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Suppression of PP2A is critical for protection of melanoma cells upon endoplasmic reticulum stress

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Endoplasmic reticulum (ER) stress triggers apoptosis by activating Bim in diverse types of cells, which involves dephosphorylation of Bim_{EL} by protein phosphatase 2A (PP2A). However, melanoma cells are largely resistant to ER stress-induced apoptosis, suggesting that Bim activation is suppressed in melanoma cells undergoing ER stress. We show here that ER stress reduces PP2A activity leading to increased ERK activation and subsequent phosphorylation and proteasomal degradation of Bim_{EL}. Despite sustained upregulation of Bim at the transcriptional level, the Bim_{EL} protein expression was downregulated after an initial increase in melanoma cells subjected to pharmacological ER stress. This was mediated by increased activity of ERK, whereas the phosphatase activity of PP2A was reduced by ER stress in melanoma cells. The increase in ERK activation was, at least in part, due to reduced dephosphorylation by PP2A, which was associated with downregulation of the PP2A catalytic C subunit. Notably, instead of direct dephosphorylation of Bim_{EL}, PP2A inhibited its phosphorylation indirectly through dephosphorylation of ERK in melanoma cells. Taken together, these results identify downregualtion of PP2A activity as an important protective mechanism of melanoma cells against ER stress-induced apoptosis.

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A number of cellular stress conditions, such as nutrient deprivation, hypoxia, alterations in glycosylation status, and disturbances of calcium flux, trigger accumulation and aggregation of unfolded and/or misfolded proteins in the endoplasmic reticulum (ER) lumen leading to so-called ER stress.[1,2] The ER responds to the stress conditions by activation of a range of signaling pathways to alter transcriptional and translational programs, which couples the ER protein folding load with the ER protein folding capacity and is termed the ER stress response or the unfolded protein response (UPR).[1,2] The UPR is fundamentally a cytoprotective response, but excessive or prolonged UPR resulting from irrevocable ER stress can cause apoptosis.[1–4] This involves many of the same molecules that have important roles in other apoptotic cascades.[3–5] Among them, the BH3-only protein Bim appears of particular importance because it mediates ER stress-induced apoptosis in diverse types of cells.[6]

Activation of Bim in cells undergoing ER stress is regulated by both transcriptional and post-translational mechanisms.[6] Transcriptional upregulation of Bim by ER stress is mediated by the transcription factor CHOP and its cofactor C/EBPα that form heterodimers, which activate a non-conventional promoter within the first intron of the *bim* gene.[6] At the post-translational level, ER stress triggers protein phosphatase 2A (PP2A)-mediated dephosphorylation of Bim, in particular, the most abundant Bim isoform, Bim_{EL}, which prevents its ubiquitination and proteasomal degradation, thus leading to its increase in expression.[6] Bim_{EL} is known to subject to phosphorylation by the MEK/ERK pathway that targets it for degradation.[7,8] It can also be phosphorylated by JNK resulting in its disassociation from the dynein light chain of the microtubule and induction of apoptosis.[7]

PP2A represents a family of serine/threonine phosphatases that regulate numerous intracellular signaling cascades.[9,10] Typically, PP2A contains a highly active core dimer composed of a catalytic C subunit (PP2A-C) and a structural A subunit (PP2A-A) that recruits one of multiple regulatory B subunits (PP2A-B) to form the PP2A heterotrimeric complex.[9,10] The substrate specificity of PP2A is determined by the B subunit in the complex, whereas the dynamic exchange of B subunits in the complex is regulated by reversible methylation and phosphorylation of the C-terminal tail of PP2A-C.[9,10] Noticeably, there is increasing evidence showing that PP2A has an important tumor-suppressive role, and various PP2A subunits has also been reported to be downregulated in a number of cancers including melanoma.[11–13]

Most cultured human melanoma cell lines are not sensitive to apoptosis induced by pharmacological ER stress inducers,[14,15] suggesting that melanoma cells have largely

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Abbreviations: ER, endoplasmic reticulum; OA, okadaic acid; 4-OHT, 4-hydroxytestosterone; PP2A, protein phosphatase 2A; PP2A-A, PP2A structural A subunit; PP2A-B, PP2A regulatory B subunit; PP2A-C, PP2A catalytic C subunit; TG, thapsigargin; TM, tunicamycin; UPR, unfolded protein response
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adapted to ER stress conditions. In support, the UPR is constitutively activated in melanoma cells *in vitro* and *in vivo*.[16,17] Although the adaptive mechanism(s) developed by melanoma cells are not fully understood, we have previously found that upregulation of the anti-apoptotic protein Mcl-1 is critical for survival of melanoma cells upon ER stress.[14] When Mcl-1 is inhibited, ER stress induces apoptosis by activation of the BH3-only proteins Noxa and PUMA.[14] In contrast, Bim does not have a role in ER stress-induced apoptosis of melanoma cells deficient in Mcl-1.[14] However, the mechanism by which Bim is inhibited in melanoma cells undergoing ER stress remains undefined.

We show in this report that the Bim_{EL} protein expression is rapidly downregulated after an initial increase, despite sustained upregulation of the Bim transcript, in melanoma cells submitted to ER stress, and that the decrease in Bim_{EL} is due to reduction in PP2A activity leading to increased activation of ERK and subsequent phosphorylation and proteasomal degradation of the protein. In addition, we demonstrate that, instead of directly dephosphorylating Bim, PP2A reduces Bim phosphorylation indirectly by dephosphorylation of ERK in melanoma cells.

Results

ER stress does not induce sustained upregulation of Bim_{EL} in melanoma cells. ER stress triggers apoptosis through activation of Bim in diverse types of cells,[6] but treatment with the ER stress inducer tunicamycin (TM) induced only a moderate, transient increase in the Bim_{EL} protein expression, which was followed by a decrease in Mel-RM cells (Figure 1a). By 36 h after treatment, the Bim_{EL} protein level was even lower than that in untreated cells (Figure 1a). The decrease in the Bim_{EL} expression at relatively late stages (24 and 36 h) after exposure to TM was also observed in another 3 melanoma cell lines (Figure 1b). In contrast, TM triggered sustained upregulation of Bim in MCF-7 breast cancer cells (Figure 1a).[6] Consistent with previous reports,[6,14,15] melanoma cells were relatively resistant ($<$20% apoptotic cells at 48 h after treatment), whereas MCF-7 cells were sensitive to TM-induced apoptosis (Figure 1c).

Downregulation of the Bim_{EL} protein after its initial increase by ER stress in melanoma cells was further confirmed with another ER stress inducer thapsigargin (TG) (Figure 1d). The ER stress-induced changes in the Bim_{EL} expression in melanoma cells appeared specific because, as reported before,[14] ER stress triggered sustained upregulation of the related Bcl-2 family proteins, Mcl-1 and PUMA, and caused no change in the expression of Bid and Bcl-X_L in melanoma cells (Supplementary Figure 1). Induction of ER stress by TM and TG was corroborated by upregulation of GRP78, CHOP, and the active form of the XBP1 mRNA (Figure 1e and Supplementary Figure 2).

