Apoptosis of human melanoma cells induced by inhibition of B-RAFV600E involves preferential splicing of bimS

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Bim is known to be critical in killing of melanoma cells by inhibition of the RAF/MEK/ERK pathway. However, the potential role of the most potent apoptosis-inducing isoform of Bim, BimS, remains largely unappreciated. Here, we show that inhibition of the mutant B-RAFV600E triggers preferential splicing to produce BimS, which is particularly important in induction of apoptosis in B-RAFV600E melanoma cells. Although the specific B-RAFV600E inhibitor PLX4720 upregulates all three major isoforms of Bim, BimEL, BimL, and BimS, at the protein and mRNA levels in B-RAFV600E melanoma cells, the increase in the ratios of BimS mRNA to BimEL and BimL mRNA indicates that it favours BimS splicing. Consistently, enforced expression of B-RAFV600E in wild-type B-RAF melanoma cells and melanocytes inhibits BimS expression. The splicing factor SRp55 appears necessary for the increase in BimS splicing, as SRp55 is upregulated, and its inhibition by small interfering RNA blocks induction of BimS and apoptosis induced by PLX4720. The PLX4720-induced, SRp55-mediated increase in BimS splicing is also mirrored in freshly isolated B-RAFV600E melanoma cells. These results identify a key mechanism for induction of apoptosis by PLX4720, and are instructive for sensitizing melanoma cells to B-RAFV600E inhibitors.

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Results from clinical studies with small molecule inhibitors of the mutant B-RAFV600E have been very encouraging, and promise to provide a much needed breakthrough in the treatment of melanoma by targeting B-RAFV600E.1,2 The latter is found in ~50% of melanomas, leading to constitutive activation of the RAF/MEK/ERK pathway that is important for melanoma cell growth and survival, and is involved in resistance to many therapeutic approaches.3,4 However, a number of questions have already been raised from these studies, such as the durability of responses and why some melanomas with mutant B-RAF have not shown major responses.1,2

It is well established that blockade of the RAF/MEK/ERK pathway inhibits melanoma cell growth.5 In addition, a more desirable outcome, induction of apoptosis, has also been shown in varying in vitro systems, in particular, in B-RAFV600E melanoma cells.6–10 Apoptosis of such cells was clearly demonstrated in an ex vivo model after administration of the B-RAF inhibitor, PLX4720, that is selective for the mutant B-RAFV600E.11 Consistently, regression of metastatic mutant B-RAF melanomas is a frequent sign of the response to administration of PLX4032, a close analogue to PLX4720,1,2 suggesting that induction of apoptosis may be a major biological consequence of inhibition of mutant B-RAF.

Several mechanisms have been reported to contribute to apoptosis induced by inhibition of the RAF/MEK/ERK pathway. These include dephosphorylation of Bad, translocation of Bmf, upregulation of BimEL, and downregulation of Mcl-1.7–11 Among them, upregulation of BimEL via inhibition of its phosphorylation and subsequent proapoptotic degradation may be the best documented7,8 and is of particular interest, in that Bim, unlike other more selective Bcl-2 homology 3 (BH3)-only proteins such as Bad and Bmf, can bind with high affinity to and inhibit all prosurvival Bcl-2 family proteins.12 In addition, Bim can directly bind to and activate Bax.12 It is of note that besides posttranslational changes, inhibition of the RAF/MEK/ERK pathway has also been shown to cause upregulation of Bim mRNA.13

There are three major isoforms of Bim, BimEL, BimL, and BimS, that are generated by alternative splicing.14 Although BimS is encoded by exons 2, 5, and 6, BimS is encoded by exons 2, 4, 5, and 6, and BimEL by exons 2, 3, 4, 5, and 6. Both BimL and BimEL contain a binding site for dynein light chain 1,14 hence, their proapoptotic activity is controlled by sequestration to the cytoskeleton-associated dynein motor complex.15 Because exon 3 encodes an ERK1/2-docking domain and ERK1/2 phosphorylation sites, BimEL is subject to phosphorylation by the MEK/ERK pathway that targets it for proteosomal degradation and also prevents its binding to...
Bax. Bim\(_S\) is not subject to any known posttranslational regulation and is the most potent apoptosis inducer among the three isofoms.\(^{13,16,17}\)

Alternative splicing is a tightly regulated process that generates multiple functional variants from individual genes, thus enhancing protein diversity.\(^{18}\) Alternative splicing patterns are frequently altered in cancer cells, resulting in aberrant expression of mRNA and protein variants that have been proposed to have unique properties to confer biological characteristics of the cells.\(^{19–22}\) The splicing process is catalyzed by the spliceosome that is composed of cis-acting elements, such as splicing enhancers and silencers, and trans-acting factors, including the serine/arginine-rich (SR) and heterogeneous ribonucleoprotein particle (hnRNP)

