

PTEN Loss Confers BRAF Inhibitor Resistance to Melanoma Cells through the Suppression of BIM Expression

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Abstract

This study addresses the role of PTEN loss in intrinsic resistance to the BRAF inhibitor PLX4720. Immunohistochemical staining of a tissue array covering all stages of melanocytic neoplasia ($n = 192$) revealed PTEN expression to be lost in >10% of all melanoma cases. Although PTEN expression status did not predict for sensitivity to the growth inhibitory effects of PLX4720, it was predictive for apoptosis, with only limited cell death observed in melanomas lacking PTEN expression (PTEN⁻). Mechanistically, PLX4720 was found to stimulate AKT signaling in the PTEN⁻ but not the PTEN⁺ cell lines. Liquid chromatography multiple reaction monitoring mass spectrometry (LC-MRM) was performed to identify differences in apoptosis signaling between the two cell line groups. PLX4720 treatment significantly increased BIM expression in the PTEN⁺ (>14-fold) compared with the PTEN⁻ cell lines (four-fold). A role for PTEN in the regulation of PLX4720-mediated BIM expression was confirmed by siRNA knockdown of PTEN and through reintroduction of PTEN into cells that were PTEN⁻. Further studies showed that siRNA knockdown of BIM significantly blunted the apoptotic response in PTEN⁺ melanoma cells. Dual treatment of PTEN⁻ cells with PLX4720 and a PI3K inhibitor enhanced BIM expression at both the mRNA and protein level and increased the level of apoptosis through a mechanism involving AKT3 and the activation of FOXO3a. In conclusion, we have shown for the first time that loss of PTEN contributes to intrinsic BRAF inhibitor resistance via the suppression of BIM-mediated apoptosis. *Cancer Res*; 71(7); 2750–60. ©2011 AACR.

Introduction

One defining moment in our understanding of melanoma initiation and progression was the discovery of activating V600E mutations in *BRAF* in >50% of melanomas (1, 2). There is now good evidence that mutated *BRAF* is a *bona fide* therapeutic target in melanoma (3–5). A number of BRAF-specific small molecule kinase inhibitors have been developed

that are now undergoing intense preclinical and clinical investigation (6, 7). In preclinical studies, the BRAF inhibitors PLX4720 and PLX4032 potently inhibited BRAF kinase activity in melanoma cells harboring the *BRAF* V600E mutation and were cytostatic and cytotoxic in both *in vitro* cell culture systems and *in vivo* xenograft melanoma models (5, 6, 8). This promising preclinical activity was mirrored by a recent phase I clinical trial of PLX4032 in advanced melanoma in which >80% of patients showed some level of tumor regression (4). Although most patients with *BRAF* V600E mutated melanoma showed some response to PLX4032, ~20% of those treated did not meet the RECIST criteria threshold for a response (4, 9). Although the mechanisms of intrinsic BRAF inhibitor resistance are not well understood, increased cyclin D1 expression (in ~17% of *BRAF* mutated melanomas) allows for cell cycle entry when MAPK signaling is abrogated (10, 11). It is also likely that constitutive activity in other pathways, such as phospho-inositide 3-kinase (PI3K)/AKT, may contribute to intrinsic resistance by limiting the apoptotic response (12, 13). One of the most critical negative regulators of AKT activity is the phosphatase and tensin homologue (PTEN), which hydrolyses PI-3,4,5-P₃ to PI-4,5-P₂, ultimately preventing the phosphorylation of AKT (14). In this study, we identify loss of PTEN expression, observed in >10% of melanoma specimens, as being responsible for increased PI3K/AKT signaling when

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BRAF is inhibited. We further show that PTEN loss contributes to the intrinsic resistance of *BRAF* V600E-mutated melanoma cell lines to PLX4720 by suppressing the expression of the proapoptotic protein BIM.

Cell culture and MTT assay

Melanoma cell lines were a gift from Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA) and were grown as described in (8). MTT assays were performed as described in ref. (15). The identity of the Wistar Institute cell lines was confirmed using the Coriell Institute (Camden, NJ) cell identity mapping kit. The M233 cell line was derived as described in (16) and its identity confirmed by Biosynthesis Inc. by STR validation analysis.

Generation of WM793TR-PTEN cell lines

Wild-type and G129E PTEN (phosphatase deficient) human cDNAs were a gift from Dr. Bill Sellers (Dana-Farber Cancer Institute; ref. 17). WM793TR-PTEN-wt, WM793TR-PTEN-G129E, and WM793 cells overexpressing wild-type BAD were a kind gift from Dr Andrew Aplin (Kimmel Cancer Center, Philadelphia, PA). Inducible expression of PTEN was obtained by treatment of cultures with doxycycline at a final concentration of 100 ng/mL. The WM793 cells stably expressing wild-type BAD were generated as described in ref. (18).

Western blotting

Proteins were blotted for as described in ref. (15). The antibodies to phospho-AKT (Ser473 and T308), total AKT, phospho-BAD (S75 and S99), Bcl-2, BIM, BRAF, FOXO3a, phospho-PDK1, total PDK1, PTEN, phospho-S6, and total S6 were from Cell Signaling Technology.

Flow cytometry

Cells were treated with 3 or 10 $\mu\text{mol/L}$ PLX4720 for 24 or 48 hours or treated with PLX4720 (3 or 10 $\mu\text{mol/L}$) in the absence or presence of GDC-0941 (3 $\mu\text{mol/L}$, Selleck Chemical Co.) and harvested after 48 hours. Annexin-V/TMRM staining was performed as described in ref. (19).

RNA interference

Cells were grown overnight in RPMI complete media. The following day, complete media was replaced with Opti-MEM (Invitrogen) and one of the following siRNA sequences in complex with Lipofectamine 2,000 (Invitrogen): 50 nmol/L BRAF (Dharmacon), 20 nmol/L PTEN, 25 nmol/L BIM (Cell Signaling Technology). Scrambled siRNAs at each concentration were also added as nontargeting controls. A final concentration of 5% FBS in complete RPMI was added the next day. Cells were transfected for a total of 48 to 72 hours before treatment with PLX4720 (3–10 $\mu\text{mol/L}$).

Quantitative real-time PCR

Total RNA was isolated using Qiagen's RNeasy mini kit. The following TaqMan Gene Expression Assays primer/probes were used: Hs00197982_m1 (BIM), P/N 4319413E (18S), and

Hs99999905_m1 (GAPDH). The 18S + GAPDH data were used for normalizing BIM. After a 2-minute incubation at 50°C, AmpliTaq Gold was activated by a 10-minute incubation at 95°C, followed by 40 PCR cycles consisting of 15 seconds of denaturation at 95°C and hybridization of probe and primers for 1 minute at 60°C. All standards and samples were tested in triplicate wells and data were analyzed using SDS software version 2.3.

Immunofluorescence staining

Cells were plated onto coverslips and treated with PLX4720 for 48 hours before being fixed and permeabilized as previously described (15) and imaged with a Leica confocal microscope at 40 \times magnification.