Suppression of Bim has an important role in resistance of melanoma cells to ER stress-induced apoptosis. Having established that ER stress does not induce sustained upregulation of Bim_{EL} in melanoma cells, we examined whether this has a role in protecting melanoma cells from ER stress-induced apoptosis. To this end, we used a lentivirus-based inducible gene expression system to establish a Mel-RM sub-line (Mel-RM.Bim) to overexpress Bim_{EL} conditionally in response to 4-hydroxytestosterone (4-OHT) (Figure 2a).[18] Induction of Bim_{EL} by 4-OHT triggered apoptosis in Mel-RM.Bim cells (Figure 2b), which was markedly enhanced by cotreatment with TM (Figures 2a and b), suggesting that melanoma cells are sensitive to ER stress-induced apoptosis provided Bim is expressed at relatively high levels. The effect of overexpression of Bim_{EL} on sensitivity of melanoma cells to TM-induced apoptosis was confirmed in another two melanoma lines transiently transfected with a construct expressing Bim_{EL} (Figures 2c and d).

ER stress induces transcriptional upregulation of Bim in melanoma cells. Like in other cell types,[6] ER stress induced sustained upregulation of the Bim transcript in Mel-RM cells (Figure 3a). This upregulation was due to a transcriptional increase mediated by the transcription factor CHOP,[6] as small interference RNA (siRNA) knockdown of CHOP markedly inhibited the increase in Bim mRNA levels, partially recapitulating the effect of the transcription inhibitor actinomycin D (Figures 3b and c). Upregulation of Bim mRNA by ER stress was confirmed in another three melanoma cell lines (Supplementary Figure 3).

To examine whether disassociation between the Bim protein expression and its transcriptional upregulation is specific to melanoma cells under ER stress, we established a Mel-RM sub-line (Mel-RM.CHOP) that expressed CHOP conditionally in response to 4-OHT (Figure 3d). Induction of CHOP resulted in persistent increases in Bim at both the mRNA and protein levels and caused apoptosis (Figures 3d and e), suggesting that downregulation of the Bim protein after its initial upregulation despite its sustained transcriptional increase mediated by CHOP is specific to melanoma cells undergoing ER stress.

The decrease in Bim in melanoma cells undergoing ER stress is due to proteasomal degradation mediated by MEK/ERK signaling. Treatment with the proteasome inhibitor MG132 inhibited downregulation of the Bim_{EL} protein in Mel-RM cells exposed to TM (Figure 4a), indicating that its decrease in melanoma cells under ER stress is associated with proteasomal degradation. In support, protein half-life analysis showed that the Bim_{EL} turnover rate was more rapid in melanoma cells treated with TM compared with those without exposure to TM (Figure 4b), in contrast to the increased Bim_{EL} half-life time in MCF-7 cells undergoing ER stress (Supplementary Figure 4).[6] Similarly, the Bim_{EL} protein underwent a retarded mobility shift (slower migration in SDS-PAGE), consistent with increased phosphorylation of Bim_{EL},[7,8,19] in melanoma cells during ER stress (Figures 1a, b and d), whereas it underwent an opposite pattern of mobility shift (faster migration in SDS-PAGE) (Figure 1a), consistent with its dephosphorylation,[6] in MCF-7 cells. The increase in Bim_{EL} phosphorylation in melanoma cells undergoing ER stress was confirmed using an antibody that specifically recognizes Bim_{EL} phosphorylated at Ser69 (Figures 4c and d). Consistently, although there was an

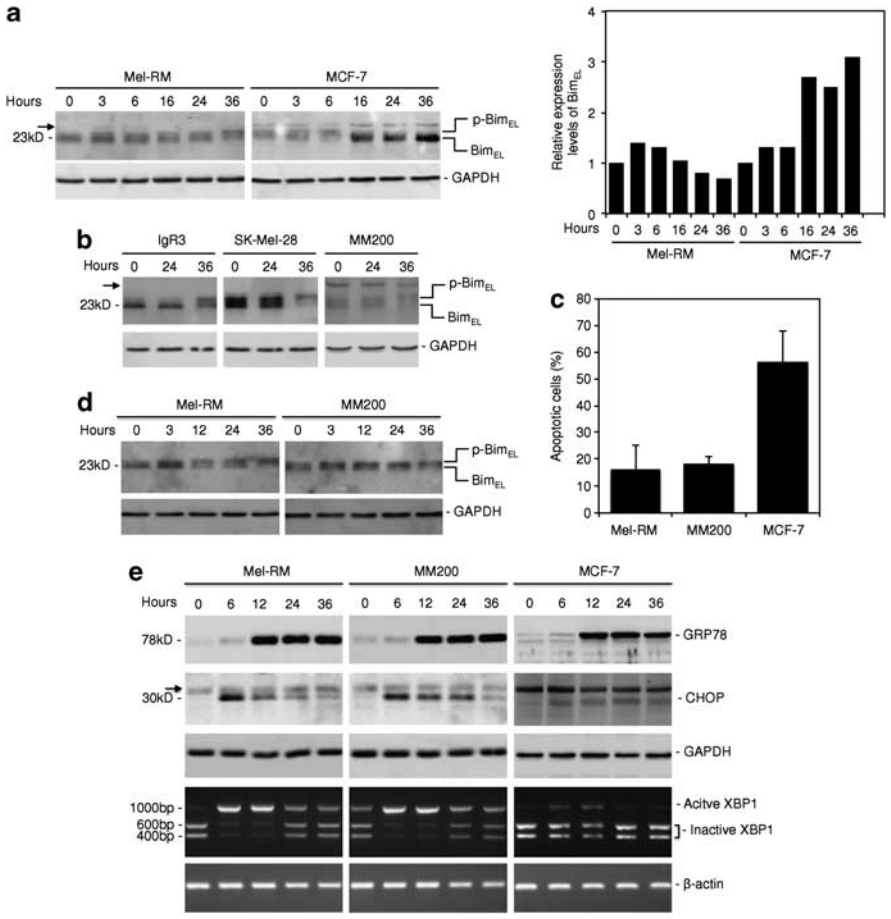

Figure 1 ER stress does not induce sustained upregulation of Bim$_{EL}$ in melanoma cells. (**a**) TM induces sustained upregulation of Bim$_{EL}$ in MCF-7 breast cancer cells but not in Mel-RM melanoma cells. Left: 30μg of total protein of whole-cell lysates from Mel-RM and MCF-7 cells treated with TM (3 μM) for indicated periods were subjected to western blot analysis of Bim and GAPDH (as a loading control). The arrowhead points to a band of unknown origin generated with the antibody against Bim. Right: quantitative expression levels of Bim$_{EL}$ as shown the left panel that were normalized to GAPDH. Quantitation of each band was carried out with ImageReader LAS-4000. The data shown are representative of three individual experiments. (**b**) TM does not induce sustained upregulation of Bim$_{EL}$ in IgR3, Sk-Mel-28, and MM200 melanoma cells. Thirty microgram of total protein of whole-cell lysates from IgR3, Sk-Mel-28, and MM200 treated with TM (3 μM) for indicated periods were subjected to western blot analysis of Bim and GAPDH (as a loading control). The arrowhead points to a band of unknown origin generated with the antibody against Bim. The data shown are representative of three individual experiments. (**c**) Melanoma cells are not sensitive to ER stress-induced apoptosis. Mel-RM and MM200 melanoma cells, and MCF-7 breast cancer cells were treated with TM (3 μM) for 48 h before apoptosis was measured by the PI method using flow cytometry. The data shown are the mean $\pm$ S.E. of three individual experiments. (**d**) TG does not induce sustained upregulation of Bim$_{EL}$ in melanoma cells. Thirty microgram of total protein of whole-cell lysates from Mel-RM and MM200 melanoma cells treated with TG (1 μM) for indicated periods were subjected to western blot analysis of Bim and GAPDH (as a loading control). The data shown are representative of three individual experiments. (**e**) Induction of ER stress by TM in Mel-RM and MM200 melanoma cells and MCF-7 breast cancer cells. Mel-RM, MM200, and MCF-7 cells were treated with TM (3 μM) for indicated periods. For examining the expression of GRP78 and CHOP, 30 μg of total protein of whole-cell lysates were subjected to western blot analysis of GRP78, CHOP, and GAPDH (as a loading control). The arrowhead points to a non-specific band generated with the antibody against CHOP. For examining activation of XBP1, RT-PCR products of XBP1 mRNA and β-actin mRNA (as a control) from total RNA extracts were digested with ApaLI for 90 min followed by electrophoresis. The longer fragment derived from the active form of XBP1 mRNA and two shorter bands derived from the inactive form are indicated. The data shown are representative of three individual experiments