**Figure 1** PLX4720 inhibits proliferation and induces apoptosis in mutant B-RAF melanoma cells. (a) Whole cell lysates from Mel-RM (wild-type B-RAF) and Mel-RMu (B-RAF\(^{V600E}\)) cells treated with PLX4720 at indicated concentrations for 72 h (upper panel) or at 10 \(\mu\)M for indicated periods (lower panel), were subjected to western blot analysis of phosphorylated ERK1/2 and ERK1/2. (b) Mel-RM and Mel-RMu cells were treated with PLX4720 at indicated concentrations for 72 h. Cell viability (left panel) and apoptosis (right panel) were quantitated by the MTS assay and propidium iodide (PI) method, respectively. The data shown are the mean \(\pm\) S.E. of three individual experiments. (c) Mel-RM and Mel-RMu cells were treated with PLX4720 at 10 \(\mu\)M for indicated periods. Cell viability (left panel) and apoptosis (right panel) were quantitated by the MTS assay and PI method, respectively. The data shown are the mean \(\pm\) S.E. of three individual experiments. (d) A summary of the effect of PLX4720 on cell survival in a panel of mutant and wild-type B-RAF melanoma cell lines. Cells treated with PLX4720 at 10 \(\mu\)M for 72 h were subjected to MTS assays. The data shown are the mean \(\pm\) S.E. of three individual experiments. (e) Left panel: B-RAF\(^{V600E}\) Mel-RMu and Mel-CV cells were transfected with the control or B-RAF siRNA. After 24 h, whole cell lysates were subjected to western blot analysis of B-RAF, phosphorylated ERK1/2, and ERK1/2. Western blot analysis of A-RAF and C-RAF was included as controls to show the specificity of the B-RAF siRNA. Right panel: Mel-RMu and Mel-CV cells were transfected with the control or B-RAF siRNA. After 24 h, cells were treated with PLX4720 (10 \(\mu\)M) for a further 72 h. Apoptotic cells were measured by the PI method. The data shown are either representative (left panel), or the mean \(\pm\) S.E. (right panel), of three individual experiments.
protein families. SR proteins are characterized by one or two RNA recognition motifs at the N-terminal and have an important part in splice-site selection through association with splicing enhancers and silencers. Changes in the expression of a number of SR proteins have been found in various types of cancer cells.

To better understand the mechanism(s) by which inhibition of B-RAF\(^{V600E}\) induces apoptosis of melanoma cells, we have examined completely the apoptotic response of B-RAF\(^{V600E}\) melanoma cells to the B-RAF\(^{V600E}\) inhibitor PLX4720. We show in this report that preferential splicing to produce Bim\(_S\) has an important role in induction of apoptosis by PLX4720 in B-RAF\(^{V600E}\) melanoma cells. Moreover, we demonstrate that the increase in Bim\(_S\) splicing is mediated by the SR protein, SRp55.

**Results**

The B-RAF\(^{V600E}\) inhibitor PLX4720 induces apoptosis in B-RAF\(^{V600E}\) melanoma cells. Our initial studies confirmed that the small molecule compound PLX4720 is specific for inhibition of B-RAF\(^{V600E}\). This was shown by its inhibitory effect on ERK activation in B-RAF\(^{V600E}\) melanoma cell lines, but not in those carrying the wild-type B-RAF even when it was used at 10 \(\mu\)M (Figure 1A and Supplementary Figure 1A). The inhibitory effect of PLX4720 at 10 \(\mu\)M on activation of ERK was sustained till 72h after treatment (Figure 1A). Examination of the effect of PLX4720 on cell growth similarly demonstrated that it inhibited proliferation of B-RAF\(^{V600E}\) melanoma cells, but had only minimal effects on growth of those harboring the wild-type B-RAF (Figure 1B and Supplementary Figures 1B).

We examined whether induction of apoptosis was involved in PLX4720-mediated inhibition of cell growth in Mel-RMu (B-RAF\(^{V600E}\)) and Mel-RM (wild-type B-RAF) cells. At concentrations of up to 1 \(\mu\)M, PLX4720 did not induce significant apoptosis by 72h (Figure 1B). At 3 \(\mu\)M, it induced apoptosis in \(\sim\) 30% of Mel-RMu cells, but apoptosis in Mel-RM cells remained marginal. When it was used at 10 \(\mu\)M, \(>\) 65% of Mel-RMu cells and \(\sim\) 20% of Mel-RM cells underwent apoptosis, which corresponded well with the efficiency of inhibition of cell viability in both cell lines (Figure 1B). Predominant induction of apoptosis by PLX4720 at 10 \(\mu\)M was confirmed by treating the cells with the compound at the same concentration for varying time periods (Figure 1C). This was also shown by activation of caspase-3 and -7, and cleavage of the caspase-3 substrate PARP (Supplementary Figure 2).

