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Apoptosis of human melanoma cells induced by inhibition of B-RAF^{V600E} involves preferential splicing of bim$_S$

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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, Bim$_S$, remains largely unappreciated. Here, we show that inhibition of the mutant B-RAF^{V600E} triggers preferential splicing to produce Bim$_S$, which is particularly important in induction of apoptosis in B-RAF^{V600E} melanoma cells. Although the specific B-RAF^{V600E} inhibitor PLX4720 upregulates all three major isoforms of Bim, Bim$_{EL}$, Bim$_L$, and Bim$_S$, at the protein and mRNA levels in B-RAF^{V600E} melanoma cells, the increase in the ratios of Bim$_S$ mRNA to Bim$_{EL}$ and Bim$_L$ mRNA indicates that it favours Bim$_S$ splicing. Consistently, enforced expression of B-RAF^{V600E} in wild-type B-RAF melanoma cells and melanocytes inhibits Bim$_S$ expression. The splicing factor SRp55 appears necessary for the increase in Bim$_S$ splicing, as SRp55 is upregulated, and its inhibition by small interfering RNA blocks induction of Bim$_S$ and apoptosis induced by PLX4720. The PLX4720-induced, SRp55-mediated increase in Bim$_S$ splicing is also mirrored in freshly isolated B-RAF^{V600E} melanoma cells. These results identify a key mechanism for induction of apoptosis by PLX4720, and are instructive for sensitizing melanoma cells to B-RAF^{V600E} inhibitors.

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Results from clinical studies with small molecule inhibitors of the mutant B-RAF^{V600E} have been very encouraging, and promise to provide a much needed breakthrough in the treatment of melanoma by targeting B-RAF^{V600E}.[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-RAF^{V600E} 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-RAF^{V600E}[6]. 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 Bim$_{EL}$, and downregulation of Mcl-1.[7–11] Among them, upregulation of Bim$_{EL}$ via inhibition of its phosphorylation and subsequent proteasomal degradation may be the best documented[7,8] 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, Bim$_{EL}$, Bim$_L$, and Bim$_S$, that are generated by alternative splicing.[14] Although Bim$_S$ is encoded by exons 2, 5, and 6, Bim$_L$ is encoded by exons 2, 4, 5, and 6, and Bim$_{EL}$ by exons 2, 3, 4, 5, and 6. Both Bim$_L$ and Bim$_{EL}$ contain a binding site for dynein light chain 1,[14,15] 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, Bim$_{EL}$ is subject to phosphorylation by the MEK/ERK pathway that targets it for proteasomal degradation and also prevents its binding to

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Abbreviations: BH3, Bcl-2 homology 3; SR protein, serine/arginine-rich protein; SBHA, suberic bishydroxamate; SFRS6, splicing factor arginine/serine-rich 6; SFRS12, splicing factor serine/arginine-rich 12
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Bax.[16] 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)

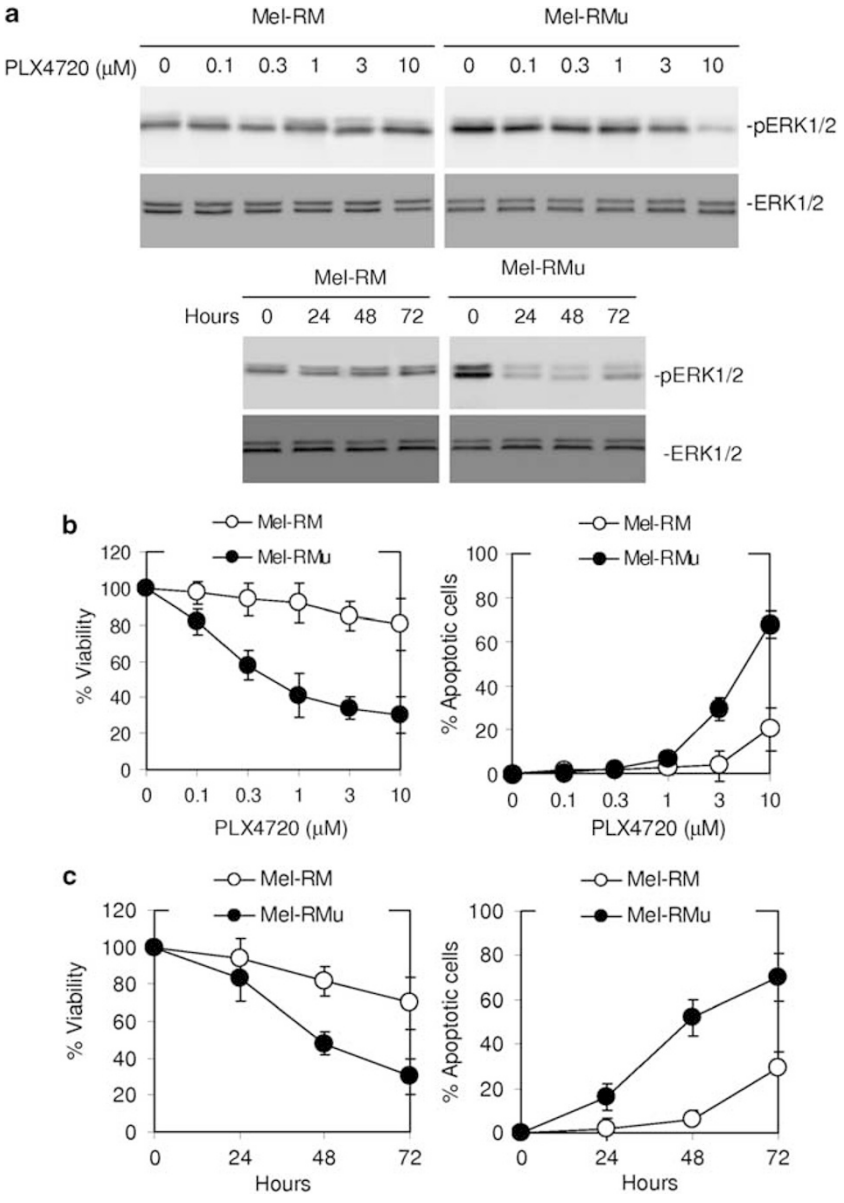

Figure 1 PLX4720 inhibits proliferation and induces apoptosis in mutant B-RAF melanoma cells. (**a**) Whole cell lysates from Mel-RM (wild-type B-RAF) and Mel-RMu (B-RAF^{V600E}) cells treated with PLX4720 at indicated concentrations for 72 h (upper panel) or at 10 μM for indicated periods (lower panel), 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 ± S.E. of three individual experiments. (**c**) Mel-RM and Mel-RMu cells were treated with PLX4720 at 10 μM 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 ± 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 μM for 72 h were subjected to MTS assays. The data shown are the mean ± 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 μM) for a further 72 h. Apoptotic cells were measured by the PI method. The data shown are either representative (left panel), or the mean ± S.E. (right panel), of three individual experiments