increase in ubiquitination of Bim in melanoma cells after treatment with TM, Bim ubiquitination was reduced in MCF-7 cells undergoing ER stress as reported by others (Figure 4e).[6]

As MEK/ERK activity can phosphorylate Bim$_{EL}$ leading to its ubiquitination and proteasomal degradation,[7,8] we examined whether this is involved in Bim$_{EL}$ phosphorylation in melanoma cells upon ER stress. Treatment with the MEK inhibitor U0126 upregulated Bim$_{EL}$ in melanoma cells exposed to TM, which was associated with reduction in its phosphorylation (Figure 4f), indicating that MEK/ERK signaling has an important role in phosphorylation of Bim$_{EL}$ and downregulation of its expression. Notably, phosphorylation of ERK was increased, albeit moderately, by induction of ER stress (Figure 4f and Supplementary Figure 5). The effect of MEK/ERK signaling on phosphorylation of Bim in melanoma cells under ER stress was further confirmed by knockdown of MEK1 (Figure 4g). Moreover, the effect was similarly observed in additional two melanoma lines treated with U0126 plus TM (Supplementary Figure 6).

ER stress reduces the phosphatase activity of PP2A in melanoma cells. The PP2A is responsible for dephosphorylation of Bim and contributes to its upregulation in many

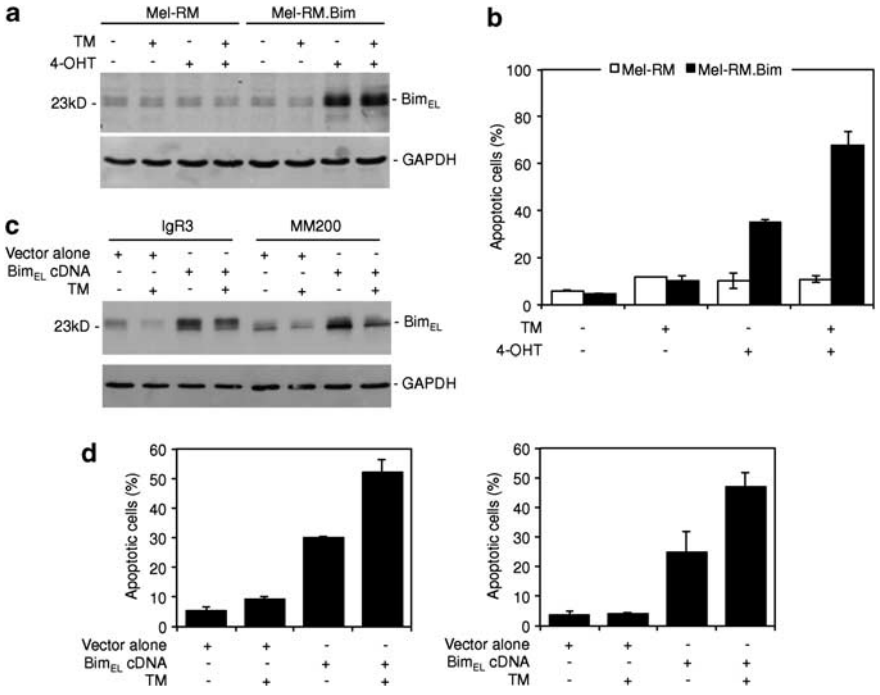

Figure 2 ER stress induces increased apoptosis when Bim_{EL} is expressed at high levels. (**a**) Induction of Bim_{EL} expression in Mel-RM.Bim cells that carried a lentivirus-based 4-OHT-responsive inducible Bim_{EL} expression system. Mel-RM cells and Mel-RM.Bim cells were treated with 4-OHT (10 nM), TM (3 μM), or 4-OHT plus TM for 24 h. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis of Bim and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses. (**b**) Induction of Bim_{EL} sensitizes Mel-RM.Bim to TM-induced apoptosis. Mel-RM cells and Mel-RM.Bim cells that carried a lentivirus-based 4-OHT-responsive inducible Bim_{EL} expression system were treated with 4-OHT (10 nM), TM (3 μM), or 4-OHT plus TM for 48 h. Apoptosis was measured by the PI method using flow cytometry. The data shown are the mean ± S.E. of three individual experiments. (**c**) Overexpression of Bim_{EL} in IgR3 and MM200 cells transiently transfected with cDNA encoding Bim_{EL}. IgR3 and MM200 cells were transfected with vector alone or Bim_{EL} cDNA. Twenty-four hours later, cells were treated with TM (3 μM) for a further 24 h. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis of Bim and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses. (**d**) Overexpression of Bim sensitizes IgR3 and MM200 cells to ER stress-induced apoptosis. IgR3 (left) and MM200 (right) cells were transfected with vector alone or Bim_{EL} cDNA. Twenty-four hours later, cells were treated with TM (3 μM) for a further 48 h. Apoptosis was measured by the PI method using flow cytometry. The data shown are the mean ± S.E. of three individual experiments

types of cells undergoing ER stress.[6] Indeed, treatment of Mel-RM cells with the pharmacological PP2A activator, FTY720 (fingolimod),[15] resulted in upregulation of Bim_{EL} in melanoma cells undergoing ER stress (Figure 5a and Supplementary Figure 7), whereas treatment with okadaic acid (OA) at a concentration that specifically inhibits PP2A led to increased phosphorylation of Bim_{EL} (Figures 5b and c),[6] indicating that PP2A has the similar regulatory effect on the Bim_{EL} expression in melanoma cells as in other cell types.[6] Nevertheless, phosphorylation of Bim was increased in melanoma cells undergoing ER stress (Figures 1a, b, d and 4c), suggesting that PP2A activity is repressed by ER stress in melanoma cells. This was confirmed by reduction in the phosphatase activity of PP2A in melanoma cells after treatment with TM, which was in contrast to the moderate increase in PP2A activity in MCF-7 cells induced by the same treatment (Figure 5d).