Studies in a panel of melanoma cell lines indicated that B-RAF\(^{V600E}\) lines were, in general, significantly more sensitive to PLX4720 than those harboring the wild-type B-RAF (\(P<0.01\); two-tailed student’s t-test) (Figure 1D). Similar to PLX4720, Small interfering RNA (siRNA) knockdown of B-RAF induced apoptosis in two B-RAF\(^{V600E}\) melanoma cell lines, indicating that induction of apoptosis by PLX4720 is due to inhibition of B-RAF\(^{V600E}\) (Figure 1E).

PLX4720 preferentially enhances splicing of Bim\(_S\). Overexpression of Bcl-2 inhibited induction of apoptosis by PLX4720 in B-RAF\(^{V600E}\) melanoma cells, indicating that the
mitochondrial apoptotic pathway is essential in PLX4720-induced apoptosis (Figure 2a). In support of this, treatment with PLX4720 resulted in activation of Bax, and mitochondrial release of cytochrome c and apoptosis-inducing factor (AIF) (Supplementary Figure 3). These results suggest that activation of one or more BH3-only proteins of the Bcl-2 family is important in initiating PLX4720-mediated apoptotic signaling.27 As shown in Figure 2b, PLX4720 caused upregulation of the Bim isoforms, BimEL, BimL, and BimS, in B-RAF<sup>V600E</sup> Mel-RMu cells, but not in wild-type B-RAF Mel-RM cells. In particular, the increase in Bim S was most prominent and sustained. The changes in Bim EL expression was associated with reduction in the levels of an extra band, with reduced electrophoretic motility that corresponds to phosphorylated BimEL.13 Of note, PLX4720 also induced a novel protein product with an apparent molecular weight between Bim<sub>L</sub> and Bim<sub>S</sub> at 36 h after treatment (Figure 2b). In contrast to regulation of Bim, PLX4720 did not cause any significant changes in other Bcl-2 family proteins analyzed, except for downregulation of the anti-apoptotic proteins Mcl-1 and Bcl-2 at relatively late stages (36 h after treatment) in Mel-RMu cells (Figure 2b). Regulation of Bim by PLX4720 was confirmed in another three B-RAF-mutant melanoma cell lines (Supplementary Figure 4).

The marked increase in Bim<sub>S</sub> induced by PLX4720 was intriguing because, unlike Bim<sub>EL</sub> and Bim<sub>L</sub>, this isoform is not regulated by any known posttranslational mechanisms.13,15 We reasoned that upregulation of Bim<sub>S</sub> is a consequence of enhanced Bim transcription and a subsequent increase in splicing to produce Bim<sub>S</sub>. To test this, we first quantitated the Bim mRNA expression before and after treatment with actinomycin D (Act-D) (3 μg/ml) for 1 h were subjected to real-time PCR analysis. The relative abundance of mRNA expression before treatment was arbitrarily designated as 1. The data shown are the mean ± S.E. of three individual experiments.

Figure 2  PLX4720 upregulates Bim. (a) Upper panel: overexpression of Bcl-2 in Mel-RMu and Mel-CV cells stably transfected with cDNA encoding Bcl-2. Whole cell lysates were subjected to western blot analysis of Bcl-2 and GAPDH (as a loading control). Lower panel: Mel-RMu and Mel-CV cells overexpressing Bcl-2 were treated with PLX4720 (10 μM) for 72 h before apoptosis was quantitated by the propidium iodide (PI) method. The data shown are either representative (upper panel) or mean ± S.E. (lower panel) of three individual experiments. (b) Whole cell lysates from Mel-RM and Mel-RMu cells treated with PLX4720 (10 μM) for indicated time periods were subjected to western blot analysis of Bim, Bid, PUMA, Noxa, Bak, Bak, Mcl-1, Bcl-2, and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses. (c) Left panel: total RNA from Mel-RM and Mel-RMu cells treated with PLX4720 (10 μM) for indicated time periods was isolated and subjected to real-time PCR analysis for Bim mRNA expression. The relative abundance of mRNA expression before treatment was arbitrarily designated as 1. Right panel: total RNA from Mel-RM and Mel-RMu cells treated with PLX4720 (10 μM) for 16 h with or without pretreatment with actinomycin D (Act-D) (3 μg/ml) for 1 h were subjected to real-time PCR analysis. The relative abundance of mRNA expression before treatment was arbitrarily designated as 1. The data shown are the mean ± S.E. of three individual experiments.
of BimS mRNA to BimEL mRNA in Mel-RMu cells after treatment for 16 h was four times higher than that before treatment (Figure 3b). Similarly, the ratio of BimS mRNA to BimL mRNA was also increased, although to a lesser extent (Figure 3b). The increase in BimS mRNA relative to BimEL and BimL mRNA was confirmed in an additional three B-RAF-mutant melanoma cell lines (Supplementary Figure 5).