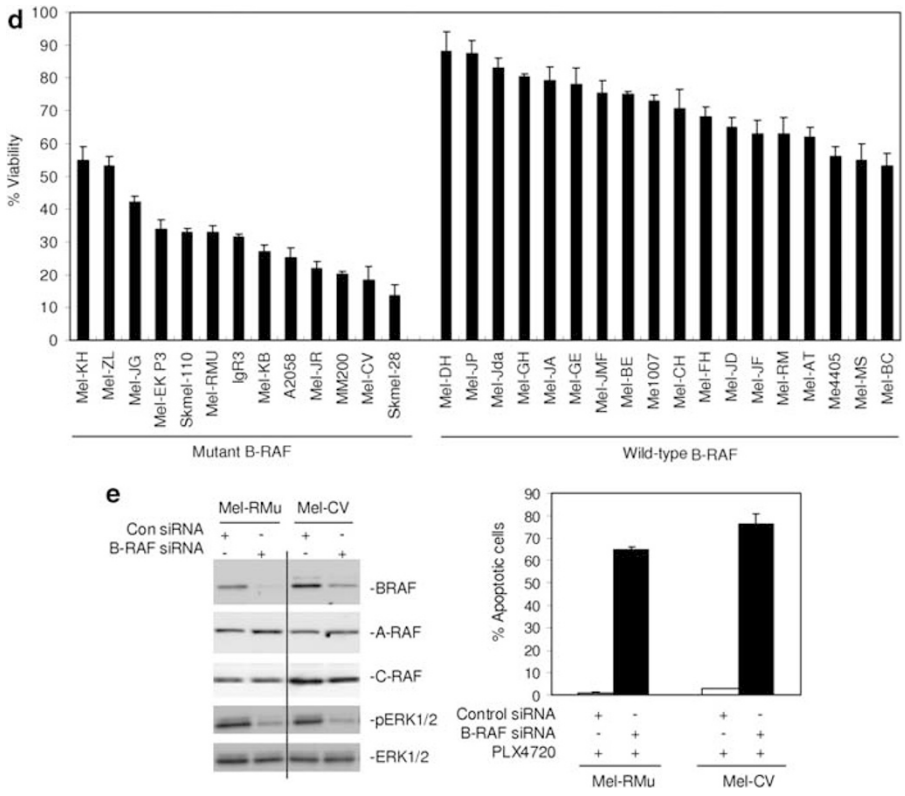

Figure 1 Continued

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.[18] Changes in the expression of a number of SR proteins have been found in various types of cancer cells.[23–26]

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 μM (Figure 1a and Supplementary Figure 1A). The inhibitory effect of PLX4720 at 10 μM on activation of ERK was sustained till 72 h 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 μM, PLX4720 did not induce significant apoptosis by 72 h (Figure 1b). At 3 μM, it induced apoptosis in $\sim$30% of Mel-RMu cells, but apoptosis in Mel-RM cells remained marginal. When it was used at 10 μM, $>$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 μM 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

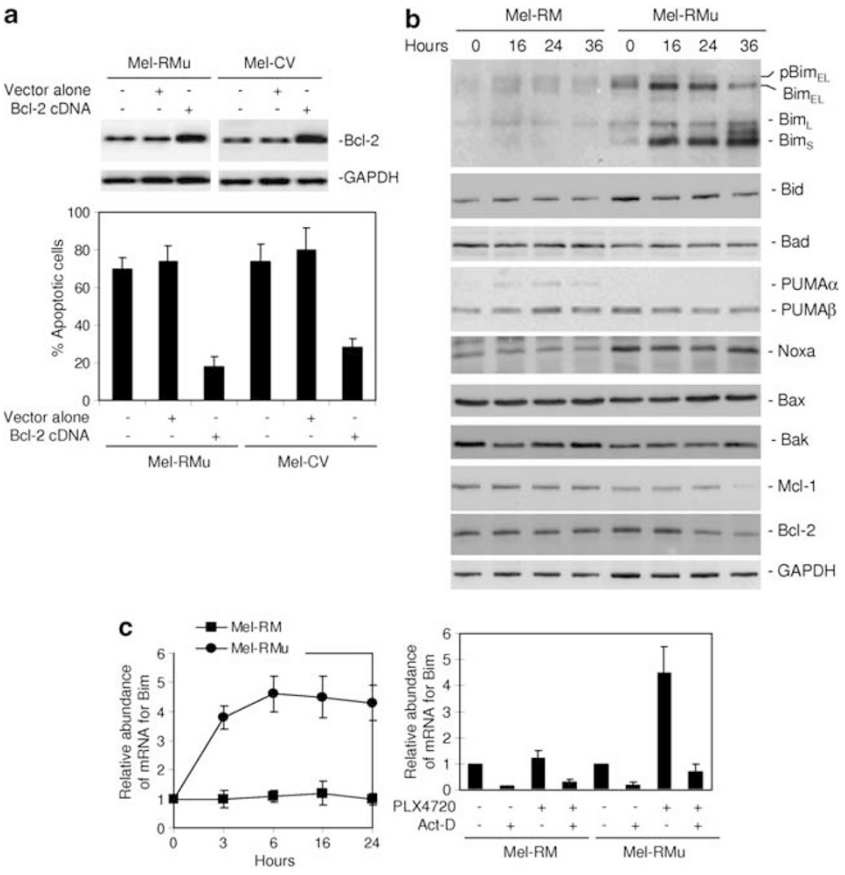

Figure 2 PLX4720 upregulates Bim. (**a**) Upper panel: overexpression of Bcl-2 in Mel-RMu and Mel-CV cells stably transfected with cDNA encoding Bcl-2. Whole cell lysates were subjected to western blot analysis of Bcl-2 and GAPDH (as a loading control). Lower panel: Mel-RMu and Mel-CV cells overexpressing 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, Bax, 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

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, Bim$_{EL}$, Bim$_L$, and Bim$_S$, in B-RAF^{V600E} 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 Bim$_{EL}$.[13] Of note, PLX4720 also induced a novel protein product with an apparent molecular weight between Bim$_L$ and Bim$_S$ 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$_S$ induced by PLX4720 was intriguing because, unlike Bim$_{EL}$ and Bim$_L$, this isoform is not regulated by any known posttranslational mechanisms.[13,15] We reasoned that upregulation of Bim$_S$ is a consequence of enhanced Bim transcription and a subsequent increase in splicing to produce Bim$_S$. To test this, we first quantitated the Bim mRNA expression before and after exposure to PLX4720. As shown in Figure 2c, PLX4720 triggered a rapid and sustained increase in Bim mRNA in Mel-RMu cells, which could be efficiently inhibited by pretreatment with actinomycin D (Figure 2c), suggesting that this was due to a transcriptional increase, rather than a change in the mRNA stability.

We next monitored the levels of the three major Bim mRNA species in Mel-RMu cells before and after exposure to PLX4720 in qPCR analysis. Figure 3a shows that, although they were all increased by treatment with PLX4720, the ratio