We monitored the expression of the catalytic C subunit of PP2A in Mel-RM in comparison with MCF-7 cells undergoing ER stress. Strikingly, PP2A-C was progressively downregulated in Mel-RM cells, whereas it was markedly increased in MCF-7 cells, after treatment with TM (Figure 5e). Downregulation of PP2A-C was also observed in another two melanoma lines (Figure 5e). The role of downregulation of PP2A-C in reduction of PP2A activity in melanoma cells under ER stress was confirmed by transfection of a PP2A-C-expressing construct into Mel-RM cells, which showed that overexpression of PP2A-C caused an increase in PP2A activity and upregulation of Bim_{EL} in cells with or without treatment with TM, and enhanced ER stress-induced apoptosis (Figures 5f and g, and Supplementary Figure 8).

Dephosphorylation of ERK is necessary for PP2A-mediated inhibition of Bim_{EL} phosphorylation in melanoma cells. Interestingly, treatment with FTY720 or overexpression of PP2A-C downregulated ERK phosphorylation, whereas exposure to OA caused an increase in the phosphorylated ERK (pERK) levels (Figures 5a, b and f), suggesting that PP2A has a role in dephosphorylating ERK in melanoma cells. In contrast, neither FTY720 nor OA caused noticeable changes in the ERK phosphorylation status in MCF-7 cells (Figures 5a and b). These results raised a possibility that dephosphorylation of ERK may have an indirect role in PP2A-mediated inhibition of Bim_{EL} phosphorylation in melanoma cells,[7,8] even though it is known that PP2A directly targets Bim_{EL} for dephosphorylation in MCF-7 cells and other types of cells.[6]

Although the role of MEK/ERK signaling in phosphorylation of Bim_{EL} in melanoma cells under ER stress had been established by inhibition of MEK with U0126 and siRNA

knockdown of MEK1 (Figures 4e and f), we sought to further clarify the role of dephosphorylation of ERK in PP2A-mediated inhibition of Bim phosphorylation in melanoma cells by knocking down ERK1/2 with siRNA in Mel-RM cells. As knockdown of ERK1/2 alone markedly inhibited phosphorylation Bim_{EL} (Figure 6a), it was not feasible to test the effect of combination of activation of PP2A and knockdown of ERK1/2 on the Bim_{EL} phosphorylation status. Nevertheless, inhibition of ERK by siRNA partially blocked phosphorylation of Bim induced by OA, in particular, in the presence of TM (Figure 6a), suggesting that PP2A-mediated dephosphorylation of Bim is associated with its dephosphorylating effect on ERK. Consistently, treatment of melanoma cells with U0126 attenuated phosphorylation of Bim_{EL} by OA (Figure 6b). Of note, OA partially restored the levels of pERK in Mel-RM cells with ERK knockdown by siRNA, suggesting that the minimal residue of ERK was phosphorylated when PP2A was inhibited (Figure 6a). Similarly, OA caused partial recovery in ERK phosphorylation in melanoma cells treated with U0126 (Figure 6b). These results further substantiate the effect of PP2A on dephosphorylation of ERK in melanoma cells.

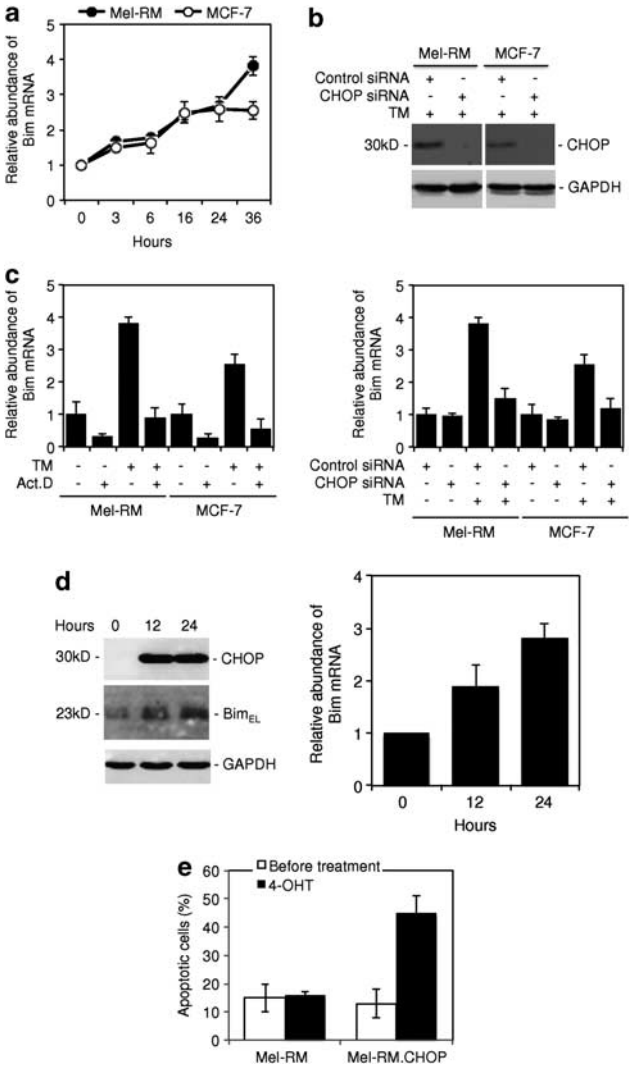

Figure 3 ER stress induces transcriptional upregulation of Bim in melanoma cells. (**a**) TM induces sustained upregulation of Bim mRNA in melanoma cells. Mel-RM melanoma cells and MCF-7 breast cancer cells were treated with TM (3 μM) for indicated periods before total RNA was isolated and subjected to qPCR analysis for the Bim mRNA expression. The relative abundance of the Bim mRNA in each cell line before treatment was arbitrarily designated as 1. The data shown are the mean ± S.E. of three individual experiments. (**b**) CHOP is required for transcriptional upregulation of Bim by ER stress in melanoma cells. Upper panel: Mel-RM melanoma cells and MCF-7 breast cancer cells were transfected with the control or CHOP siRNA. Twenty-four hours later, cells were treated with TM (3 μM) for a further 6 h. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis of CHOP and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses. Lower panel: Mel-RM melanoma cells and MCF-7 breast cancer cells were transfected with the control or CHOP siRNA. Twenty-four hours later, cells were treated with TM (3 μM) for a further 36 h before total RNA was isolated and subjected to qPCR analysis for the Bim mRNA expression. The relative abundance of the Bim mRNA in each cell line transfected with the control siRNA without treatment was arbitrarily designated as 1. The data shown are the mean ± S.E. of three individual experiments. (**c**) The general transcription inhibitor actinomycin D (Act.D) efficiently blocks transcriptional upregulation of Bim in Mel-RM melanoma cells and MCF-7 breast cancer cells. Mel-RM and MCF-7 cells were treated with Act.D (100 ng/ml) for 1 h before the addition of TM (3 μM) for a further 24 h. Total RNA was then isolated and subjected to qPCR analysis for the Bim mRNA expression. The relative abundance of the Bim mRNA in each cell line before treatment was arbitrarily designated as 1. The data shown are the mean ± S.E. of three individual experiments. (**d**) Overexpression of CHOP induces upregulation of the Bim mRNA and the Bim_{EL} protein in melanoma cells. Left panel: Mel-RM.CHOP cells that carried a lentivirus-based 4-OHT-responsive inducible CHOP expression system were treated with 4-OHT (10 nM) for indicated periods. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis of CHOP, Bim, and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses. Right panel: Mel-RM.CHOP cells that carried a lentivirus-based 4-OHT-responsive inducible CHOP expression system were treated with 4-OHT (10 nM) for indicated periods. Total RNA was isolated and subjected to qPCR analysis for the Bim mRNA expression. The relative abundance of the Bim mRNA in cells without 4-OHT treatment was arbitrarily designated as 1. The data shown are the mean ± S.E. of three individual experiments. (**e**) Overexpression of CHOP induces apoptosis in melanoma cells. Mel-RM.CHOP cells that carried a lentivirus-based 4-OHT-responsive inducible CHOP expression system were treated with 4-OHT (10 nM) for 48 h before apoptosis was measured by the PI method using flow cytometry. The data shown are the mean ± S.E. of three individual experiments