3D spheroid assays

Collagen implanted spheroids were prepared using the liquid overlay method (12) and were treated with 3 $\mu\text{mol/L}$ of PLX4720, 10 $\mu\text{mol/L}$ LY294002, or both drugs in combination for 72 hours before being analyzed by fluorescence microscopy as described in ref. (11). In other studies, spheroids were treated for 72 hours, washed 3 times in fresh media and allowed to recover for 120 hours before analysis.

Immunohistochemical staining

A melanoma tissue array was generated from de-identified formalin-fixed paraffin-embedded tissue samples from the Moffitt Pathology archives under a protocol approved by the Institutional Review Board of the University of South Florida. Slides were stained using the Ventana Discovery XT automated system (Ventana Medical Systems) as per manufacturer's protocol. The PTEN antibody (1:200, #E4250, Spring Bioscience) was incubated for 32 minutes and the pAKT antibody (1:20, #4051, Cell Signaling) was incubated for 16 minutes. Slides were analyzed by 2 independent observers and consensus scored on a scale from 0 to +3.

Liquid chromatography, multiple reaction monitoring mass spectrometry (LC-MRM) analysis

Whole cell proteins extracts were separated by SDS-PAGE, visualized with Coomassie Brilliant Blue G-250 (Bio-Rad) and selected bands were excised. Following digestion, the internal standard peptides were added in 2% acetonitrile. LC-MRM analysis was performed as described in ref. (20) with 3 replicate analyses for each peptide. Quantification was achieved by using the sum of the peak areas for all detected transitions using Xcalibur QuanBrowser (Thermo). Relative protein expression is determined using the ratio of peak area of the native peptide to corresponding internal standard (Supplementary Table S2).

Statistical analysis

Data show the mean of at least 3 independent experiments \pm the SE mean, unless stated otherwise. GraphPad Prism 5 statistical software was used to perform the Student's *t* test where asterisk (*) indicates $P \leq 0.05$.

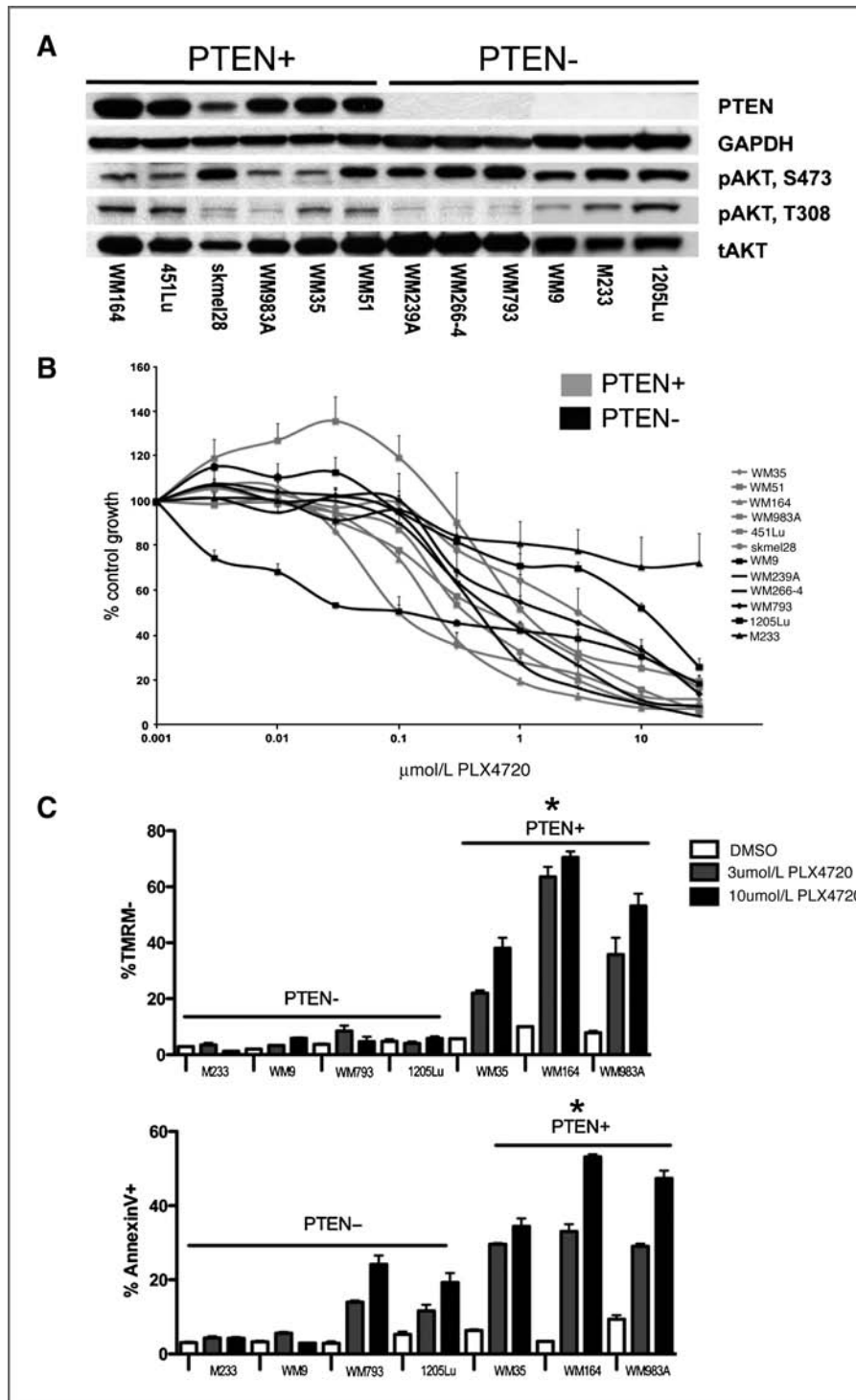


Figure 1. PTEN predicts for PLX4720-induced apoptosis. **A**, basal PTEN and phospho-AKT (pAKT; S473, T308) expression in PTEN+ (WM164, 451Lu, SK-mel-28, WM983A, WM35, WM51) and PTEN- (WM239A, WM266-4, WM793, M233, WM9, 1205Lu) melanoma cell lines. **B**, MTT assay of PTEN+ (gray)-expressing versus PTEN- (black) cell lines. **C**, PTEN+ cells are more sensitive than PTEN- cells to PLX4720-mediated apoptosis. Cells treated for 48 hours with 3 or 10 μmol/L PLX4720 before being stained for TMRM and Annexin-V. Apoptosis was measured by flow cytometry. Data shows mean ± SE mean of 3 independent experiments. *, PTEN+ cohort significantly different from PTEN- cohort ($P < 0.05$).