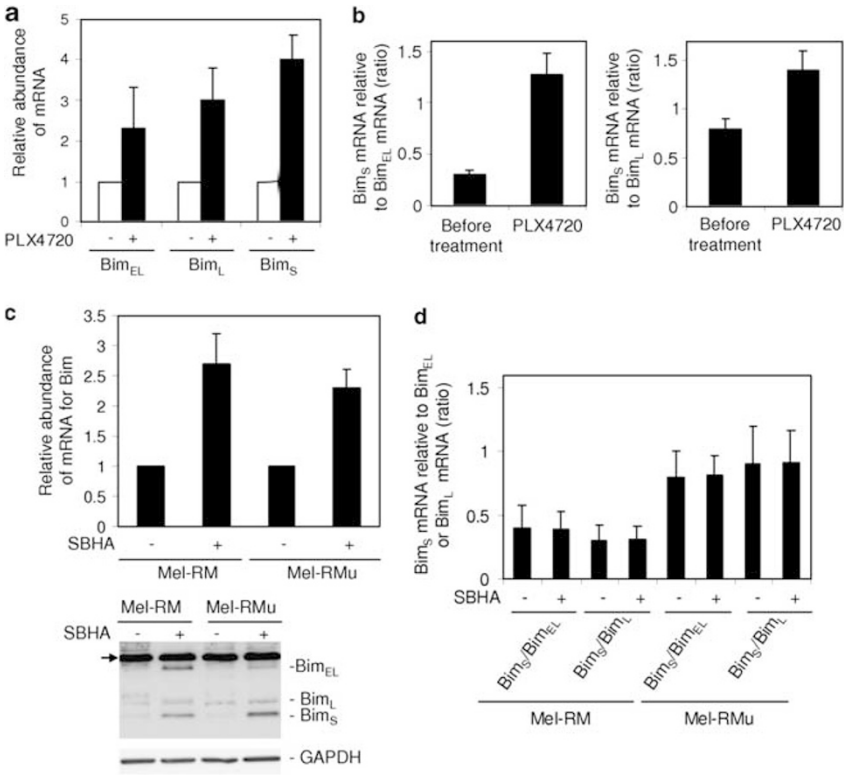

Figure 3 PLX4720 preferentially increases splicing to produce Bim$_S$. (**a**) Total RNA from Mel-RMu cells treated with PLX4720 (10 μM) for 16 h was subjected to real-time PCR analysis for Bim$_{EL}$, Bim$_L$, and Bim$_S$ mRNA expression. The levels of the expression of individual species before treatment were arbitrarily designated as 1. The data shown are the mean ± S.E. of three individual experiments. (**b**) Total RNA from Mel-RMu cells treated with PLX4720 (10 μM) for 16 h was subjected to Bim$_{EL}$, Bim$_L$, and Bim$_S$ mRNA expression as in **a**. Left panel: the ratios between the levels of Bim$_S$ mRNA and Bim$_{EL}$ mRNA before and after treatment, respectively, were calculated as ($\Delta\Delta C$t of Bim$_S$/$\Delta\Delta C$t of Bim$_{EL}$). Right panel: the ratios between the levels of Bim$_S$ mRNA and Bim$_L$ mRNA before and after treatment, respectively, were calculated as ($\Delta\Delta C$t of Bim$_S$/$\Delta\Delta C$t of Bim$_L$). The data shown are the mean ± S.E. of three individual experiments. (**c**) Upper panel: total RNA from Mel-RM and Mel-RMu cells treated with SBHA (10 μg/ml) for 16 h were subjected to real-time PCR analysis for Bim mRNA expression. The relative abundance of mRNA expression before treatment was arbitrarily designated as 1. Lower panel: whole cell lysates from cells treated as above were subjected to western blot analysis of Bim and GAPDH (as a loading control). The strong band denoted by the arrowhead is nonspecific, and was associated with the particular batch of the antibody against Bim used in the experiment. The data shown are either representative (lower panel) or the mean ± S.E. (upper panel) of three individual experiments. (**d**) Total RNA from Mel-RM and Mel-RMu cells treated with SBHA (10 μg/ml) for 16 h were subjected to real-time PCR analysis for Bim$_{EL}$, Bim$_L$, and Bim$_S$ mRNA expression. The ratios of Bim$_S$ mRNA to Bim$_{EL}$ and Bim$_L$ mRNA before and after treatment, respectively, were calculated as in **b**. The data shown are the mean ± S.E. of three individual experiments

of Bim$_S$ mRNA to Bim$_{EL}$ mRNA in Mel-RMu cells after treatment for 16 h was four times higher than that before treatment (Figure 3b). Similarly, the ratio of Bim$_S$ mRNA to Bim$_L$ mRNA was also increased, although to a lesser extent (Figure 3b). The increase in Bim$_S$ mRNA relative to Bim$_{EL}$ and Bim$_L$ 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 Bim$_S$.

To confirm that the increase in Bim$_S$ 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 Bim$_S$ mRNA to the Bim$_{EL}$ and Bim$_L$ mRNA in both cell lines before and after treatment remained unaltered (Figure 3d).

Enforced expression of B-RAF^{V600E} inhibits Bim$_S$ 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 Bim$_S$ mRNA induced by SBHA. Inhibition of SBHA-mediated induction of Bim$_S$ 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 Bim$_{EL}$, Bim$_L$, and Bim$_S$ proteins were all constitutively expressed at detectable, although moderate,

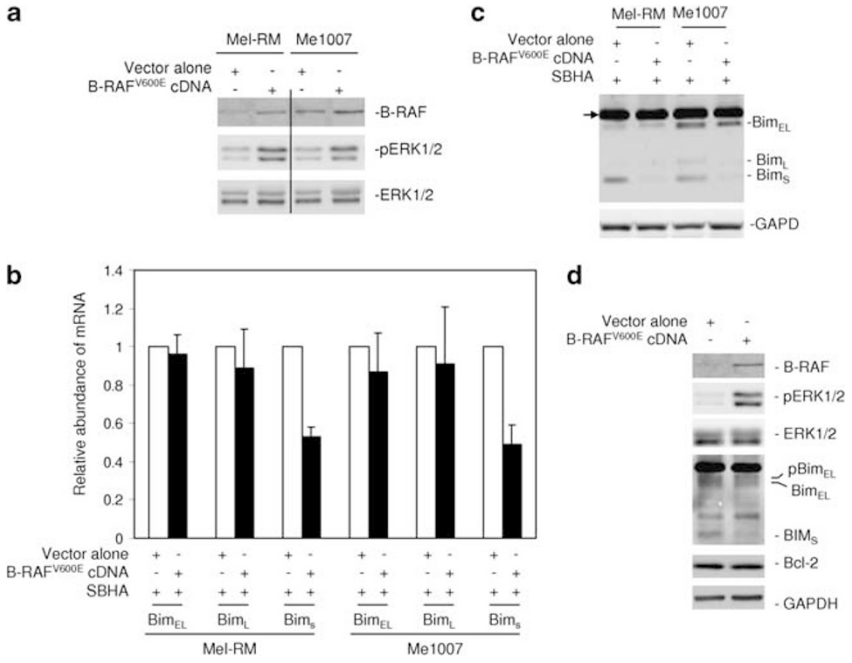

Figure 4 Enforced expression of B-RAF^{V600E} inhibited Bim$_S$ expression in wild-type B-RAF melanoma cells and melanocytes. (**a**) Mel-RM and Me1007 cells were stably transfected with cDNA encoding B-RAF carrying the V600E mutation. Whole cell lysates were subjected to western blot analysis of B-RAF, pERK1/2, and ERK1/2. The data shown are representative of three individual western blot analyses. (**b**) Mel-RM and Me1007 cells were stably transfected with cDNA encoding B-RAF carrying the V600E mutation. Cells were treated with SBHA (10 μg/ml) for a further 16 h. Total RNA was isolated and subjected to real-time PCR analysis for Bim$_{EL}$, Bim$_L$, and Bim$_S$ mRNA expression. The levels of the expression of individual species before treatment were arbitrarily designated as 1. The data shown are the mean ± S.E. of three individual experiments. (**c**) Whole cell lysates from Mel-RM and Me1007 cells treated as in **b** were subjected to western blot analysis of Bim and GAPDH (as a loading control). The arrowhead points to nonspecific bands. The data shown are representative of three individual western blot analyses. (**d**) Melanocytes were transiently transfected with cDNA encoding B-RAF carrying the V600E mutation. After 24 h, whole cell lysates were subjected to western blot analysis of B-RAF, pERK1/2, ERK1/2, Bim, and Bcl-2. The arrowhead points to nonspecific bands. The data shown are representative of three individual western blot analyses

levels in cultured melanocytes (Figure 4d). Survival of melanocytes in the presence of these Bim isoforms is conceivable owing to sequestration of Bim$_{EL}$ and Bim$_L$ in the cytoskeleton,[13,14] and neutralization of Bim$_S$ by anti-apoptotic Bcl-2 family proteins (Figure 4d).[12] Consistent with its inhibitory effect on the expression of Bim$_S$ in melanoma cells, enforced expression of B-RAF^{V600E} caused a decrease in this isoform in melanocytes (Figure 4d). Intriguingly, there was an increase in the levels of Bim$_L$ in melanocytes transfected with B-RAF^{V600E}, suggesting that the effect of B-RAF^{V600E} on regulation of Bim expression may be more complex than just impinging on Bim$_{EL}$ and Bim$_S$, and may vary between different types of cells.