PP2A is physically associated with ERK but not Bim in melanoma cells. PP2A exerts its phosphatase effect by physical interaction with its substrates.[20,21] We therefore examined the potential physical association of PP2A with ERK and Bim_{EL} in melanoma cells in comparison with MCF-7 cells. Strikingly, although Bim_{EL} was readily co-precipitated with PP2A-C in protein extracts from MCF-7 cells under ER stress, no Bim_{EL} was detected in PP2A-C precipitates from Mel-RM cells with or without treatment with TM (Figure 6c). In contrast, little ERK was co-precipitated with PP2A in MCF-7 cells, but it was readily co-precipitated with PP2A in Mel-RM cells with or without undergoing ER stress (Figure 6d). These results further suggest that, in contrast to dephosphorylating Bim directly,[6] PP2A inhibits phosphorylation of Bim indirectly through dephosphorylating ERK in melanoma cells.

Discussion

Past studies have shown that upregulation of the anti-apoptotic protein Mcl-1 is critical for survival of melanoma

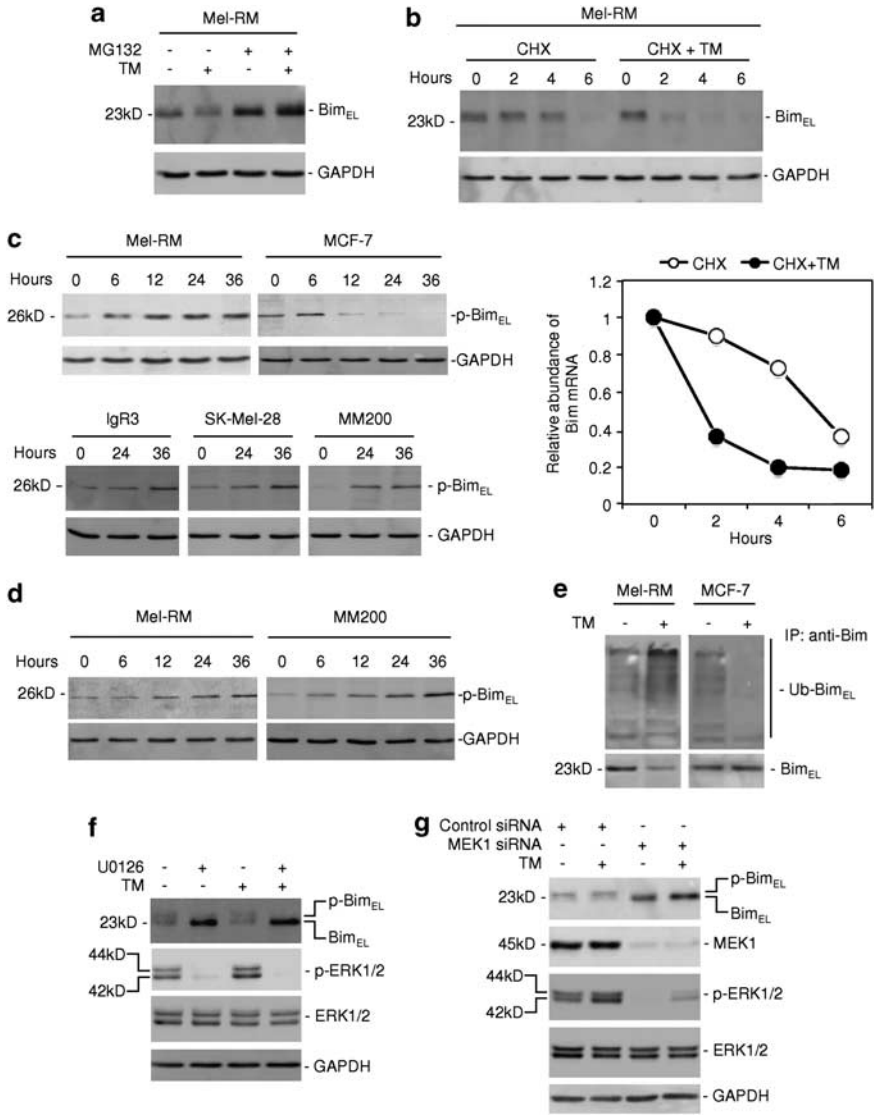

Figure 4 Downregulation of the Bim$_{EL}$ protein in melanoma cells undergoing ER stress is mediated by MEK/ERK signaling. (**a**) The proteasome inhibitor MG132 reverses downregulation of the Bim$_{EL}$ in melanoma cells under ER stress. Mel-RM cells with or without pretreatment with the proteasome inhibitor MG132 (10 μM) for 1 h were treated with TM (3 μM) for 24 h. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis of Bim and GAPDH (as a loading control). The data shown are representative of three individual experiments. (**b**) ER stress accelerates the turnover rate of Bim$_{EL}$ in melanoma cells. Upper panel: Mel-RM cells were treated with the protein synthesis inhibitor cycloheximide (CHX) (10 μg/ml) with or without the addition of TM (3 μM) for indicated periods. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis of Bim and GAPDH (as a loading control). The data shown are representative of three individual experiments. Lower panel: quantitative expression levels of Bim$_{EL}$ as shown the upper panel that were normalized to GAPDH. Quantitation of each band was determined using ImageReader LAS-4000. The data shown are representative of three individual experiments. (**c**) TM phosphorylates Bim$_{EL}$ in melanoma cells but dephosphorylates it in MCF-7 cells. Mel-RM and MCF-7 (upper panel) and IgR3, Sk-Mel-28, and MM200 (lower panel) cells were treated TM (3 μM) for indicated periods. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis using an antibody that specifically recognizes Bim$_{EL}$ phosphorylated at Ser69. Western blot analysis of GAPDH was included as a loading control. The data shown are representative of three individual experiments. (**d**) TG induces phosphorylation of Bim$_{EL}$ in melanoma cells. Mel-RM and MM200 cells were treated with TG (1 μM) for indicated periods. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis using an antibody that specifically recognizes Bim$_{EL}$ phosphorylated at Ser69. Western blot analysis of GAPDH was included as a loading control. The data shown are representative of three individual experiments. (**e**) TM increases an increase in ubiquitination of Bim$_{EL}$ in Mel-RM melanoma cells, but a decrease in MCF-7 breast cancer cells. Whole-cell lysates from Mel-RM melanoma cells and MCF-7 breast cancer cells with or without treatment with TM (3 μM) for 36 h were subjected to immunoprecipitation using an antibody against Bim. Thirty microgram of total protein of the resulting precipitates were subjected to SDS-PAGE and probed with an antibody against ubiquitin and an antibody against Bim. The data shown are representative of three individual experiments. (**f**) The MEK inhibitor U0126 inhibits phosphorylation of Bim$_{EL}$ and increases its expression in melanoma cells undergoing ER stress. Thirty microgram of total protein of whole-cell lysates from Mel-RM cells treated with U0126 (20 μM), TM (3 μM), or U0126 plus TM for 24 h were subjected to western blot analysis of Bim, pERK, ERK, and GAPDH (as a loading control). The data shown are representative of three individual experiments. (**g**) Knockdown of MEK1 by siRNA inhibits phosphorylation of Bim$_{EL}$ and increases its expression in melanoma cells undergoing ER stress. Mel-RM cells were transfected with the control or MEK1 siRNA. Twenty-four hours later, cells were treated with TM (3 μM) for a further 24 h. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis of Bim, MEK1, pERK (pERK), ERK, and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses