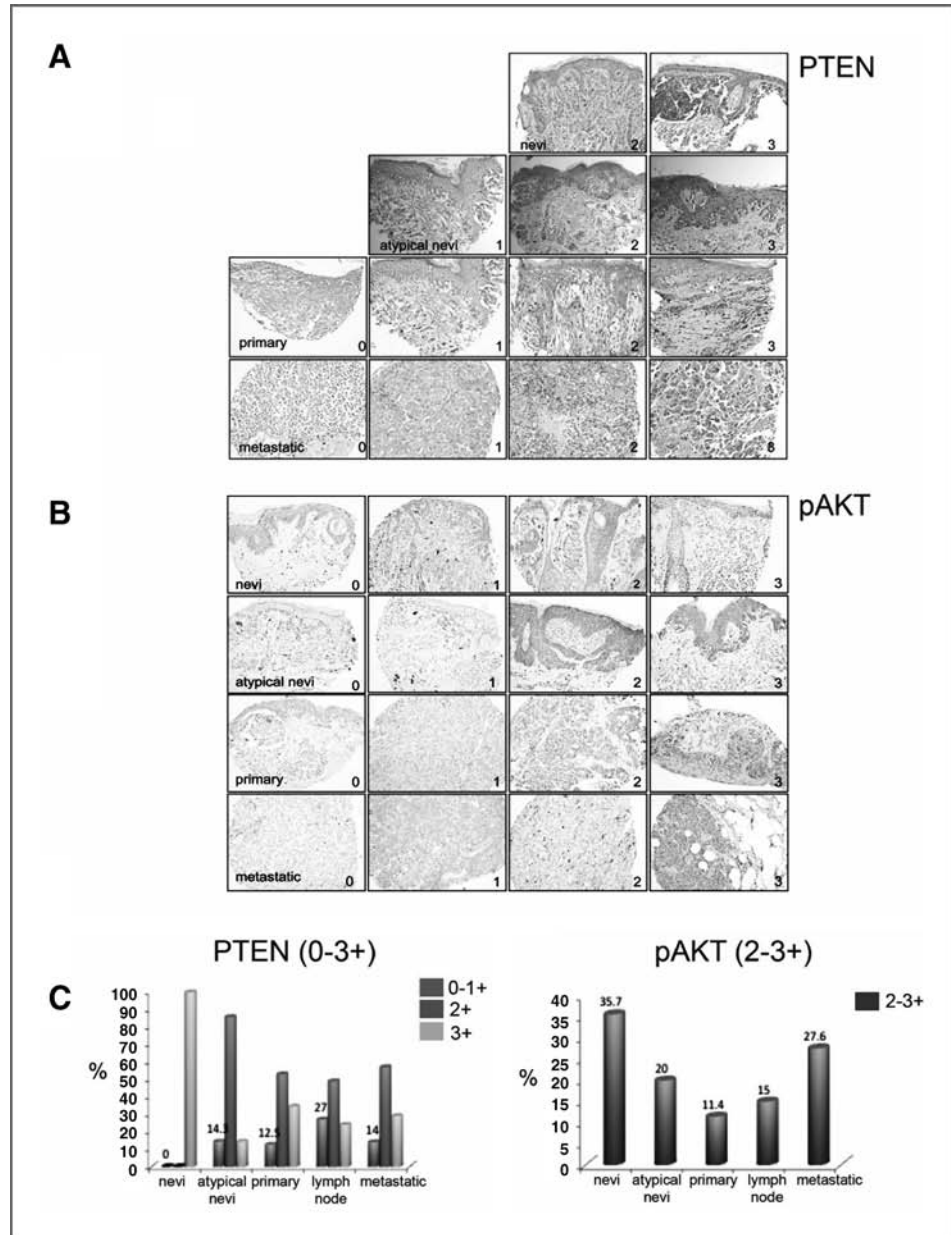
Results

The role of PTEN loss in the response to PLX4720

Initial studies identified 6 *BRAF* mutated melanoma cell lines that retained PTEN expression (PTEN+; WM35, WM51, WM164, WM983A, 451Lu, SK-Mel-28) and 6 that lacked PTEN

expression (PTEN-; WM239A, WM266-4, WM793, 1205Lu, WM9, M233; Fig. 1A and Supplemental Table S1). Genomic analysis showed the WM9 and M233 cell lines to be homozygously deleted for PTEN and the WM793 and 1205lu cell lines be hemizyously deleted for PTEN in conjunction with a PTEN mutation (Supplementary Table S1). The PTEN+ cell

Figure 2. PTEN expression is lost at all stages of melanoma progression. A, images showing representative immunohistochemical staining of PTEN. B, pAKT expression in a tissue array of nevi, atypical nevi, primary, and metastatic melanoma patient tumor samples. 0 and 1 indicate no to low PTEN expression and +3 indicates the highest expression whereas +2 to 3 relate to high expression of pAKT. Magnification 200 \times . C, left panel shows percentage loss of PTEN expression in each subset of patient samples as indicated in first of three columns per grouping whereas the right panel shows AKT activity in matched samples.



lines had lower constitutive levels of pAKT (Ser473) compared with the PTEN⁻ (Fig. 1A). Similar levels of pAKT (Thr308) were observed in the PTEN⁻ and PTEN⁺ cell lines. Analysis of the growth inhibitory effects of PLX4720 by the MTT and Alamar Blue assays (Fig. 1B and Supplemental Fig. S1) did not reveal any statistically significant differences in the GI₅₀ values between the PTEN⁺ and PTEN⁻ cell lines ($P = 0.48$; Supplementary Fig. S2).

As increased PI3K/AKT signaling is known to limit apoptosis, we next measured PLX4720-induced apoptosis in our PTEN⁻/PTEN⁺ melanoma cell line panel (Fig. 1C). Here we observed that following PLX4720 treatment (3 and 10 $\mu\text{mol/L}$, 48 hours), the PTEN⁻ melanoma cell lines showed significantly less apoptosis than the PTEN⁺ (*, $P < 0.05$; Fig. 1C).

PLX4720 mediated apoptosis was blocked by high doses (>75 $\mu\text{mol/L}$) of the caspase inhibitor z-vad-fmak (Supplementary Fig. S3).

Loss of PTEN expression is independent of melanoma stage

We confirmed the incidence of PTEN loss in a tissue microarray containing a large sample of melanocytic neoplasms ($n = 192$) drawn from all stages of tumor progression (Fig. 2A–C). Results of immunohistochemical staining were graded from 0 to 3+ based on strength of the staining. It was observed that although nonatypical nevi rarely demonstrated loss of PTEN, >10% of atypical nevi and every stage of melanoma demonstrated loss of PTEN expression (either 0 or +1). Significantly,