Collectively, these results suggest that PLX4720 may cause preferential splicing to produce BimS.

To confirm that the increase in BimS splicing is specific to inhibition of B-RAF V600E by PLX4720, we treated Mel-RMu and Mel-RM cells with the histone deacetylase inhibitor suberic bishydroxamate (SBHA), which is known to upregulate Bim at the transcriptional level.28 Figure 3c shows that, as reported before, the Bim mRNA and protein levels were upregulated by SBHA in melanoma cells, regardless of their B-RAF mutational status. Although this increase was reflected at the levels of the three Bim mRNA species, the ratios of the BimS mRNA to BimEL and BimL mRNA in both cell lines before and after treatment remained unaltered (Figure 3d).

Enforced expression of B-RAF V600E inhibits BimS expression in melanocytes and melanoma cells. We transfected cDNA encoding B-RAF V600E into wild-type B-RAF Mel-RM and Me1007 cells. Enforced expression of B-RAF V600E resulted in increases in activation of ERK (Figure 4a). Because the three Bim protein variants were all constitutively expressed at low levels in both cell lines, it was not feasible to judge whether enforced expression of B-RAF V600E resulted in downregulation of the proteins. To overcome this limitation, we treated the cells transfected with B-RAF V600E with SBHA, and monitored changes in the three mRNA species. Figure 4b shows that enforced expression of B-RAF V600E blocked increases in BimS mRNA induced by SBHA. Inhibition of SBHA-mediated induction of BimS by enforced expression of B-RAF V600E was also mirrored at the protein level (Figure 4c).

The effect of B-RAF V600E on the expression of the three Bim isoforms was also examined in a cultured melanocyte line that was transfected with cDNA encoding B-RAF V600E. It was notable that the BimEL, BimL, and BimS proteins were all constitutively expressed at detectable, although moderate,
levels in cultured melanocytes (Figure 4d). Survival of melanocytes in the presence of these Bim isoforms is conceivable owing to sequestration of BimEL and BimL in the cytoskeleton, and neutralization of BimS by anti-apoptotic Bcl-2 family proteins (Figure 4d). Consistent with its inhibitory effect on the expression of BimS in melanoma cells, enforced expression of B-RAFV600E caused a decrease in this isoform in melanocytes (Figure 4d). Intriguingly, there was an increase in the levels of BimL in melanocytes transfected with B-RAFV600E, suggesting that the effect of B-RAFV600E on regulation of Bim expression may be more complex than just impinging on BimEL and BimS, and may vary between different types of cells.

**BimS has a dominant role in apoptosis of B-RAFV600E melanoma cells induced by PLX4720.** To examine the relative importance of BimEL and BimS in PLX4720-induced apoptosis, we transfected siRNA specific for Bim in general, BimEL, and BimS into Mel-RMu cells (Figures 5a–c). Although siRNA knockdown of BimEL inhibited PLX4720-induced apoptosis by 30%, inhibition of BimS by siRNA blocked apoptosis induction by PLX4720 by 56% (Figure 5d). These results indicate that both BimEL and BimS are involved in induction of apoptosis of B-RAFV600E melanoma cells by PLX4720, but BimS has a greater part than BimEL. Although we did not specifically measure the role of BimL, it is conceivable that this isoform also contributes to PLX4720-induced apoptosis. More potent inhibition of PLX4720-induced apoptosis by knockdown of BimS was also demonstrated in another two B-RAFV600E melanoma cell lines (Supplementary Figure 6).