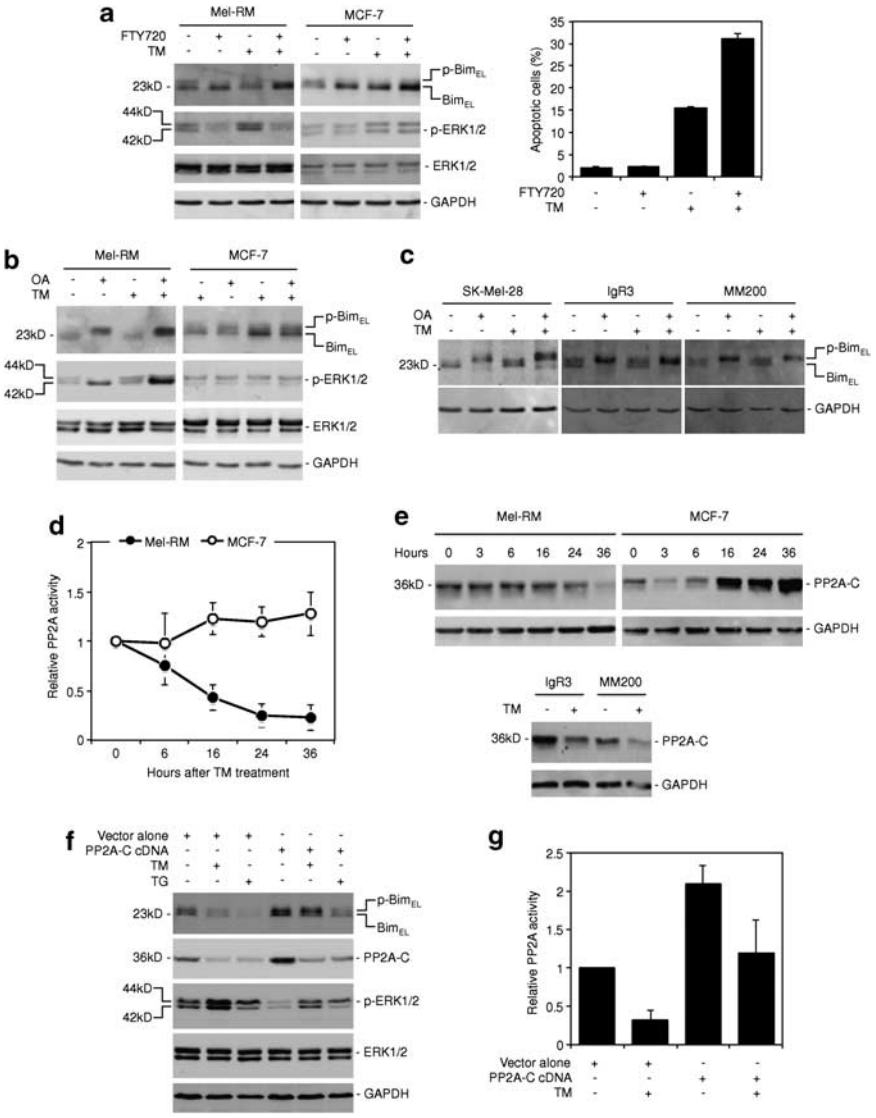

Figure 5 Downregulation of the Bim$_{EL}$ protein in melanoma cells under ER stress is associated with reduction in PP2A activity that is, at least in part, due to downregulation of the PP2A catalytic C subunit (PP2A-C). (**a**) Pharmacological activation of PP2A reverses downregulation of Bim$_{EL}$ by ER stress in melanoma cells. Left panel: 30 μg of total protein of whole-cell lysates from Mel-RM melanoma cells or MCF-7 breast cancer cells treated with the pharmacological PP2A activator FTY720 (2.5 μM), TM (3 μM), or FTY720 plus TM for 24 h were subjected to western blot analysis of Bim, pERK, ERK, and GAPDH (as a loading control). The data shown are representative of three individual experiments. Right panel: Mel-RM cells were treated with FTY720 (2.5 μM), TM (3 μM), or FTY720 plus TM for 48 h before apoptosis was measured by the PI method using flow cytometry. The data shown are the mean $\pm$ S.E. of three individual experiments. (**b**) Inhibition of PP2A further promotes phosphorylation of Bim$_{EL}$ and increases phosphorylation of ERK in Mel-RM cells under ER stress. Thirty microgram of total protein of whole-cell lysates from Mel-RM melanoma cells or MCF-7 breast cancer cells treated with the OA (50 nM), TM (3 μM), or OA plus TM for 24 h were subjected to western blot analysis of Bim, pERK, ERK, and GAPDH (as a loading control). The data shown are representative of three individual experiments. (**c**) Inhibition of PP2A further promotes phosphorylation of Bim$_{EL}$ in Sk-Mel-28, IgR3, and MM200 melanoma cells. Thirty microgram of total protein of whole-cell lysates from Sk-Mel-28, IgR3, and MM200 cells treated with the OA (50 nM), TM (3 μM), or OA plus TM for 24 h were subjected to western blot analysis of Bim and GAPDH (as a loading control). The data shown are representative of three individual experiments. (**d**) ER stress reduces PP2A activity in Mel-RM melanoma cells, but increases PP2A activity in MCF-7 breast cancer cells. Mel-RM cells and MCF-7 cells were treated with TM (3 μM) for indicated periods before the phosphatase activity of PP2A was quantitated using a PP2A-C immunoprecipitation phosphatase assay kit. The PP2A phosphatase activity in cells without treatment was arbitrarily designated as 1. The data shown are the mean $\pm$ S.E. of three individual experiments. (**e**) ER stress downregulates PP2A-C in melanoma cells but upregulates its expression in MCF-7 breast cancer cells. Upper panel: Thirty microgram of total protein of whole-cell lysates from Mel-RM melanoma cells or MCF-7 breast cancer cells treated with TM (3 μM) for indicated periods were subjected to western blot analysis of PP2A-C and GAPDH (as a loading control). The data shown are representative of three individual experiments. Lower panel: Thirty microgram of total protein of whole-cell lysates from IgR3 and MM200 melanoma cells treated with TM (3 μM) for 24 h were subjected to western blot analysis of PP2A-C and GAPDH (as a loading control). The data shown are representative of three individual experiments. (**f**) Overexpression of PP2A-C upregulates Bim$_{EL}$ and decrease ERK phosphorylation in melanoma cells. Mel-RM cells were transiently transfected with vector alone or cDNA encoding PP2A-C. Twenty-four hours later, cells were treated with TM (3 μM) or TG (1 μM) for a further 24 h. Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis of Bim, PP2A-C, pERK, ERK, and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses. (**g**) Overexpression of PP2A-C causes increased PP2A activity in melanoma cells with or without undergoing ER stress. Mel-RM cells were transiently transfected with vector alone or cDNA encoding PP2A-C. Twenty-four hours later, cells were treated with TM (3 μM) for a further 24 h. The phosphatase activity of PP2A was then quantitated using a PP2A-C immunoprecipitation phosphatase assay kit. The PP2A phosphatase activity in cells transfected with vector alone without TM treatment was arbitrarily designated as 1. The data shown are the mean $\pm$ S.E. of three individual experiments