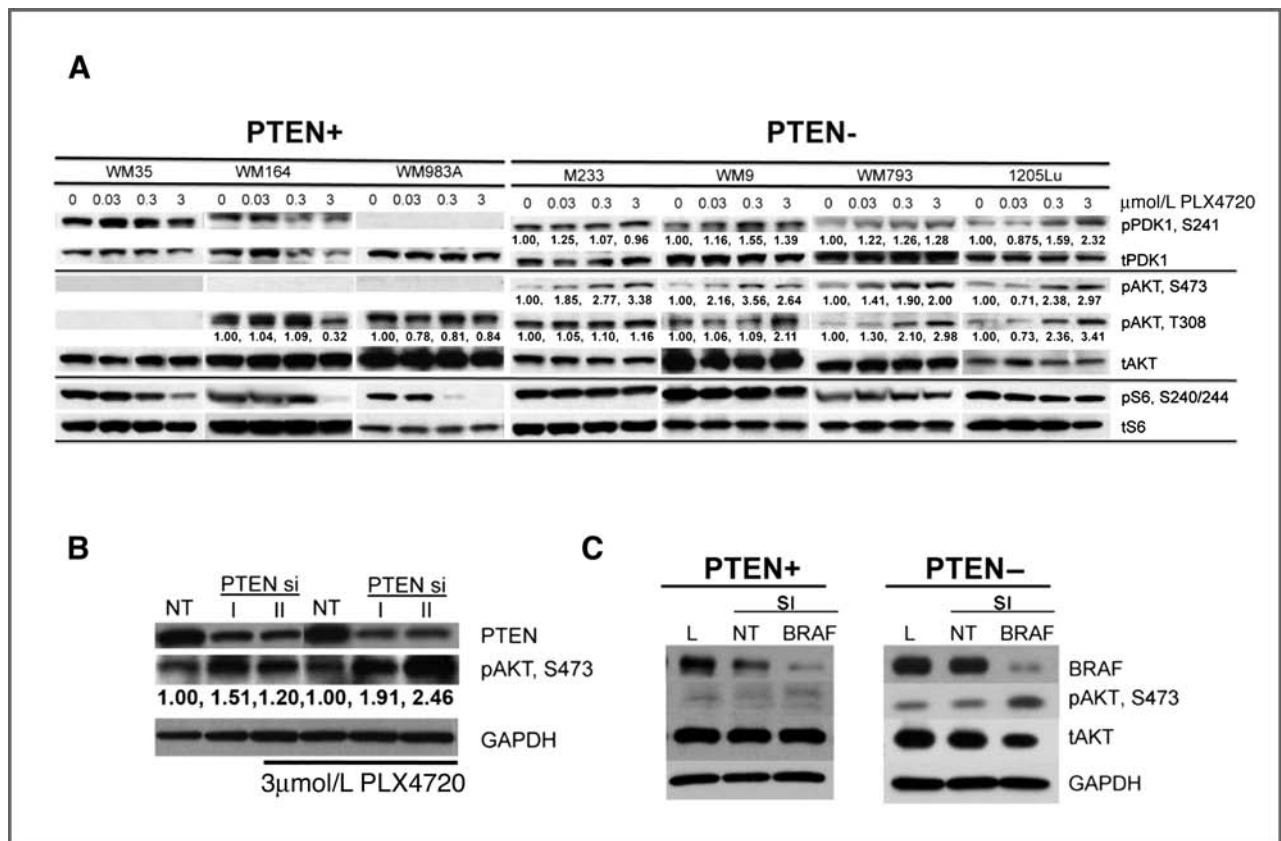


Figure 3. Loss of PTEN is associated with PI3K/AKT signaling following BRAF inhibition. **A**, PTEN+ (WM35, WM164, WM983A) and PTEN- (M233, WM9, WM793, 1205Lu) cells were treated with PLX4720 (24 hours: 0.03–3 μmol/L) and probed for phospho-PDK1 (pPDK1), total PDK1, phospho-AKT (pAKT), total AKT (tAKT), phospho-S6 (pS6), and total S6. Numbers indicate relative intensity of pPDK1 normalized to PDK1 and pAKT normalized to tAKT. **B**, PLX4720 increases pAKT following PTEN knockdown. WM35 cells were incubated with nontargeting siRNA (NT) or 2 different PTEN-specific siRNA's (PTEN I, PTEN II) before treatment with either vehicle or PLX4720 (3 μmol/L). **C**, siRNA knockdown of BRAF increases pAKT in melanoma cell lines that are PTEN-. WM164 (PTEN+) and WM793 (PTEN-) cells were incubated with lipofectamine alone (L), nontargeting siRNA (NT), or BRAF-specific siRNA (BRAF). Protein was extracted, resolved, and probed for BRAF, pAKT, total AKT, and GAPDH.

primary melanoma, lymph node metastases, and distant metastases melanoma demonstrated loss of PTEN in 12.5%, 27%, and 14% of cases each (Fig. 2A and C). Staining of the same TMA for pAKT demonstrated an increase in AKT activation as the tumors progressed from primary melanoma to distant metastasis (Fig. 2B). The level of pAKT positivity only partially correlated with PTEN expression status (Fig. 2C).

PLX4720 and BRAF siRNA leads to AKT signaling in BRAF V600E-mutated/PTEN- melanoma cell lines

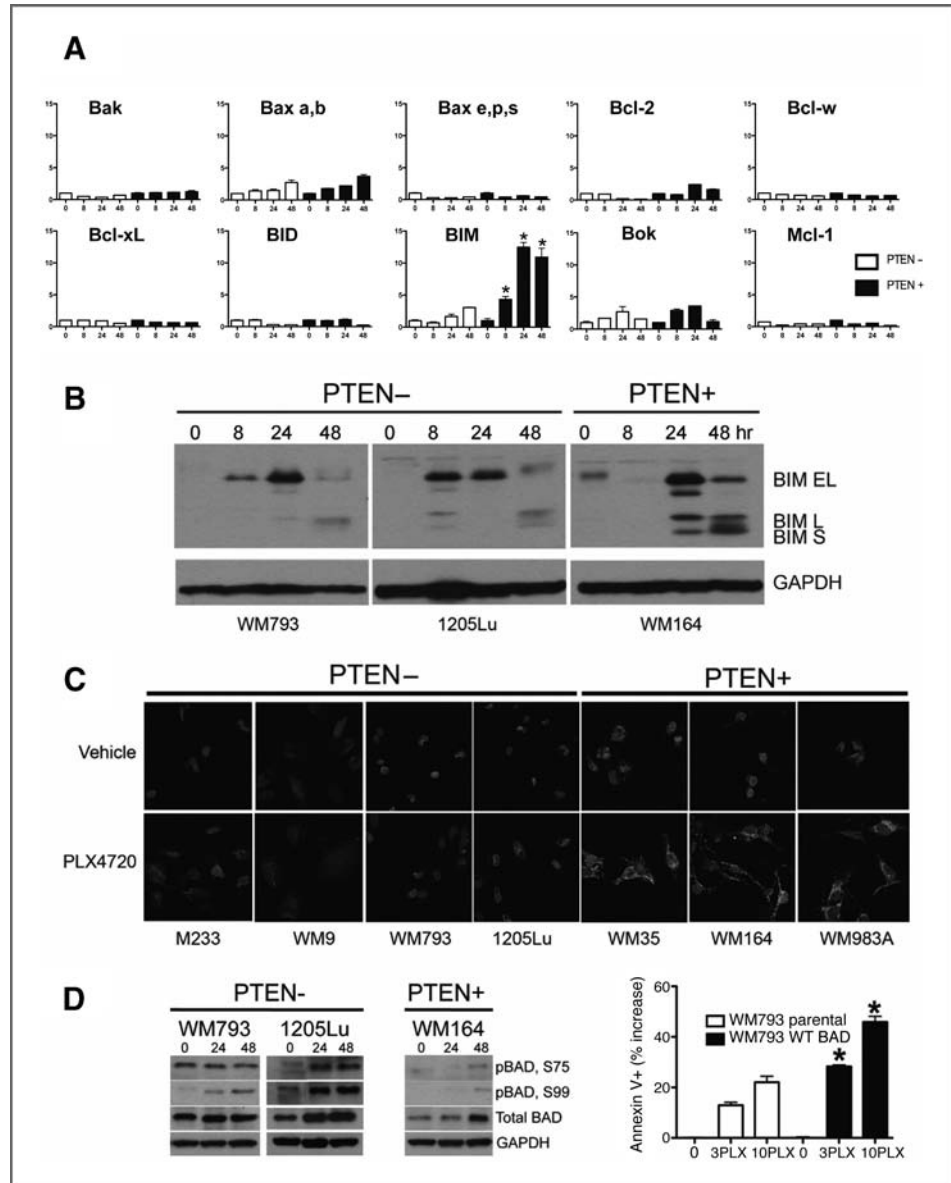
Treatment of the PTEN+/- cell line panels with PLX4720 increased pPDK1 and pAKT signaling only in the melanoma cell lines lacking PTEN expression (Fig. 3A). In contrast, PLX4720 inhibited BRAF activity in both PTEN- and PTEN+ cell lines with a similar potency (Supplementary Fig. S4) and prevented BrdU uptake in both PTEN+ and PTEN- cell lines (Supplementary Fig. S5). Addition of PLX4720 also led to the inhibition of mTOR activity in the PTEN+ cell lines only (Fig. 3A) and was associated with stimulation of LKB1 and AMPK signaling (Supplementary Fig. S6). The requirement for PTEN in the increased AKT signaling was confirmed by studies showing