To further consolidate the role of BimS in induction of apoptosis of mutant B-RAF melanoma cells, the GFP-tagged open reading frame of human BimS cDNA cloned into the pCMV6-AC vector (pCMV6-AC-BimS-GFP) was transiently transfected into Mel-RMu and Mel-CV cells (Figure 5e). Figure 5f shows that overexpression of BimS induced apoptosis of the cells that could be detected as early as 16 h after transfection. By 48 h, 50% of the cells in both cell lines had committed to apoptosis. It is of note that BimS-GFP is readily detected in mitochondrial fractions at 24 h (Figure 5e), consistent with previous reports that BimS-induced apoptosis requires its mitochondrial localization. As shown in Figure 5g, exposure to PLX4720 similarly resulted in marked relocation of endogenous BimS onto mitochondria in Mel-RMu and Mel-CV cells.

**The SR protein SRp55 is involved in increased splicing of BimS triggered by PLX4720 in B-RAFV600E melanoma cells.** The gene encoding the SR protein SRp55, splicing factor arginine/serine-rich 6 (SFRS6), has been shown to be upregulated in B-RAFV600E melanoma cells. We therefore studied whether SRp55 is involved in regulation of alternative
splicing of Bim in B-RAF\textsuperscript{V600E} melanoma cells by inhibition of B-RAF\textsuperscript{V600E}. Surprisingly, the levels of the SRp55 protein appeared similar between B-RAF\textsuperscript{V600E} melanoma cell lines and those in the wild-type B-RAF (Figure 6a). However, in response to treatment with PLX4720, the levels were increased in B-RAF\textsuperscript{V600E} Mel-RMu cells, but not in wild-type Mel-RM cells (Figure 6a). Similarly, treatment with PLX4720 resulted in a marked increase (fivefold) in the expression levels of the SFRS6 mRNA in Mel-RMu but not in Mel-RM cells (Figure 6b).

We next transfected a siRNA pool for SFRS6 into Mel-RMu and Mel-CV cells. Transfection of a siRNA pool for splicing factor arginine/serine-rich 12 (SFRS12) was included as a control (Figures 6c and d). Inhibition of SRp55 but not of
SRp86, blocked the increase in the BimS mRNA and protein, and the increases in the ratios of the BimS mRNA to the BimEL and BimL mRNAs was induced by PLX4720 (Figures 6e–g). This was associated with attenuation of PLX4720-induced killing in both Mel-RMu and Mel-CV cells (Figure 6h). Figure 6i shows that overexpression of SRp55 resulted in moderate levels of apoptosis in Mel-RMu and Mel-CV cells in the absence of any further treatment. This was associated with an increase in BimS to varying degrees in Mel-RMu and Mel-CV cells (Figure 6i).

PLX4720 preferentially increases BimS and induces apoptosis in fresh melanoma isolates carrying B-RAFV600E. As shown in Figure 7a, PLX4720 inhibited activation of ERK1/2 in two fresh isolates with B-RAFV600E but not in the one with wild-type B-RAF. Consistently, PLX4720 markedly reduced the viability of the B-RAFV600E cells but not of wild-type B-RAF cells (Figure 7b). Two B-RAFV600E fresh isolates were used for further studies. PLX4720 upregulated the three Bim mRNA species in both fresh isolates (Figure 7c). It also upregulated all three protein variants, except for BimL, in Mel-JG cells (Figure 7c). Notably, there were also discrepancies in the levels of other mRNA species and corresponding protein variants after treatment with PLX4720. This suggests that mechanisms other than those mediated by inhibition of B-RAFV600E may be involved in regulation of the expression of Bim protein variants. Nevertheless, similar to results with melanoma cell lines, PLX4720 triggered increases in the ratios of the BimS mRNA to the BimEL and BimL mRNAs in both fresh isolates (Figure 8a). This was associated with an increase in the levels of the SRp55 protein and the SFRS6 mRNA (Figure 8b). Inhibition of BimS by siRNA partially restored viability of the cells, whereas inhibition of SRp55 by siRNA blocked the increase in BimS and similarly inhibited killing induced by PLX4720 (Figure 8c).

Discussion

The above results extend the role of Bim in apoptosis induced by inhibition of B-RAFV600E beyond upregulation of BimEL by showing that PLX4720 triggers preferential splicing to produce BimS, which has a greater part in induction of apoptosis than BimEL. In addition, the results demonstrate that the increase in splicing of BimS is due to a mechanism that is regulated by the splicing factor SRp55, which is increased in B-RAFV600E melanoma cells by PLX4720.