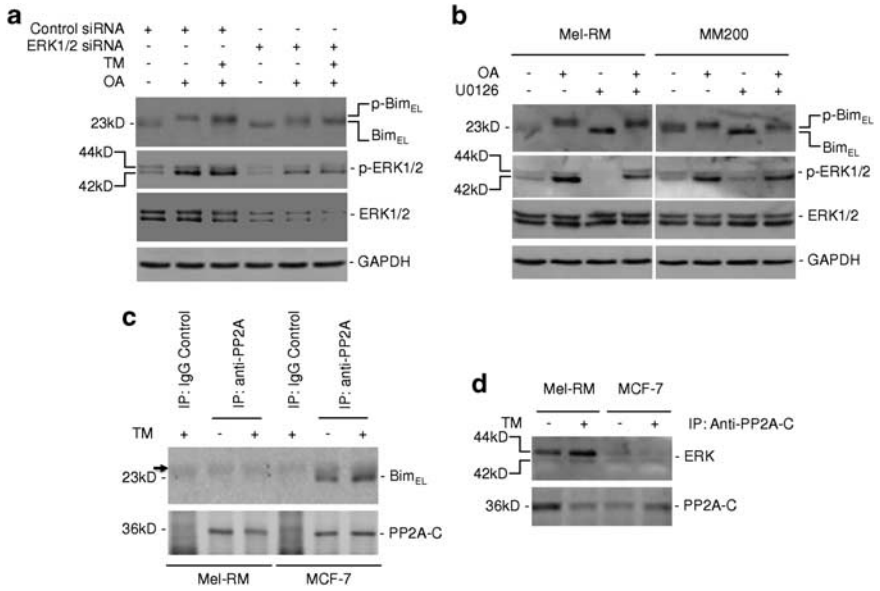

Figure 6 PP2A inhibits Bim_{EL} phosphorylation through dephosphorylation of ERK in melanoma cells. (**a**) Knockdown of ERK1/2 blocks phosphorylation of Bim_{EL} in melanoma cells with or without undergoing ER stress. Mel-RM cells were transfected with the control siRNA or ERK1 siRNA plus ERK2 siRNA. Twenty-four hours later, cells were treated with OA (50 nM) for 24 h in the presence or absence of TM (3 μM). Thirty microgram of total protein of whole-cell lysates were then subjected to western blot analysis of Bim, pERK, ERK, and GAPDH (as a loading control). The data shown are representative of three individual western blot analyses. (**b**) The MEK inhibitor U0126 blocks phosphorylation of Bim_{EL} mediated by OA in melanoma cells. Thirty microgram of total protein of whole-cell lysates from Mel-RM and MM200 cells treated with OA (50 nM), U0126 (20 μM), or OA plus U0126 for 24 h were subjected to western blot analysis of Bim, pERK, ERK, and GAPDH (as a loading control). The data shown are representative of three individual experiments. (**c**) PP2A-C is physically associated with Bim_{EL} in MCF-7 breast cancer cells but not in melanoma cells. Whole-cell lysates from Mel-RM melanoma cells with or without treatment with TM (3 μM) for 6 h and those from MCF-7 breast cancer cells with or without treatment with TM (3 μM) for 16 h were subjected to immunoprecipitation with a mouse antibody against PP2A-C. Immunoprecipitation of whole-cell lysates from cells treated with TM with purified mouse IgG was included as a control. Thirty microgram of total protein of the resulting precipitates were subjected to SDS-PAGE and probed with an antibody against Bim and an antibody against PP2A-C. The data shown are representative of three individual experiments. (**d**) PP2A-C is physically associated with ERK in melanoma cells but not in MCF-7 breast cancer cells. Whole-cell lysates from Mel-RM melanoma cells and MCF-7 breast cancer cells with or without treatment with TM (3 μM) for 6 h were subjected for immunoprecipitation with a mouse antibody against PP2A-C. Thirty microgram of total protein of the resulting precipitates were subjected to SDS-PAGE and probed with an antibody against ERK and an antibody against PP2A-C. The data shown are representative of three individual experiments

cells upon ER stress.[14] When Mcl-1 is inhibited, ER stress induces apoptosis of melanoma cells by activation of the BH3-only proteins Noxa and PUMA, whereas Bim, which is responsible for induction of apoptosis by ER stress in many other cell types, is not involved.[14] Nevertheless, Bim has an important role in killing of melanoma cells by other apoptotic stimuli such as histone deacetylase inhibitors and inhibitors of the RAF/MEK/ERK pathway.[19,22,23] These results collectively suggest that Bim is suppressed in melanoma cells under ER stress. In this study, we identify reduction in PP2A activity as an important mechanism to keep Bim_{EL} in check by enhancing its post-translational degradation in melanoma cells subjected to pharmacological ER stress inducers.

We found that, in agreement with observations in a number of other cell types including MCF-7 cells, the Bim transcript was increased in melanoma cells by ER stress through the transcription factor CHOP.[6] However, in contrast to the sustained increase in the Bim mRNA, the Bim_{EL} protein was downregulated after an initial increase in melanoma cells upon ER stress. This downregulation was important in protection of melanoma cells, as ER stress induced markedly increased apoptosis when Bim was overexpressed. These results, along with our previous finding that PUMA and Noxa that are upregulated in melanoma cells upon ER stress are inhibited by Mcl-1,[9] demonstrated that melanoma cells have developed multiple mechanisms to maintain the balance between pro- and anti-apoptotic Bcl-2 family proteins to survive ER stress.[14,15,24,25]

Downregulation of Bim at the protein level despite the sustained increase in its transcript mediated by CHOP is specific to melanoma cells under ER stress, as overexpression of CHOP resulted in upregulation of the Bim mRNA as well as the Bim_{EL} protein. It appears therefore that in response to ER stress, melanoma cells activate a powerful post-transcriptional mechanism(s) leading to robust suppression of the Bim_{EL} protein expression. Indeed, proteasomal degradation of the Bim_{EL} protein was accelerated in melanoma cells treated with TM. This was in sharp contrast to the observation in MCF-7 cells in which the half-life time of the protein was prolonged upon ER stress.[6] Consistently, although phosphorylation and ubiquitination of Bim_{EL} were reduced in MCF-7 cells,[6] these post-translational modifications of the protein were enhanced in melanoma cells under ER stress.