that PLX4720 stimulated pAKT in WM35 cells (PTEN+) when PTEN was knocked down by siRNA (Fig. 3B). The effects of PLX4720 upon pAKT signaling were BRAF specific, with BRAF siRNA knockdown found to increase pAKT in PTEN- cells only (Fig. 3C). Mechanistically, PLX4720 increased IGF-I signaling in the PTEN- cells, with the IGF1R inhibitor NVP-ADW-742 (21) being found to abrogate the PLX4720-mediated increase in pAKT signaling (Supplementary Fig. S7).

PLX4720 treatment differentially regulates BIM in PTEN+ and PTEN- cells

We next used LC-MRM to quantify the PLX4720-induced changes in the expression of 17 members of the Bcl-2 protein family (Fig. 4A shows results for 9 proteins). The only proapoptotic protein to demonstrate significant differences between the PTEN- and PTEN+ cell lines was BIM (Fig. 4A; 14- and 4-fold increases in the PTEN+ and PTEN- cell lines, respectively). Western blots and immunofluorescence staining confirmed the LC-MRM data and showed a greater degree of PLX4720-induced (3 and 10 μmol/L) BIM expression in the PTEN+ cell lines compared to PTEN- cell

Figure 4. LC-MRM identifies differential regulation of BIM in PTEN⁺ and PTEN⁻ cell lines following PLX4720 treatment. **A**, representative LC-MRM data showing the fold changes in the expression of Bak, Bax, Bcl-2, Bcl-w, Bcl-xL, BID, BIM, Bok, and Mcl-1 over internal standard in the WM164 (PTEN⁺) and 1205Lu (PTEN⁻) cell lines following treatment with PLX4720 (10 μ mol/L, 0–48 hours). Statistical analysis of BIM fold change in PTEN⁻ versus PTEN⁺. *, $P < 0.05$. **B**, Western blot showing BIM expression following PLX4720 treatment (10 μ mol/L, 0–48 hours) in PTEN⁻ (WM793, 1205Lu) and WM164 cell lines (PTEN⁺). **C**, immunofluorescence staining, showing expression of BIM and DAPI staining of PTEN⁻ (M233, WM9, WM793, 1205Lu) and PTEN⁺ (WM35, WM164, WM983A) cells following PLX4720 treatment (3 μ mol/L, 48 hours). **D**, Western blot showing BAD phosphorylation following treatment with PLX4720 (0–48 hours) in PTEN⁻ (WM793, 1205Lu) and PTEN⁺ WM164. Annexin V binding following treatment with 3 or 10 μ mol/L PLX4720 (48 hours) showing increased apoptosis in WM793 stably overexpressing WT BAD. *, $P < 0.05$.



lines (Fig. 4B,C and Supplementary Fig. S8). In parallel, we observed that PLX4720 also increased the inactivation of BAD (as shown by increased phospho-BAD) in the PTEN⁻ cells (Fig. 4D) and that overexpression of BAD in the PTEN⁻ cells enhanced PLX4720-mediated apoptosis (Fig. 4D). PLX4720 treatment also increased total BAD expression in both the PTEN⁺ and PTEN⁻ cell lines. Little PLX4720-induced changes in Mcl-1 expression were observed in the PTEN⁺ and PTEN⁻ cell lines (Supplementary Fig. S9).

PTEN is required for efficient BIM upregulation following BRAF inhibition

We next explored the link between PTEN expression status and PLX4720-mediated induction of BIM. siRNA knockdown of PTEN using 2 siRNA sequences led to the inhibition of PLX4720-induced BIM expression in PTEN⁺ cells (Fig. 5A).

We next determined whether reintroduction of wild-type PTEN (PTEN-wt) or lipid phosphatase mutated PTEN (PTEN-G129E) into a PTEN⁻ cell line enhanced BIM expression when BRAF was inhibited. In these studies, we used an isogenic pair of WM793 melanoma cell lines that expressed either doxycycline inducible PTEN-wt or PTEN-G129E mutant. Control studies showed that doxycycline (100 ng/mL, 48 hours) increased expression of PTEN in both cell lines (Fig. 5B). The impaired lipid phosphatase function of the G129E mutant was confirmed by the fact that only the induction of PTEN-wt suppressed pAKT activation (Fig. 5B). The role of PTEN in the PLX4720-mediated induction of BIM was confirmed by the enhanced expression of BIM seen when PTEN-wt was induced compared to when PTEN-G129E was induced (Fig. 5B) and was paralleled by a significant increase in PLX4720-mediated apoptosis ($P < 0.05$; not

