm well) using Fugene HD reagent (Promega, Madison, WI). After 24 hours, cells were harvested and subcultured equally into 8 wells. Next day, 4 wells were treated with ±TNFα and ±PLX4720, respectively, for another 24 hours and lysed for the luciferase assay using the Dual-Luciferase Reporter Assay Kit (Promega). The other four wells were treated in the same manner and lysed for the Bradford Assay. The luciferase readings were normalized against Bradford Assay readings accordingly and plotted against each treatment condition.

Quantitative reverse transcriptase–PCR

Total RNA isolation and quantitative reverse transcriptase–PCR were performed as previously described (Shao and Aplin, 2010). Primer sequences are provided in the Supplementary Information online.

Propidium iodide staining

Cells were treated with indicated drugs for 24 hours and then washed in phosphate-buffered saline and fixed in 70% ethanol for 2 hours. Fixed cells were washed in phosphate-buffered saline, pelleted, and resuspended in 500 μl propidium iodide staining buffer (phosphate-buffered saline with 40 $\mu g\,ml^{-1}$ propidium iodide (Sigma-Aldrich), 100 $\mu g\,ml^{-1}$ RNaseA (Thermo Scientific), and 0.1% Triton X-100). Cells were stained for 30 minutes at room temperature and analyzed by flow cytometry on a FACS Calibur. Data were analyzed using Flowjo software (Three Star, Ashland, OR).

RNA interference

Cells were transfected for 72 hours with 12.5 nM siRNAs and Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA). The siRNAs for p50 (5′-GCAAUAGCCUGCCAUGUUU-3′), p65 (5′-GGAUUG AGGAGAAACGUAA-3′), c-IAP2 (5′-UCAAUGAUCUUGUGUUAG A-3′), c-FLIP (5′-GAUGUGUCCUCAUUAAUUU-3′), and nontargeting siRNA control (5′-UAGCGACUAAACACAUCAA-3′) were purchased from Dharmacon (Lafayette, CO).

Lentiviral complementary DNA constructs

c-FLIP complementary DNA isoforms (HA-c-FLIP-L and HA-c-FLIP-S) were amplified from a melanoma complementary DNA pool to incorporate a HA tag using the KOD hot Start DNA polymerase kit (EMD Millipore, Billerica, MA). DNA fragments were cloned into pENTR/D-TOPO vector (Invitrogen), and the resultant entry plasmids were then recombined with pLentipuro/TO/V5-DEST. Lentivirus were produced in 293FT cells (Shao and Aplin, 2010), and melanoma cells were infected with lentivirus for 72 hours before selection with puromycin.

Lentiviral shRNA constructs

DNA oligonucleotides were annealed and ligated into pENTR/H1/TO using the manufacturer's kit and instructions (Invitrogen). The sequences are provided in the Supplementary Information online. The shRNA cassettes were recombined into a destination vector with puromycin resistance. Lentiviruses were produced and melanoma cells were transduced, as above.

Animal studies

A375TR cells (1×10^6/mouse) were intradermally injected into female athymic mice (NU/J: Jackson, Bar Harbour, ME) and allowed to grow for 7 days to reach a palpable tumor size (40–100 mm^3). Mice were then exposed to drinking water containing doxycycline (2 $mg\,ml^{-1}$) and 3 days later fed with control chow or PLX4720 chow (90 $mg\,kg^{-1}$ PLX4720). The first measure of tumor size was taken (day 0). Subsequently, measurements were taken twice a week using digital calipers, and tumor volume was determined by the following formula: $volume = (length \times width^2) \times 0.52$. Mice were killed when tumor volume reached 1,000 mm^3.

Statistical analysis

For *in vitro* experiments, statistical significance of differences between the results was evaluated using student two-tailed *t*-test assuming nonequal variance. For *in vivo* data, statistical analysis was conducted using a mixed-effects model and Tukey's corrected pairwise comparisons of mean fold change in volume between treatment groups (SAS statistical software, Cary, NC).

Animal study approval

All animal experiments were approved by the Institutional Animal Care and Use Committee and conducted in an Association for the Assessment and Accreditation of Laboratory Animal Care accredited facility at Thomas Jefferson University (Philadelphia, PA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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