In support of previous observations, BimEL was upregulated by PLX4720 in B-RAF-mutant melanoma cells, which was associated with a reduction in the levels of an extra band with reduced electrophoretic motility that corresponds to Figure 5 Continued
phosphorylated BimEL.\cite{13} This was consistent with inhibition of activation of ERK1/2 by PLX4720,\cite{5} in that ERK1/2 can phosphorylate BimEL, thereby causing its ubiquitination and degradation by the proteasome system.\cite{13} Inhibition of this pathway has been suggested to account for a major part of the accumulation of BimEL.\cite{13,30} Strikingly, BimL, and in particular, Figure 6

SRp55 has a role in upregulation of BimS by PLX4720. (a) Left panel: whole cell lysates from a panel of mutant and wild-type B-RAF melanoma cell lines were subjected to western blot analysis of SRp55 and GAPDH (as a loading control). Right panel: whole cell lysates from Mel-RM and Mel-RMu cells treated with PLX4720 (10 \( \mu \)M) for indicated time periods were subjected to western blot analysis of SRp55 and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses. (b) Total RNA from Mel-RM and Mel-RMu cells treated with PLX4720 (10 \( \mu \)M) for 16 h was isolated and subjected to real-time PCR analysis for SFRS6. The relative abundance of mRNA expression before treatment was arbitrarily designated as 1. The data shown are the mean ± S.E. of three individual experiments. (c) Mutant B-RAF Mel-RMu and Mel-CV cells were transfected with the control, SFRS6, and SFRS12 siRNAs. After 24 h, total RNA was isolated and subjected to real-time PCR analysis for SFRS6 and SFRS12 mRNA expression. The relative abundance of mRNA expression in cells transfected with the control siRNA was arbitrarily designated as 1. The data shown are the mean ± S.E. of three individual experiments. (d) Whole cell lysates from cells treated as in c were subjected to western blot analysis of SRp55, SRrp86, and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses. (e) Mel-RMu and Mel-CV cells were transfected with the control, SFRS6, and SFRS12 siRNAs. After 24 h, cells were treated with PLX4720 (10 \( \mu \)M) for a further 16 h. Total RNA was isolated and subjected to real-time PCR analysis for BimS mRNA expression. The relative abundance of mRNA expression in cells transfected with the control siRNA without treatment with PLX4720 was arbitrarily designated as 1, which was not shown. The data shown are the mean ± S.E. of three individual experiments. (f) Mel-RMu and Mel-CV cells were transfected with the control, SFRS6, and SFRS12 siRNA. After 24 h, total RNA was isolated and subjected to real-time PCR analysis for BimEL, BimL, and BimS mRNA expression. Left panel: the ratios between the levels of BimS mRNA and BimEL mRNA before and after treatment, respectively, were calculated as \((\Delta \Delta CT)\) of BimS/\((\Delta \Delta CT)\) of BimEL. Right panel: the ratios between the levels of BimS mRNA and BimL mRNA before and after treatment, respectively, were calculated as \((\Delta \Delta CT)\) of BimS/\((\Delta \Delta CT)\) of BimL. The data shown are the mean ± S.E. of three individual experiments. (g) Mel-RMu and Mel-CV cells were transfected with the control and SFRS6 siRNA, respectively. After 24 h later, whole cell lysates were subjected to western blot analysis of SRp55-GFP and BimS. Western blot analysis of GAPDH was then performed as a loading control. The data shown are representative of three individual western blot analyses.
BimS, were also increased by PLX4720 in B-RAFV600E melanoma cells. Nevertheless, the kinetics and sustainability of upregulation of the three Bim isoforms varied from one another, suggesting that the mechanisms responsible for upregulation of the individual proteins may be different.

The marked increase in BimS induced by PLX4720 is of particular interest, in that BimS is rarely detectable at the protein level in cells.13,16 This is presumably associated with its stronger potency in induction of apoptosis than other isoforms, as BimS is not subject to any posttranslational regulation and is instantly activated once it is expressed.13 When overexpressed, BimS can rapidly translocate onto the mitochondrial outer membrane where it recruits and activates Bax independently of inhibition of anti-apoptotic Bcl-2 family proteins.17 Further studies by real-time revealed that the BimS transcript was preferentially induced by PLX4720 in B-RAFV600E melanoma cells. Moreover, the preferential induction of splicing to produce BimS resulted in a greater degree of inhibition of PLX4720-induced killing than selective inhibition of BimEL.