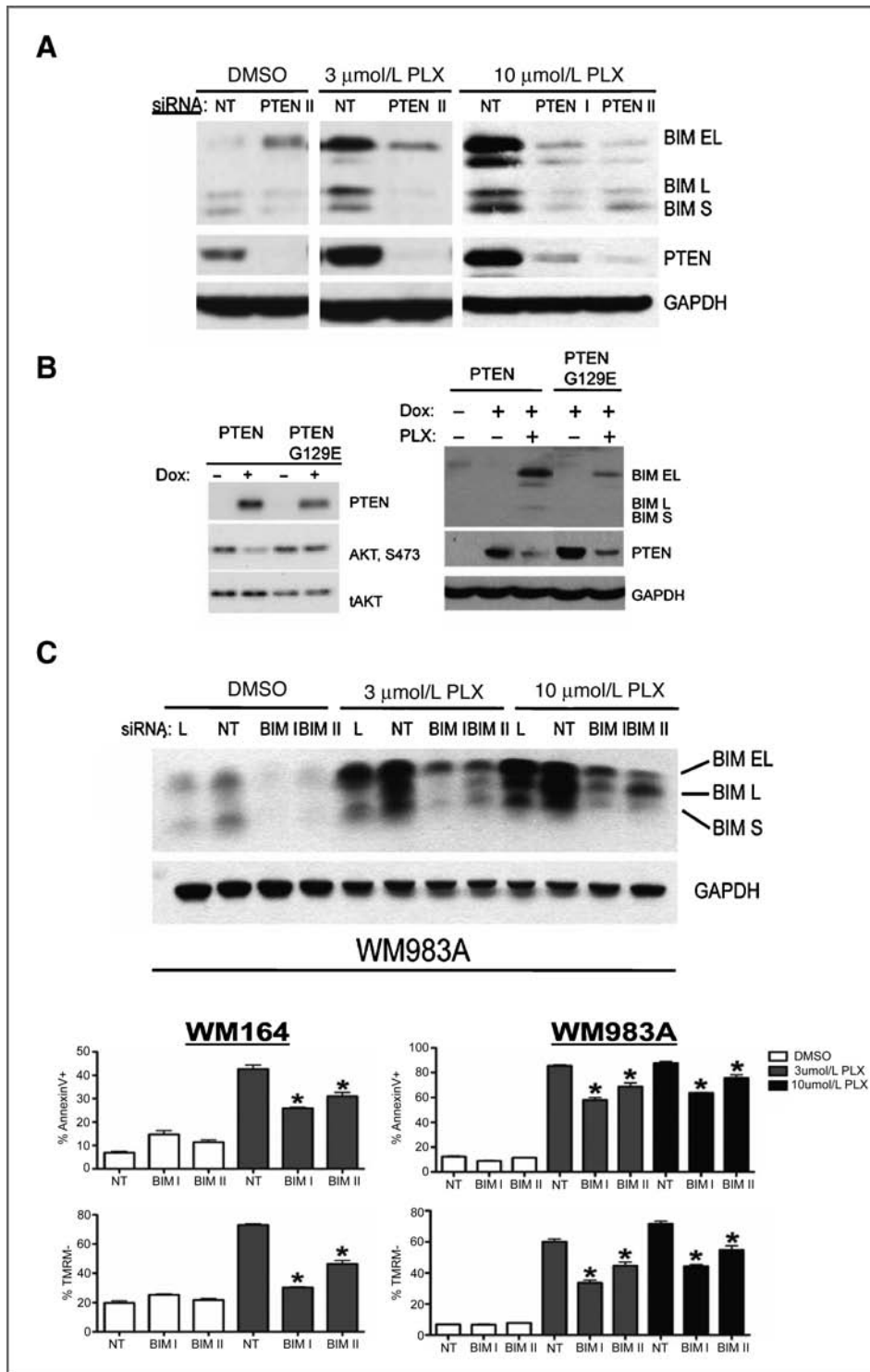


Figure 5. PTEN is required for efficient upregulation of BIM following BRAF inhibition. **A**, WM164 cells (PTEN+) were incubated with nontargeting siRNA (NT) or transfected with 2 PTEN-specific siRNAs (I and II) before treatment with PLX4720 (3 or 10 $\mu\text{mol/L}$, 48 hours). Protein was resolved and probed for expression of BIM, GAPDH, and PTEN. **B**, induction of PTEN-wt but not PTEN-G129E in WM793 (PTEN-) cells was sufficient to increase BIM expression when BRAF was inhibited. Left, Western blot shows induction of PTEN-wt and PTEN-G129E following doxycycline treatment. Right, induction of PTEN-wt + PLX4720 induces BIM more efficiently than PTEN-G129E + PLX4720. **C**, BIM is required for PLX4720-induced apoptosis in PTEN+ cells. WM164 and WM983A cells were incubated with nontargeting siRNA (NT) or transfected with 2 BIM-specific siRNAs (BIM I and BIM II) before treatment with PLX4720 (3 or 10 $\mu\text{mol/L}$, 48 hours). Flow cytometry studies showed a significant reduction in TMRM loss and Annexin V binding when cells were transfected with BIM siRNA compared with nontargeting control siRNA (* $P < 0.05$).

shown). Interestingly, the addition of PLX4720 decreased the expression of PTEN through mechanisms that are not currently clear. The effects of PI3K/AKT signaling upon the suppression of BIM were mostly mediated through AKT3, with siRNA knockdown of AKT3 found to increase BIM

expression when BRAF was inhibited (Supplementary Fig. S10). As a final test of the relevance of BIM induction in the PLX4720-induced apoptotic response, we showed that siRNA knockdown of BIM led to an impairment of PLX4720 (3 and 10 $\mu\text{mol/L}$) induced apoptosis (Fig. 5D).