Bim$_S$ has a dominant role in apoptosis of B-RAF^{V600E} melanoma cells induced by PLX4720. To examine the relative importance of Bim$_{EL}$ and Bim$_S$ in PLX4720-induced apoptosis, we transfected siRNA specific for Bim in general, Bim$_{EL}$, and Bim$_S$ into Mel-RMu cells (Figures 5a–c). Although siRNA knockdown of Bim$_{EL}$ inhibited PLX4720-induced apoptosis by 30%, inhibition of Bim$_S$ by siRNA blocked apoptosis induction by PLX4720 by 56% (Figure 5d). These results indicate that both Bim$_{EL}$ and Bim$_S$ are involved in induction of apoptosis of B-RAF^{V600E} melanoma cells by PLX4720, but Bim$_S$ has a greater part than Bim$_{EL}$. Although we did not specifically measure the role of Bim$_L$ because of technical limitations in designing siRNA that specifically silences Bim$_L$, it is conceivable that this isoform also contributes to PLX4720-induced apoptosis. More potent inhibition of PLX4720-induced apoptosis by knockdown of Bim$_S$ was also demonstrated in another two B-RAF^{V600E} melanoma cell lines (Supplementary Figure 6).

To further consolidate the role of Bim$_S$ in induction of apoptosis of mutant B-RAF melanoma cells, the GFP-tagged open reading frame of human Bim$_S$ cDNA cloned into the pCMV6-AC vector (pCMV6-AC-Bim$_S$-GFP) was transiently transfected into Mel-RMu and Mel-CV cells (Figure 5e). Figure 5f shows that overexpression of Bim$_S$ 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 Bim$_S$-GFP is readily detected in mitochondrial fractions at 24 h (Figure 5e), consistent with previous reports that Bim$_S$-induced apoptosis requires its mitochondrial localization.[17] As shown in Figure 5g, exposure to PLX4720 similarly resulted in marked relocation of endogenous Bim$_S$ onto mitochondria in Mel-RMu and Mel-CV cells.

The SR protein SRp55 is involved in increased splicing of Bim$_S$ triggered by PLX4720 in B-RAF^{V600E} melanoma cells. The gene encoding the SR protein SRp55, splicing factor arginine/serine-rich 6 (*SFRS6*), has been shown to be upregulated in B-RAF^{V600E} melanoma cells.[29] We therefore studied whether SRp55 is involved in regulation of alternative

splicing of Bim in B-RAF^{V600E} melanoma cells by inhibition of B-RAF^{V600E}. Surprisingly, the levels of the SRp55 protein appeared similar between B-RAF^{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^{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

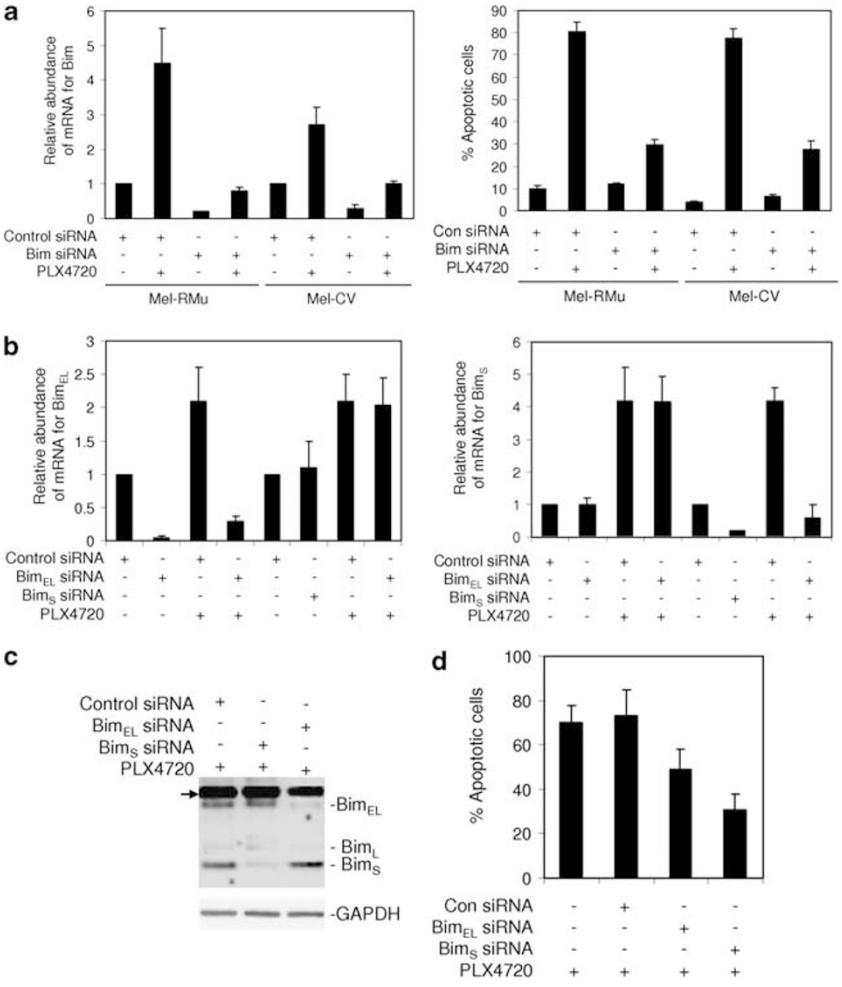

Figure 5 Bim$_S$ has a critical role in PLX4720-induced apoptosis of mutant B-RAF melanoma cells. (**a**) Left panel: Mel-RMu and Mel-CV cells (B-RAF^{V600E}) were transfected with the control or Bim siRNA. After 24 h, cells were treated with PLX4720 for 16 h. Total RNA was isolated and subjected to real-time PCR analysis for Bim mRNA expression. The relative abundance of mRNA expression in cells transfected with the control siRNA before treatment was arbitrarily designated as 1. Right panel: Mel-RMu and Mel-CV cells were transfected with the control or Bim siRNA. After 24 h, cells were treated with PLX4720 for a further 72 h. Apoptosis was quantitated by the propidium iodide (PI) method. The data shown are the mean ± S.E. of three individual experiments. (**b**) Mel-RMu cells were transfected with the control, Bim$_{EL}$, or Bim$_S$ siRNA. After 24 h, cells were treated with PLX4720 (10 μM) for 16 h. Total RNA was isolated and subjected to real-time PCR analysis for Bim$_{EL}$ (left panel) and Bim$_S$ (right panel) mRNA expression. The levels of the expression of individual species in cells transfected with the control siRNA without treatment were arbitrarily designated as 1. The data shown are the mean ± S.E. of three individual experiments. (**c**) Whole cell lysates from Mel-RMu cells treated as in **b** were subjected to western blot analysis of Bim and GAPDH (as a loading control). The arrowhead points to nonspecific bands. The data shown are representative of three individual western blot analyses. (**d**) Mel-RMu cells with Bim$_{EL}$ or Bim$_S$ knocked down as in **b** were treated with PLX 4720 at 10 μM for 72 h. Apoptosis was quantitated by the PI method. The data shown are the mean ± S.E. of three individual experiments. (**e**) Left panel: Mel-RMu and Mel-CV cells were transfected with pCMV6-AC-GFP or pCMV6-AC-Bim$_S$-GFP. After 24 h, whole cell lysates were subjected to western blot analysis of Bim$_S$-GFP using an antibody against GFP. Western blot analysis of GAPDH was then performed as a loading control. Right panel: Mel-RMu and Mel-CV cells were transfected with pCMV6-AC-GFP or pCMV6-AC-Bim$_S$-GFP. After 24 h, mitochondrial fractions were subjected to western blot analysis of Bim$_S$-GFP using an antibody against GFP. Western blot analysis of COX IV was then performed as a loading control. (**f**) Left panel: Representative flow cytometric histograms of measurement of apoptosis using PE-conjugated Annexin-V in Mel-RMu and Mel-CV cells transfected with pCMV6-AC-GFP or pCMV6-AC-Bim$_S$-GFP. PE-positive cells were quantitated in gated GFP-positive cell populations. The numbers represent percentages of positive cells. Right panel: Mel-RMu and Mel-CV cells were transfected with pCMV6-AC-GFP or pCMV6-AC-Bim$_S$-GFP for indicated time periods. Apoptotic cells were quantitated with PE-conjugated Annexin-V in gated GFP-positive cell populations. The data shown are representative of two experiments. (**g**) Mitochondrial fractions from Mel-RMu and Mel-CV cells treated with PLX4720 (10 μM) for indicated time periods were subjected to western blot analysis of Bim and COX IV (as a control). The data shown are representative of three individual western blot analyses