The preferential induction of splicing of BimS suggests that the mutant B-RAFV600E may regulate Bim alternative splicing in melanoma cells, and in particular, may suppress splicing to produce BimS. This was supported by the finding that enforced expression of B-RAFV600E in wild-type B-RAF melanoma cells blocked upregulation of the BimS transcript by SBHA, but had no effect on upregulation of the BimEL and BimL mRNA. Furthermore, enforced expression of B-RAFV600E in melanocytes resulted in decreases in the BimS mRNA and protein, but intriguingly, the levels of the BimL mRNA and protein were increased by enforced expression of B-RAFV600E. These results suggest that regulation of Bim splicing by mutant B-RAF is more complex than inhibition of splicing of BimS, and may vary between different cell types. It is of interest that, in contrast to melanoma cells, melanocytes expressed readily detectable levels of BimS together with BimEL and BimL. This may indicate that BimS expression is
lost during melanoma development as a consequence of mutations in B-RAF. Bim has been shown to be decreased with melanoma progression.31 Altered splicing patterns, in particular, changes in splicing patterns of apoptosis-related genes, are frequently found in various cancers.20,21,23 Although the mechanisms underlying this remain unclear, it is well established that alternative splicing is tightly regulated by splicing factors, including the SR and hnRNP protein families.25,27,32 There is a growing body of evidence showing that some protein kinases such as
Inhibition of B-RAF<sup>V600E</sup> triggers splicing of bim<sub>S</sub>

CC Jiang et al

Figure 8 Inhibition of Bim<sub>S</sub> by siRNA reverses the reduction in cell viability induced by PLX4720, whereas inhibition of SRp55 blocks induction of Bim<sub>S</sub> and induction of apoptosis by PLX4720 in mutant B-RAF fresh melanoma isolates. (a) Left panel: Mel-JR and Mel-JG cells were transfected with the control and Bim<sub>S</sub> siRNA, respectively. After 24 h, whole cell lysates were subjected to western blot analysis of Bim and GAPDH (as a loading control). The arrowhead points to nonspecific bands. Right panel: Mel-JR and Mel-JG cells were transfected with the control and Bim<sub>S</sub> siRNA, respectively. After 24 h, cells were treated with PLX4720 (10 μM) for a further 72 h. Apoptosis was measured by the propidium iodide (PI) method. The data shown are either representative (left panel) or the mean ± S.E. of three individual experiments. (b) Mel-JR and Mel-JG cells were transfected with the control and SFRS6 siRNA, respectively. After 24 h, cells were treated with PLX4720 (10 μM) for a further 16 h. Whole cell lysates were subjected to western blot analysis of SRp55, Bim, and GAPDH (as a loading control). The arrowhead points to nonspecific bands. The data shown are representative of three individual western blot analyses. (c) Total RNA from Mel-JR and Mel-JG cells treated as in b was subjected to real-time PCR analysis for Bim<sub>S</sub> mRNA expression. The relative abundance of Bim<sub>S</sub> mRNA in cells transfected with the control siRNA without treatment with PLX4720 was designated as 1, which was not shown. The data shown are the mean ± S.E. of three individual experiments. (d) Mel-JR and Mel-JG cells were transfected with the control and SFRS6 siRNA, respectively. After 24 h, cells were treated with PLX4720 (10 μM) for a further 72 h. Apoptosis was quantitated by the PI method. The data shown are either the mean ± S.E. (right panel of a, c, and d) or representative (left panel of a and b) of three individual experiments. The data shown are the mean ± S.E. of three individual experiments.

Akt and ERK1/2 that have important roles in cancer development also have roles in regulation of activity of SR proteins, probably by modulating their phosphorylation status.33–35 In this study, we found that one of the SR proteins, SRp55, was associated with preferential splicing to produce Bim<sub>S</sub> after inhibition of mutant B-RAF by PLX4720. This was demonstrated by the findings that PLX4720 upregulated SRp55, and that inhibition of SRp55 with siRNA blocked upregulation of the Bim<sub>S</sub> transcript and reversed the increases in ratios of the Bim<sub>EL</sub> mRNA to the Bim<sub>EL</sub> and Bim<sub>S</sub> mRNA induced by PLX4720. Consistently, knockdown of SRp55 partially inhibited apoptosis induced by PLX4720 in mutant B-RAF melanoma cells. Therefore, the mutant B-RAF<sup>V600E</sup> appears to regulate the expression of SRp55 that in turn has a role in regulating alternative splicing of Bim, in particular, in promoting splicing to produce Bim<sub>S</sub>

The finding that killing of mutant B-RAF fresh melanoma isolates by PLX4720 was similarly associated with upregulation of Bim, in particular, Bim<sub>S</sub>, is of particular importance, in that this may reflect more closely the reaction of melanoma cells in vivo to treatment with B-RAF inhibitors. Together, the results reported in this study identify induction of Bim<sub>S</sub> as a key mechanism for induction of apoptosis by PLX4720 in melanoma cells carrying B-RAF<sup>V600E</sup>. We speculate that this may be critical for long-term clinical responses to the inhibitor.