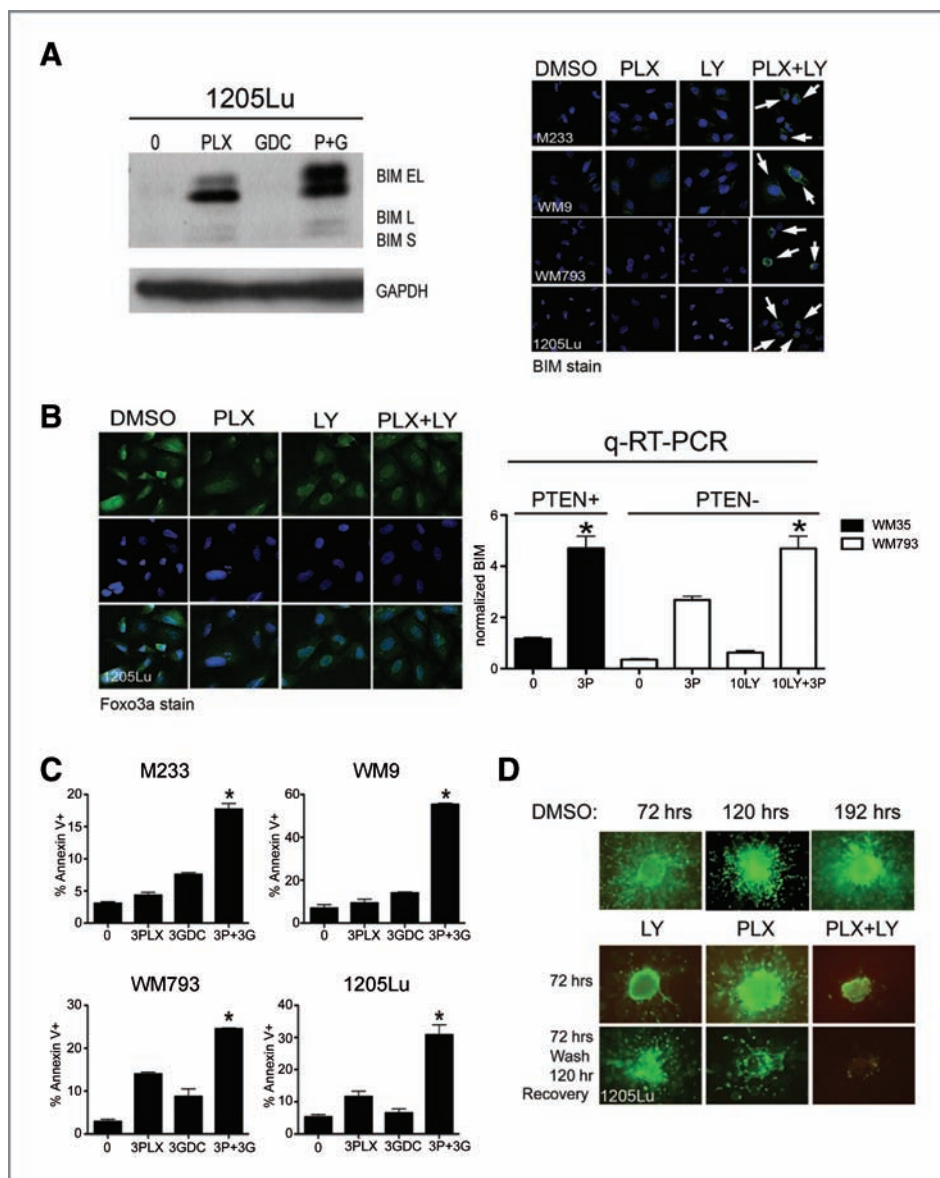


Figure 6. Dual PI3K/BRAF inhibition upregulates BIM and enhances apoptosis in PTEN⁻ cells. **A**, left, Western blot of 1205Lu cells treated with PLX4720 (3 μ mol/L, 48 hours), the PI3K inhibitor GDC-0941 (3 μ mol/L, 48 hours), or both drugs in combination (P+G); right, immunofluorescence staining of BIM (green) and DAPI (blue) in PTEN⁻ cells following PLX4720 treatment (3 μ mol/L, 48 hours), the PI3K inhibitor LY294002 (10 μ mol/L, 48 hours), or both drugs in combination (PLX+LY). **B**, left, immunofluorescence staining of PTEN⁻ 1205Lu following combined inhibition (3 μ mol/L PLX4720 + 10 μ mol/L LY294002, 48 hours) increases nuclear localization of FOXO3a (green). DAPI is shown in blue. Magnification 40 \times . Right, combined inhibition (3 μ mol/L PLX4720 + 10 μ mol/L LY294002, 48 hours) increases PTEN⁻ WM793 BIM mRNA levels to those observed with single BRAF inhibition (3 μ mol/L PLX4720, 48 hours) in the PTEN⁺ WM35. **C**, PTEN⁻ cells were treated with PLX4720 (3 μ mol/L, 48 hours), GDC-0941 (3 μ mol/L, 48 hours), or a combination of the 2 drugs (3P+3G) before Annexin-V staining was analyzed by flow cytometry (*, $P < 0.05$ between the drug combination and each inhibitor alone). **D**, combined BRAF/PI3K inhibitor treatment blocks the escape of 1205Lu cells (PTEN⁻) from therapy. Spheroids of 1205Lu cells were treated with either PLX4720 alone (3 and 10 μ mol/L; data shows 3 μ mol/L), LY294002 (10 μ mol/L) alone or a combination of the 2 drugs for 72 hours. In other studies, spheroids were treated with drugs for 72 hours and then allowed to recover for 120 hours. Micrograph shows viability staining (green = live cells, red = dead cells). Magnification 10 \times .

Dual BRAF/PI3K inhibition enhances BIM expression and apoptosis in PTEN⁻ cells

One of the major effects of PTEN is to limit PIP3 levels through its lipid phosphatase activity. We next treated PTEN⁻ cell lines with a PI3K inhibitor (GDC-0941, 3 μ mol/L or LY294002 10 μ mol/L), PLX4720 (3 and 10 μ mol/L), or the 2

drugs in combination, and showed that combined PI3K and BRAF inhibition increased the level of BIM expression in both Western blot and immunofluorescence studies (Fig. 6A). Both the MAPK and PI3K/AKT pathways are known to regulate BIM RNA expression levels through the transcription factor FOXO3a (22–25). In agreement with this, PLX4720 treatment

increased the nuclear accumulation of FOXO3a in the PTEN+ but not PTEN- melanoma cells (Fig. 6B; not shown). Consistent with a role for increased AKT signaling suppressing BIM expression in PTEN- cells, dual BRAF and PI3K inhibition increased nuclear FOXO3a localization in the PTEN- cell lines (Fig. 6B) and enhanced the level of BIM mRNA (Fig. 6B). siRNA knockdown of FOXO3a was further found to block PLX4720-mediated upregulation of BIM in PTEN+ cells (Supplementary Fig. S11). The observation that PLX4720 treatment led to increased PI3K/AKT signaling in PTEN- melanoma cell lines suggested that dual BRAF/PI3K inhibition could be one strategy to overcome intrinsic resistance. In agreement with this, the combination of PLX4720 with the PI3K inhibitor GDC-0941 significantly enhanced the levels of apoptosis observed in PTEN- melanoma cell lines compared to either the BRAF or PI3K inhibitor alone (Fig. 6C). Similar results were also observed in a 3D spheroid assay, where combined PLX4720 (3 μ mol/L) and LY294002 (10 μ mol/L) treatment prevented the recovery of cell growth observed when melanoma spheroids were treated with either drug alone (Fig. 6D). The proposed mechanism for BIM regulation following BRAF inhibition in PTEN+ and PTEN- melanoma cell lines is shown in Supplementary Figure S12.

Discussion

This study has focused upon the mechanisms underlying the intrinsic resistance observed in melanoma patients recently treated in the phase-I trial of PLX4032 (4). Melanomas are known to have constitutive activity in many signaling pathways whose outputs converge to regulate cell cycle entry and survival. Of these, melanoma initiation and progression is known to be dependent upon both the Ras/Raf/MEK/ERK and PI3K/AKT pathways (12, 26–28). The mechanisms underlying this signaling activity differ according to the initiating oncogenic event. Thus, melanomas with activating *NRAS* mutations rarely harbor concurrent alterations in either *BRAF* or *PTEN/AKT* as Ras stimulates both the Raf/MEK/ERK and PI3K/AKT pathways (29, 30). In contrast, melanomas with *BRAF* mutations require other mechanisms to activate their PI3K/AKT signaling and frequently show inactivation/deletion of *PTEN* or increased expression of *AKT3* (31–34).