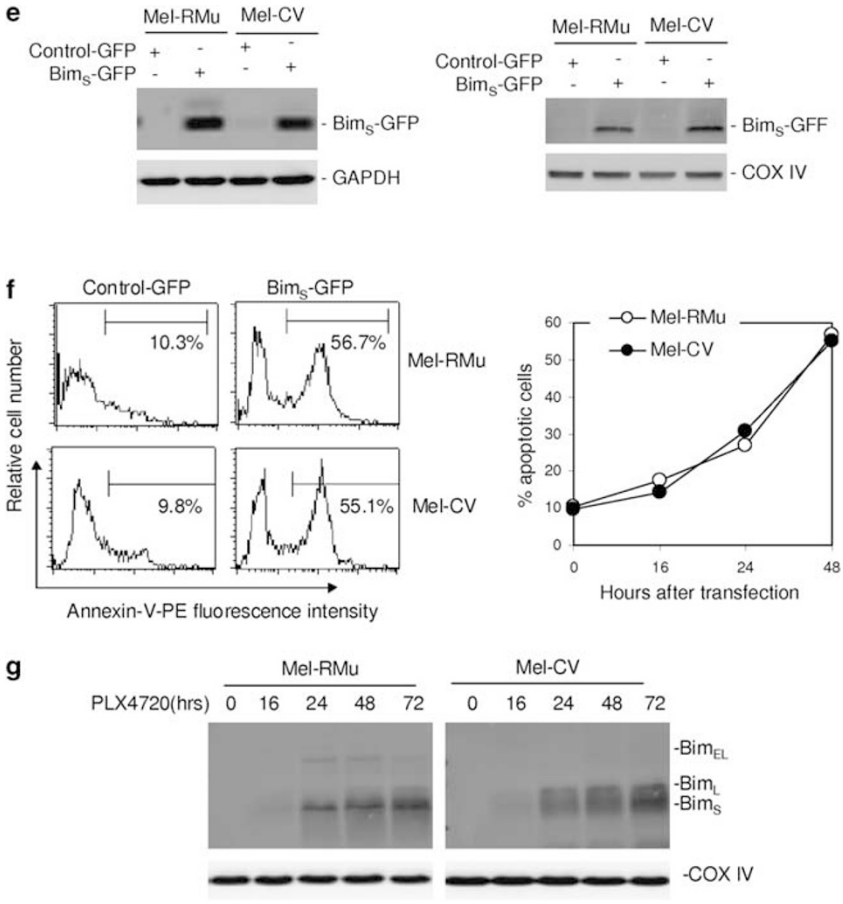

Figure 5 Continued

SRrp86, blocked the increase in the Bim_S mRNA and protein, and the increases in the ratios of the Bim_S mRNA to the Bim_{EL} and Bim_L 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 Bim_S to varying degrees in Mel-RMu and Mel-CV cells (Figure 6i).

PLX4720 preferentially increases Bim_S and induces apoptosis in fresh melanoma isolates carrying B-RAF^{V600E}. As shown in Figure 7a, PLX4720 inhibited activation of ERK1/2 in two fresh isolates with B-RAF^{V600E} but not in the one with wild-type B-RAF. Consistently, PLX4720 markedly reduced the viability of the B-RAF^{V600E} cells but not of wild-type B-RAF cells (Figure 7b). Two B-RAF^{V600E} 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 Bim_L, 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-RAF^{V600E} 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 Bim_S mRNA to the Bim_{EL} and Bim_L 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 Bim_S by siRNA partially restored viability of the cells, whereas inhibition of SRp55 by siRNA blocked the increase in Bim_S 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-RAF^{V600E} beyond upregulation of Bim_{EL} by showing that PLX4720 triggers preferential splicing to produce Bim_S, which has a greater part in induction of apoptosis than Bim_{EL}.[7,8] In addition, the results demonstrate that the increase in splicing of Bim_S is due to a mechanism that is regulated by the splicing factor SRp55, which is increased in B-RAF^{V600E} melanoma cells by PLX4720.

In support of previous observations,[30] Bim_{EL} 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

phosphorylated Bim$_{EL}$.[13] This was consistent with inhibition of activation of ERK1/2 by PLX4720,[5] in that ERK1/2 can phosphorylate Bim$_{EL}$, thereby causing its ubiquitination and degradation by the proteasome system.[13] Inhibition of this pathway has been suggested to account for a major part of the accumulation of Bim$_{EL}$.[13,30] Strikingly, Bim$_L$, and in particular,