Plasma concentrations of PLX4032 of around 60 μM were apparently not associated with significant adverse effects in phase I clinical trials with PLX4032, suggesting that the concentrations used in this study are achievable clinically.

Materials and Methods

Cell lines. Human melanoma cell lines Mel-RM, Me1007, Mel-RM50, Mel-CV, and Sk-Mel-110 have been described previously.36,37 They were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, Vic, Australia). Melanocytes were kindly provided by Dr P Parsons (Queensland Institute of Medical Research, Qld, Australia) and cultured in medium supplied by Clonetics (Edward Kellar, Vic, Australia).

Fresh melanoma isolates. Isolation of melanoma cells from fresh surgical specimens was carried out as described previously.38 Protocols were approved by the Human Research Ethics Committee of Hunter New England Health, Australia.

Antibodies, recombinant proteins, and other reagents. PLX4720 was provided from Plexikon Inc (Berkeley, CA, USA). It was dissolved in DMSO and made up in stock solutions of 4 mM. Actinomycin D and SBHA were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). SBHA was dissolved in distilled water and made up in a stock solution of 10 mg/ml. The mouse MAbs against pERK, Bcl-2, Mcl-1, Bad, and AIF, and the rabbit polyclonal antibodies (Abs) against B-RAF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The MAb against Noxa and the polyclonal Ab against Bim were purchased from Imgenex (San Diego, CA, USA). The rabbit polyclonal Abs against PUMA, ERK, COX IV, A-RAF, C-RAF, and Bid were from Cell Signalling Technology (Beverly,
Real-time PCR. G418 or puromycin (Sigma-Aldrich) selection were then passaged at 1:10 ratio into fresh medium for further 24 h, followed by according to the manufacturer's protocol. At 6 h after transfection, the cells were (Invitrogen, Carlsbad, CA, USA) with Lipofectamine 2000 reagent (Invitrogen), 

m TAMRA, and 9 mM MgCl2. To specifically detect individual Bim isoforms, forward primers were designed to span the junctions of exons 3 and 4, exons 2 and 4, and exons 4 and 5, which are unique to BimL, BimS, and BimB, respectively. BimL forward primer is 5'-GTGGGTATTTCCTTTTGCAGCACG-3'; BimS forward primer is 5'-TACACAGAGCAACCAAGACG-3'; and common reverse primer for both BimL and BimS is 5'-GTGACGCCGTCGCTTAAGAG-3'. BimB forward primer is 5'-TGACCCGAAGAGTACGAAT-3'; BimB reverse primer is 5'-GCCA TACAACTCTAGGCAG-3'. Real-time PCR for three Bim isoforms was done by Fast SYBR Green Master Mix (Applied Biosystems). For SFRS6 and SFRS12, assay-on-demand for SFRS6 (assay ID: Hs00174777_q1), SFRS12 (assay ID: Hs00377948_m1), and GAPDH (assay ID: Hs99999905_m1) were used according to manufacturer's protocol (Applied Biosystems). Analysis of cDNA for β-actin or GAPDH cycle numbers. The relative abundance of mRNA expression of a control sample was arbitrarily designated as 1, and the values of the relative abundance of mRNA of other samples were calculated accordingly.

Small interfering RNA. The siRNA constructs for Bim, B-RAF, SFRS6, and SFRS12 were obtained as the siGENOME SMARTpool reagents (Dharmacon, Lafayette, CO, USA), the siGENOME SMARTpool Bim (M-004383-01-001), the siGENOME SMARTpool B-RAF (M-003460-03-001), the siGENOME SMARTpool SFRS6 (M-00607-01-001), and the siGENOME SMARTpool SFRS12 (M-016865-01-001). The nontargeting siRNA control, SiConTRolNon-targeting SiRNA pool (D-001206-13-20) was obtained from Dharmacon.

To specifically knockdown BimL and BimS, siRNAs were designed to target exons 3 and 5, which are unique to BimL and BimS, respectively. The oligonucleotides used were BimL sense 5'-CUCCUGUGCUCAUCUCAGUGTTdTdT-3'; BimS antisense 5'-UGGAGGUGCAACGAGCAUGdIdTdT-3'; BimL sense 5'-CAUAGUGGUGUAUUGGCUAUdTdT-3'; BimS antisense 5'-AAUCCACUGAUAUGdIdTdT-3'; control sense 5'-GCCUGUAACAUAGUGCAGUdIdTdT-3', control antisense 5'-AAUAGACUGUAGUACCGCAGdTdT-3'. Transfection of siRNA pools was carried out as described previously.

Conflict of interest
The authors declare no conflict of interest.

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