We began by investigating *PTEN* expression across a large sample of melanocytic lesions and found that *PTEN* was lost in 10% to 27% of melanomas. Although *PTEN* loss overlapped with the level of pAKT staining it was not always well correlated, agreeing with previous observations that other mechanisms may underlie the increased AKT activation associated with melanoma progression (35). Our results agree with other published studies on smaller numbers of melanoma samples ($n = 16$ –39), and confirm that reduced *PTEN* expression is a significant oncogenic event for a restricted subgroup of melanomas (31, 32, 36). Although *PTEN* was retained in nonatypical nevi, a significant number (23%) of atypical nevi lacked expression, suggesting this to be an early event in melanoma development. This idea is supported by recent mouse modeling studies showing that the conditional expres-

sion of the *BRAF* V600E mutation leads to melanoma development only when *PTEN* is suppressed (28).

Although lack of *PTEN* expression did not predict for sensitivity of *BRAF* V600E-mutated melanoma cell lines to the growth inhibitory effects of PLX4720, there were significant differences in PLX4720-mediated apoptosis between PTEN+ and PTEN- melanoma cell lines. Initially, we hypothesized that PTEN- melanoma cell lines would show higher levels of AKT activity and that this would mediate resistance to PLX4720. Instead, we observed that drug treatment increased AKT signaling in the PTEN- cell lines. The effects upon AKT signaling were *PTEN* dependent, and could be recapitulated in PTEN+ melanoma cell lines when *PTEN* was knocked down using siRNA. The increase in AKT signaling observed in the PTEN- cell line panel was associated with PDK1 phosphorylation and increased expression of IGF-I. These effects were reversed following pretreatment with the IGF1R inhibitor NVD-ADW-742 (21), suggesting a link between BRAF inhibition and enhanced IGF1R-mediated PI3K signaling. Similar findings, linking BRAF/MEK inhibition to increased IGF signaling, have been recently reported by 2 other groups (37, 38).

AKT plays a critical role in cancer development through its ability to regulate cell survival through the direct phosphorylation of BAD, the stimulation of ribosomal S6 kinase signaling, the inhibition of FOXO signaling, and the inhibition of glycogen synthase 3-kinase (35). To determine the mechanism of PLX4720-induced apoptosis induction in the PTEN+ melanoma cell lines, LC-MRM analysis was used to quantify the relative expression of members of the Bcl-2 protein family (20). For the majority of proteins examined, PLX4720 treatment was associated with very similar dynamics in both the PTEN+ and PTEN- cell lines. These findings agree with previous studies and show that BRAF inhibition leads to an increase in the expression in the proapoptotic protein BIM (18, 39, 40). In contrast to these studies, which did not distinguish between PTEN+ and PTEN- cell lines, the LC-MRM analysis allowed us to identify significant *PTEN*-dependent differences in the level of PLX4720-induced BIM expression. BIM is a proapoptotic BH3-only member of the Bcl-2 protein family that exists in 3 major splice forms; extra long (BIM-EL), long (BIM-L) and short (BIM-S; refs. 41 and 42). It exerts its cytotoxic activity by binding to and antagonizing the antiapoptotic proteins Bcl-2, Bcl-w, Bcl-XL, and Mcl-1 (41, 42). Expression of BIM is regulated both transcriptionally and posttranscriptionally by a number of signaling pathways, including BRAF/MEK/ERK, JNK, p38 MAPK, and PI3K/AKT (39, 41, 43, 44). In melanoma, the *BRAF* V600E mutation regulates BIM expression through the MEK/ERK pathway-mediated phosphorylation of the extra-long form of BIM (BIM-EL) at Serine 69, leading to its subsequent degradation by the proteasome (39, 45). Our study is the first to demonstrate that the level of BIM expression following BRAF inhibition is also determined by *PTEN* status and that the differing levels of BIM induction can determine the extent of apoptosis induction when BRAF is inhibited. Apoptosis control in melanoma cells is complex and increased AKT signaling is likely to regulate survival at multiple levels. One of the best known prosurvival substrates of

AKT is the cell death inducing molecule BAD. AKT inactivates BAD via phosphorylation at Ser99, which prevents its binding to Bax and relieves the antagonism of Bax on Bcl-2 and Bcl-XL (18). A role for Bad inactivation in the escape of PTEN⁻ cells from PLX4720-induced apoptosis was suggested by the preferential inactivation of BAD when BRAF was inhibited and the fact that overexpression of BAD sensitized the same cell line to PLX4720-induced apoptosis. Another candidate proapoptotic factor up-regulated in melanoma cells following BRAF/MEK/ERK inhibition is BMF (Bcl-2 modifying factor; refs. 40 and 46). BMF, which is also regulated through the PI3K/AKT pathway, mediates its apoptotic effects through binding to Mcl-1. Although it is possible that BMF may also be differentially regulated in PTEN^{+/-} cells, we, like other groups, were unable to confirm the selectivity of commercially available BMF antibodies (40, 47).

In addition to regulating PIP3 levels in the cytoplasm through its lipid phosphatase function, PTEN also localizes to the nucleus where it exerts its tumor suppressor function through lipid phosphatase-independent effects upon the regulation of chromosomal integrity, p53 acetylation and the expression of cyclin D1 (48). As the lipid phosphatase-dependent and -independent functions of PTEN are likely to be very different, we re-expressed either wild-type PTEN or a PTEN mutant with impaired lipid phosphatase function (G129E) in melanoma cells that were PTEN⁻ (49). These studies confirmed the requirement for the lipid phosphatase function of PTEN in the suppression of BIM expression, with PLX4720 treatment inducing only a weak upregulation of BIM protein when PTEN G129E was expressed. The importance of the lipid phosphatase function in the suppression of BIM expression was supported by experiments showing that combined BRAF/PI3K inhibition and siRNA knockdown of AKT3 both enhanced the level of BIM expression and increased the level of apoptosis in the PTEN⁻ cells. In other systems, AKT downregulates BIM expression by phosphorylating and inactivating the transcription factor

FOXO3a (22, 24). In agreement with these reports, we confirmed that PLX4720 treatment led to increased nuclear accumulation of FOXO3a in the PTEN⁺ cells only and demonstrated that siRNA knockdown of FOXO3a abrogated the increase in BIM expression.

In summary, we have identified an important role for PTEN loss in the intrinsic resistance of *BRAF* V600E mutated melanoma cells to the BRAF inhibitor PLX4720. These studies further suggest that increased BIM expression may be a useful biomarker in predicting clinical response to BRAF inhibition and demonstrates that LC-MRM is a useful method for monitoring BIM expression that could be translated to patient assessment. This work also provides a rationale for dual BRAF/PI3K inhibitor treatment in the management of melanomas that are *BRAF*V600E/PTEN⁻.

Disclosure of Potential Conflicts of Interest

A. Ribas: honorarium, Roche. The other authors disclosed no potential conflicts of interest.

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PTEN Loss Confers BRAF Inhibitor Resistance to Melanoma Cells through the Suppression of BIM Expression

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