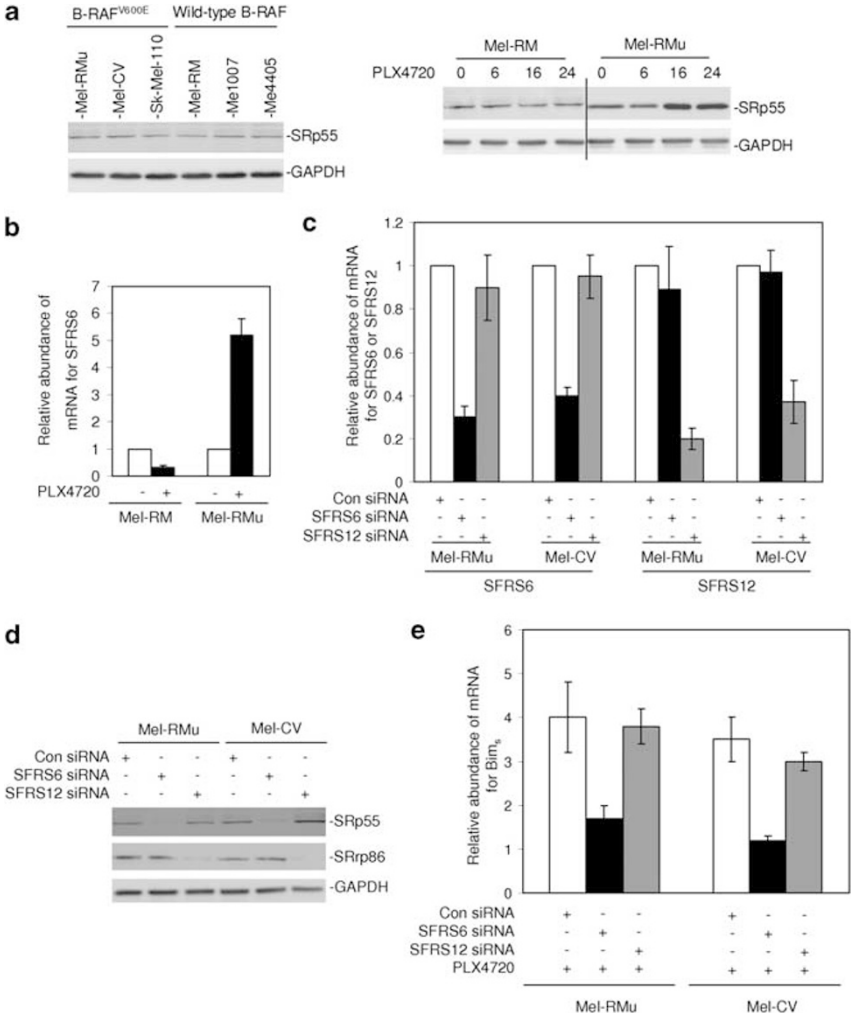

Figure 6 SRp55 has a role in upregulation of Bim$_S$ by PLX4720. (**a**) Left panel: whole cell lysates from a panel of mutant and wild-type B-RAF melanoma cell lines were subjected to western blot analysis of SRp55 and GAPDH (as a loading control). Right panel: whole cell lysates from Mel-RM and Mel-RMu cells treated with PLX4720 (10 μM) 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 μM) 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 μM) for a further 16 h. Total RNA was isolated and subjected to real-time PCR analysis for Bim$_S$ 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 Bim$_{EL}$, Bim$_L$, and Bim$_S$ mRNA expression. Left panel: the ratios between the levels of Bim$_S$ mRNA and Bim$_{EL}$ mRNA before and after treatment, respectively, were calculated as ($\Delta\Delta C$t of Bim$_S$/$\Delta\Delta C$t of Bim$_{EL}$). Right panel: the ratios between the levels of Bim$_S$ mRNA and Bim$_L$ mRNA before and after treatment, respectively, were calculated as ($\Delta\Delta C$t of Bim$_S$/$\Delta\Delta C$t of Bim$_L$). 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 Bim and GAPDH (as a loading control). The arrowhead points to nonspecific bands generated by the antibody against Bim. The data shown are representative of three individual western blot analyses. (**h**) Mel-RMu and Mel-CV cells were transfected with the control, *SFRS6*, and *SFRS12* siRNA. After 24 h, cells were treated with PLX4720 (10 μM) for a further 72 h. Apoptosis was quantitated by the propidium iodide (PI) method. The data shown are the mean ± S.E. of three individual experiments. (**i**) Left panel: Mel-Rmu and Mel-CV cells were transfected with with pCMV6-AC-GFP or pCMV6-AC-SFRS6-GFP. After 48 h, cells were harvested and apoptosis was measured with PE-conjugated Annexin-V in gated GFP-positive cell populations. The data shown are representative of two experiments. Right panel: Mel-Rmu and Mel-CV cells were transfected with with pCMV6-AC-GFP or pCMV6-AC-SFRS6-GFP. After 24 h, whole cell lysates were subjected to western blot analysis of SRp55-GFP and Bim$_S$. The arrowhead points to nonspecific bands. Western blot analysis of GAPDH was then performed as a loading control. The data shown are representative of three individual western blot analyses

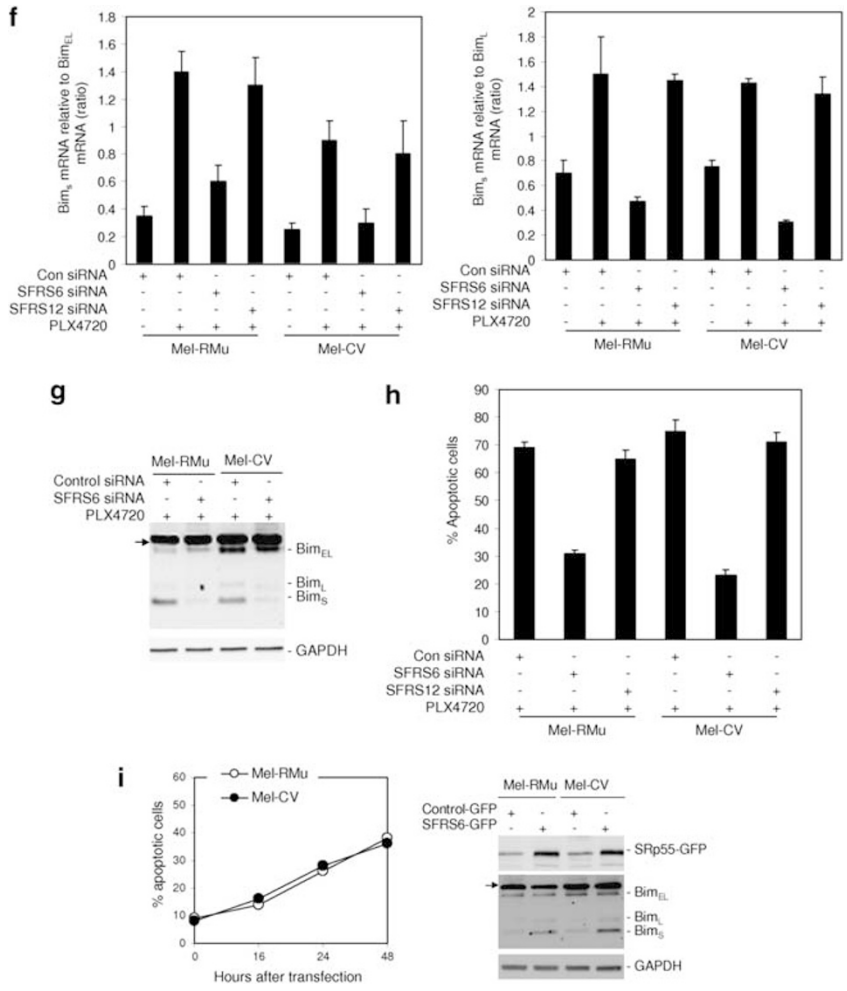

Figure 6 Continued

Bim$_S$, were also increased by PLX4720 in B-RAF^{V600E} 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 Bim$_S$ induced by PLX4720 is of particular interest, in that Bim$_S$ 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 Bim$_S$ is not subject to any posttranslational regulation and is instantly activated once it is expressed.[13] When overexpressed, Bim$_S$ 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 Bim$_S$ transcript was preferentially induced by PLX4720 in B-RAF^{V600E} melanoma cells. Moreover, the preferential induction of splicing to produce Bim$_S$ seemed to be specific for PLX4720, as treatment of B-RAF^{V600E} melanoma cells with the histone deacetylase inhibitor SBHA, which is known to increase transcription of Bim, did not cause selective upregulation of the Bim$_S$ transcript, although the levels of all three mRNA species were increased.[28] The preferential splicing to produce Bim$_S$ is of functional significance, in that specific inhibition of Bim$_S$ resulted in a greater degree of inhibition of PLX4720-induced killing than selective inhibition of Bim$_{EL}$.

The preferential induction of splicing of Bim$_S$ suggests that the mutant B-RAF^{V600E} may regulate Bim alternative splicing in melanoma cells, and in particular, may suppress splicing to produce Bim$_S$. This was supported by the finding that enforced expression of B-RAF^{V600E} in wild-type B-RAF melanoma cells blocked upregulation of the Bim$_S$ transcript by SBHA, but had no effect on upregulation of the Bim$_{EL}$ and Bim$_L$ mRNA. Furthermore, enforced expression of B-RAF^{V600E} in melanocytes resulted in decreases in the Bim$_S$ mRNA and protein, but intriguingly, the levels of the Bim$_L$ mRNA and protein were increased by enforced expression of B-RAF^{V600E} These results suggest that regulation of Bim splicing by mutant B-RAF is more complex than inhibition of splicing of Bim$_S$, and may vary between different cell types. It is of interest that, in contrast to melanoma cells, melanocytes expressed readily detectable levels of Bim$_S$ together with Bim$_{EL}$ and Bim$_L$. This may indicate that Bim$_S$ expression is

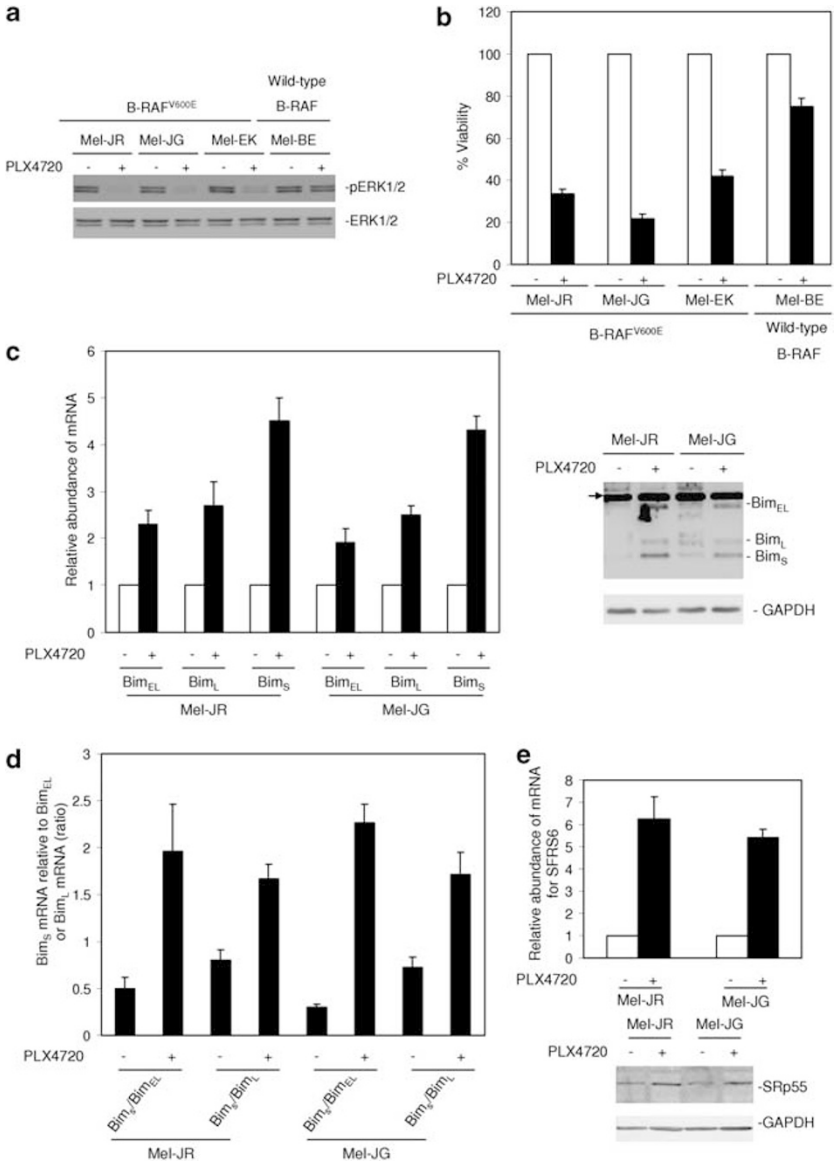

Figure 7 PLX4720 inhibits activation of ERK1/2, reduces cell viability, upregulates Bim$_S$, increases the ratios of Bim$_S$ mRNA to Bim$_{EL}$ and Bim$_L$ mRNA, and upregulates SRp55 in mutant B-RAF fresh melanoma isolates. (**a**) Whole cell lysates from fresh melanoma isolates harboring mutant B-RAF (Mel-JR, Mel-JG, and Mel-EK) or wild-type B-RAF (Mel-BE) treated with PLX4720 (10 μM) for 16 h were subjected to western blot analysis of pERK1/2 and ERK1/2. The data shown are representative of three individual western blot analyses. (**b**) Fresh melanoma isolates were treated with PLX4720 (10 μM) for 72 h before cell viability was quantitated by MTS assays. The data shown are the mean ± S.E. of three individual experiments. (**c**) Left panel: freshly isolated Mel-JR and Mel-JG cells were treated with PLX4720 (10 μM) for 16 h. Total RNA was isolated and subjected to real-time PCR analysis for Bim$_{EL}$, Bim$_L$, and Bim$_S$ mRNA expression. The levels of the expression of individual species before treatment were arbitrarily designated as 1. Right panel: whole cell lysates from freshly isolated Mel-JR and Mel-JG cells treated with PLX4720 (10 μM) for 16 h were subjected to western blot analysis of Bim and GAPDH (as a loading control). The arrowhead points to nonspecific bands. The data shown are either representative (right panel) or the mean ± S.E. (left panel) of three individual experiments. (**d**) Freshly isolated Mel-JR and Mel-JG cells were treated with PLX4720 (10 μM) for 16 h. Total RNA was isolated and subjected to real-time PCR analysis for Bim$_{EL}$, Bim$_L$, and Bim$_S$ mRNA expression. The ratios between the levels of Bim$_S$ mRNA and Bim$_{EL}$ mRNA before and after treatment, respectively, were calculated as ($\Delta\Delta Ct$ of Bim$_S$/$\Delta\Delta Ct$ of Bim$_{EL}$), and the ratios between the levels of Bim$_S$ mRNA and Bim$_L$ mRNA before and after treatment, respectively, were calculated as ($\Delta\Delta Ct$ of Bim$_S$/$\Delta\Delta Ct$ of Bim$_L$). The data shown are the mean ± S.E. of three individual experiments. (**e**) Upper panel: total RNA from Mel-JR and Mel-JG cells treated with PLX4720 as in **a** was subjected to real-time PCR analysis for *SFRS6* mRNA expression. The relative abundance of *SFRS6* mRNA in cells before treatment was arbitrarily designated as 1. Lower panel: whole cell lysates from Mel-JR and Mel-JG cells treated as given above were subjected to western blot analysis of SRp55 and GAPDH (as a loading control). The data shown are either representative (lower panel) or the mean ± S.E. (upper panel) of three individual experiments

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

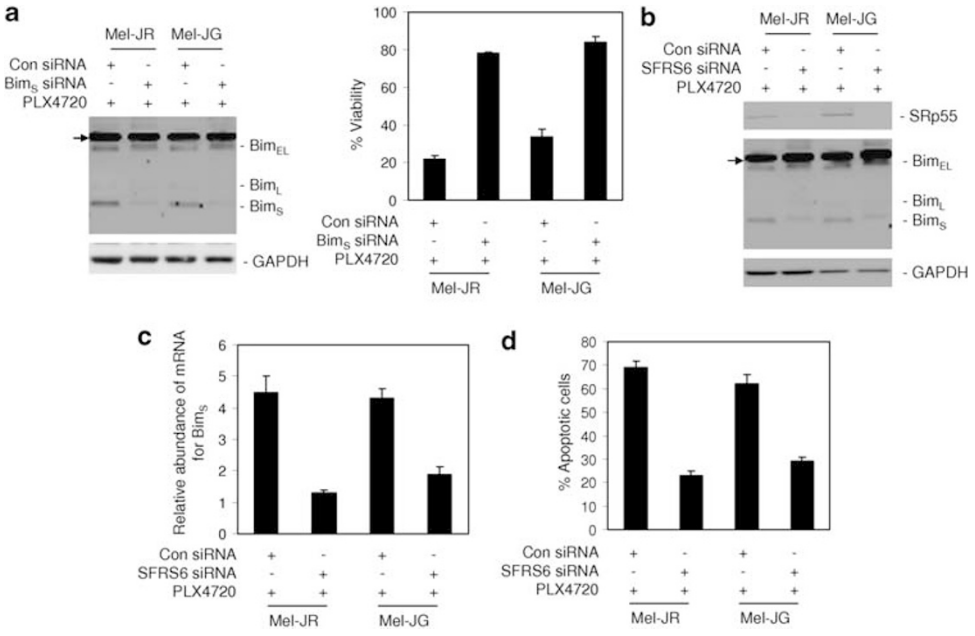

Figure 8 Inhibition of Bim_S by siRNA reverses the reduction in cell viability induced by PLX4720, whereas inhibition of SRp55 blocks induction of Bim_S and induction of apoptosis by PLX4720 in mutant B-RAF fresh melanoma isolates. (**a**) Left panel: Mel-JR and Mel-JG cells were transfected with the control and Bim_S 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_S 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. (right panel) 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_S mRNA expression. The relative abundance of Bim_S 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_S 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_S transcript and reversed the increases in ratios of the Bim_S mRNA to the Bim_{EL} and Bim_L 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^{V600E} 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_S.

The finding that killing of mutant B-RAF fresh melanoma isolates by PLX4720 was similarly associated with upregulation of Bim, in particular, Bim_S, 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_S as a key mechanism for induction of apoptosis by PLX4720 in melanoma cells carrying B-RAF^{V600E}. 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,[1] suggesting that the concentrations used in this study are achievable clinically.

Materials and Methods

Cell lines. Human melanoma cell lines Mel-RM, Me1007, Mel-RMu, MM200, 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 Plexxikon 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,

MA, USA). The rabbit polyclonal Abs against SFRS6, SFRS12, and β-actin were from Sigma-Aldrich. The mouse MAbs against cytochrome *c*, PARP were from Pharmingen (Bioclone, Marrickville, NSW, Australia). The rabbit polyclonal anti-Bax against amino acids 1 through to 20 was purchased from Upstate Biotechnology (Lake Placid, NY, USA). The mouse MAb against Bak (Ab-1) was purchased from Calbiochem (La Jolla, CA, USA). The mouse MAb against caspase-7 and the rabbit polyclonal Ab against caspase-3 were from Stressgen (Victoria, BC, Canada). The cell-permeable general caspase inhibitor Z-Val-Ala-Asp(OMe)-CH_2F (z-VAD-fmk) was purchased from Calbiochem. Isotype control Abs used were the ID4.5 (mouse IgG2a) MAb against *Salmonella typhi* supplied by Dr L Ashman (Institute for Medical and Veterinary Science, Adelaide, Australia), the 107.3 mouse IgG1 MAb purchased from PharMingen (San Diego, CA, USA), and rabbit IgG from Sigma-Aldrich .

Cell viability assays (MTS assays). The cytotoxic effect of PLX4720 on melanoma cells was determined using VisionBlue Fluorescence Cell Viability Assay Kit (Biovision Inc., Mountain View, CA, USA) as described previously.[38] Briefly, cells were seeded at 5000 cells per well onto flat-bottomed 96-well culture plates and allowed to grow for 24 h followed by the desired treatment. Cells were then labeled with the VisionBlue reagent and detected by Synergy two multi-detection microplate reader (BioTek, Winooski, VT, USA) according to the manufacturer's instructions.

Apoptosis. Quantitation of apoptotic cells by measurement of sub-G1 DNA content using the propidium iodide (Sigma-Aldrich) method or by Annexin-V staining was carried out as described elsewhere.[36,37]

Western blot analysis. Western blot analysis was carried out as described previously.[36,37] Labeled bands were detected by Immun-Star HRP Chemiluminescent Kit, and images were captured and the intensity of the bands was quantitated with the Bio-Rad VersaDoc image system (Bio-Rad, Regents Park, NSW, Australia).

Preparation of mitochondrial and cytosolic fractions. The mitochondrial and cytosolic fractions were prepared by using Qproteome Mitochondrial Isolation Kit (Qiagen, Doncaster, Vic, Australia) according to the manufacturer's instruction. In brief, after trypsinization, cells were washed with PBS and followed by 0.9% sodium chloride solution. Cell pellets were resuspended in ice-cold lysis buffer and incubated for 10 min at 4°C on an end-over-end shaker. After incubation, cell lysates were spun at $1000 \times g$ for 10 min at 4°C and the supernatant for cytosolic fraction was collected. Cell pellets were resuspended in disruption buffer, purification buffer, and followed by storage buffer with spins in between. The mitochondrial pellets were finally resuspended in lysis buffer for western blot analysis.

Plasmid vector and transfection. Stable Mel-RMu and Mel-CV transfectants of *Bcl-2* were established by electroporation of the pEF-puro vector carrying human Bcl-2 cDNA provided by Dr David Vaux (Walter and Eliza Hall Institute, Melbourne, Vic, Australia) and described elsewhere.[39] The pCDH-CMV-MCS-EF1-Puro (CD510B-1) vector carrying B-RAF^{V600E} cDNA was kindly provided by Professor Richard Marais (The Institute of Cancer Research, UK). The pCMV6-AC-Bim$_S$-GFP vector and the pCMV6-AC-SFRS6-GFP vector were purchased from OriGene (Rockville, MD, USA). Melanoma cells were seeded at 1×10^5 cells per well in 24-well plate, 24 h before transfection. Cells were transfected with 1 μg plasmid as well as the empty vector (Sigma-Aldrich) in Opti-MEM medium (Invitrogen, Carlsbad, CA, USA) with Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's protocol. At 6 h after transfection, the cells were switched into antibiotic-free medium containing 5% FCS for a further 24 h. Cells were then passaged at 1:10 ratio into fresh medium for further 24 h, followed by G418 or puromycin (Sigma-Aldrich) selection.

Real-time PCR. Real-time PCR was performed using the ABI Fast 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). For Bim mRNA detection, 25 μl mixture was used for reaction, which contains 5 μl cDNA sample (0.5–1 μg/μl), 300 nM forward primers for Bim 5′-TGCAGACATTTTG CTTGTTCAA-3′ and β-actin 5′-GGCACCCAGCACAATGAAG-3′, 300 nM reverse primers for Bim 5′-GAACCGCTGGCTGCATAATAAT-3′ and β-actin 5′-GCCGAT CCACACGGAGTACT-3′, 200 nM probes for Bim 6FAM-CCAACAAGACCCAG CACCGCG-TAMRA and β-actin 6FAM-TCAAGATCATTGCTCCTCCTGAGCGC-TAMRA, and 9 mM $MgCl_2$. 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 Bim$_{EL}$, Bim$_L$, and Bim$_S$, respectively. Bim$_{EL}$ forward primer is 5′-GTGGGTATTTCTCTTTTGACACAGAC-3′, Bim$_L$ forward primer is 5′-TACAGACAGAGCCACAAGACAG-3′, and common reverse primer for both Bim$_{EL}$ and Bim$_L$ is 5′-GTTCAGCCTGCCTCATGGAAG-3′; Bim$_S$ forward primer is 5′-TGACCGAGAAGGTAGACAAT-3′, Bim$_S$ reverse primer is 5′-GCCA TACAAATCTAAGCCAGT-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: Hs00740177_g1), 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 was included as a control. The threshold cycle value (*C*t) was normalized against 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-004383-01-0010), the siGENOME SMARTpool B-RAF (M-003460-03-0010), the siGENOME SMARTpool SFRS6 (M-016067-01-0010), and the siGENOME SMARTpool SFRS12 (M-016865-01-0010). The nontargeting siRNA control, SiConTRolNon-targeting SiRNA pool (D-001206-13-20) was also obtained from Dharmacon.

To specifically knockdown Bim$_{EL}$ and Bim$_S$, siRNAs were designed to target exons 3 and 5, which are unique to Bim$_{EL}$ and Bim$_S$, respectively. The oligonucleotides used were Bim$_{EL}$ sense 5′-CUGCUGUCUCGAUCCUCCAdTdT-3′, Bim$_{EL}$ antisense 5′-UGGAGGAUCGAGACAGCAGdTdT-3′; Bim$_S$ sense 5′-CAUAUGGUCA UUGGUGAUUdTdT-3′, Bim$_S$ antisense 5′-AAUCACCAAUGACCAUAUGdTdT-3′; control sense 5′-GGCUGUAACUUACGUGUACUUdTdT-3′, control antisense, 5′-AAGUACACGUAAGUUACAGCCdTdT-3′. Transfection of siRNA pools was carried out as described previously.[39]

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported by research grants from the NSW State Cancer Council, the Melanoma and Skin Cancer Research Institute Sydney, the Hunter Melanoma Foundation, NSW, and the National Health and Medical Research Council (NHMRC), Australia. XD Zhang, RF Thorne, and H Rizos are Cancer Institute NSW Fellows. We thank Dr. David Vaux (Walter and Eliza Hall Institute, Melbourne, Vic, Australia) for the pEF-puro vector carrying human Bcl-2 cDNA and Professor Richard Marais (The Institute of Cancer Research, UK) for the pCDH-CMV-MCS-EF1-Puro (CD510B-1) vector carrying B-RAF^{V600E} cDNA. We also greatly thank Dr. Gideon Bollag for supply of PLX4720.

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