Bim_{EL} can be phosphorylated by protein kinases such as ERK and JNK and dephosphorylated by the PP2A.[7,8] The latter is of particular importance in cells under ER stress as PP2A-mediated dephosphorylation of Bim has an essential role in upregulation of Bim in many types of cells undergoing ER stress.[6] Strikingly, while ERK activation was increased and had an essential role in phosphorylation of Bim_{EL} in

melanoma cells subjected to ER stress, the phosphatase activity of PP2A was reduced by ER stress in melanoma cells. This suggests that the increased phosphorylation and subsequent proteasomal degradation of Bim_{EL} in melanoma cells under ER stress is caused by the predominant phosphorylating effect of ERK that overrides the dephosphorylating effect of PP2A on the protein. We did not observe a role of JNK in phosphorylation of Bim by ER stress in melanoma cells (data not shown), but whether other protein kinases such as PKA are involved remains to be defined.[26,27]

ER stress-triggered reduction in PP2A activity seems to be highly specific to melanoma cells in that PP2A activity is increased in many other cell types by ER stress.[6,28,29] In the search for the mechanism(s) responsible for the differential regulation of PP2A by ER stress, we found that PP2A-C was progressively decreased in melanoma cells, but markedly increased in MCF-7 cells, after treatment with TM. Introduction of a PP2A-C-expressing construct increased PP2A activity not only in healthy melanoma cells, but also in those subjected to ER stress, indicating that downregulation of PP2A-C is responsible, at least in part, for suppression of PP2A activity in melanoma cells. Similar to the pharmacological PP2A activator, overexpression of PP2A-C reversed downregulation of Bim_{EL} in melanoma cells under ER stress, confirming the importance of reduction in PP2A activity in ER stress-induced suppression of Bim in melanoma cells.

A question remaining unaddressed is how PP2A-C is selectively downregulated by ER stress in melanoma cells. Similarly, it is unclear whether additional mechanisms such as possible alterations in the expression of other PP2A subunits are involved in regulating PP2A activity in cells undergoing ER stress. Mutations have been identified in different components of the PP2A complex, which have been linked to a variety of human cancers.[11–13] In particular, a truncated form of PP2A-B56γ has been found in the mouse melanoma cell line B16 that is not able to dephosphorylate specific targets and has a role in malignant progression.[13] Although no mutations have been found in patients, the expression of the *PP2A-B56*γ gene is frequently reduced in human melanoma compared to naevi.[30] Similarly, PP2A-B56α has recently been shown to be expressed at lower levels in metastatic compared with primary melanomas.[31] Regardless, our results clearly demonstrated that reduction in PP2A activity associated with downregulation of PP2A-C is an important mechanism triggered by ER stress in melanoma cells to suppress the Bim_{EL} expression. Notably, despite the reduction in its activity, PP2A retained part of its dephosphorylating effect on Bim, in that treatment with OA caused an increase in Bim_{EL} phosphorylation in melanoma cells treated with TM. Nevertheless, this residual effect was apparently not adequate to supersede the phosphorylating effect of ERK on Bim_{EL} to accumulate the protein to such a level required for efficient induction of apoptosis.

Activation of the MEK/ERK pathway has been well documented to protect cells from ER stress-induced apoptosis.[25,32] However, how ER stress triggers activation of the pathway remains undefined. We found that the increase in ERK activation in melanoma cells under ER stress was closely associated reduction in PP2A activity, which otherwise directly targets ERK for dephosphorylation. This was demonstrated by (1) activation of PP2A by FTY720 or overexpression of PP2A-C caused downregulation of pERK in melanoma cells with or with being subjected to ER stress; (2) inhibition of PP2A by OA upregulated ERK phosphorylation, in particular, in melanoma cells treated with TM; and (3) ERK was readily co-precipitated with PP2A-C in melanoma cells with or without subjected to ER stress. In contrast, we did not observe any noticeable effect of PP2A on ERK activation in MCF-7 cells. On the other hand, although PP2A directly targets Bim for dephosphorylation in MCF-7 and a number of other types of cells undergoing ER stress,[6] it did not appear to directly act on Bim in melanoma cells with or without subject to ER stress. Instead, the inhibitory effect of PP2A on Bim phosphorylation was mediated indirectly by its dephosphorylating effect on ERK. This was suggested by reduced phosphorylation of Bim_{EL} mediated by OA when ERK was inhibited, and was further supported by the lack of physical association between Bim_{EL} and PP2A in melanoma cells. It is known that the B subunit in the PP2A heterotrimeric complex dictates the substrate specificity of PP2A, and that the dynamic exchange of PP2A-B in the complex is regulated by reversible methylation and phosphorylation of the C-terminal tail of the C subunit, involving the PP2A specific methyelsterase PME1 and methyltransferase, LCMT1.[9,10] It is conceivable that the differential effects of PP2A on ERK and Bim are associated with cell type- and context-dependent recruitment of different B subunits into the PP2A heterotrimeric complex, but the mechanism responsible for this remains to be defined.[9,10,33,34]

In conclusion, we have shown in this study that reduction in PP2A activity is an important mechanism responsible for activation of ERK, downregulation of Bim_{EL}, and resistance to apoptosis in melanoma cells undergoing ER stress. This reduction is, at least in part, due to downregulation of PP2A-C. Moreover, we have demonstrated that the inhibitory effect of PP2A on Bim_{EL} phosphorylation is predominantly mediated by its dephosphorylating effect on ERK (Supplementary Figure 9). These results suggest that pharmacological activation of PP2A may improve treatment results of agents that induce ER stress in melanoma cells such as cisplatin and sorafenib.[35,36] In addition, the dephosphorylating effect of PP2A on ERK in melanoma cells may also have important implications in improving efficacy of targeting the RAF/MEK/ERK pathway, which is a promising strategy in the treatment of metastatic melanoma.[37,38]

Materials and Methods

Cell lines. The human melanoma cell lines Mel-RM, MM200, IgR3, and SK-Mel-28 and the human breast cancer cell line MCF-7 were described previously.[14,39] They were cultured in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, VIC, Australia).

Antibodies, recombinant proteins, and other reagents. The rabbit polyclonal antibodies (pAbs) against Bim was supplied by Imgenex (San Diego, CA, USA); the mouse monoclonal antibodies (mAbs) against CHOP, pERK, Mcl-1, and Bcl-X_L and the rabbit pAb against GRP78 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the rabbit pAbs against phosphorylated Bim, ERK, Bid, and PUMA and the mouse mAbs against ubiquitin were from Cell Signaling Technology (Beverly, MA, USA); the mouse mAb against GAPDH was from Ambion (Austin, TX, USA). The restriction enzyme ApaLI was purchased from New England Biolabs (Ipswich, MA, USA). The PP2A inhibitor OA,

Actinomycin D, TM, and TG were purchased from Sigma-Aldrich (St. Louis, MO, USA). The PP2A activator FTY720 and MG132 were from Cayman Chemicals (Ann Arbor, MI, USA). The MEK inhibitor U0126 was from Promega (Madison, WI, USA). Cycloheximide was purchased from Calbiochem (La Jolla, CA, USA).

Apoptosis analysis. Apoptotic cells were quantified by measurement of sub-G1 DNA content using propidium iodide (PI) on a flow cytometer (Becton Dickinson, Sunnyvale, CA, USA) as described elsewhere.[40]

Western blot analysis. Western blot analysis was carried out as described previously.[40] Labeled bands were detected by Luminata Crescendo Western HRP substrate (Millipore, Billerica, MA, USA) and images were captured and the intensity of the bands was quantitated with ImageReader LAS-4000 (Fujifilm Corporation, Tokyo, Japan).

Immunoprecipitation. Immunoprecipitation experiments were carried out as described previously.[40]

PP2A phosphatase activity assay. PP2A activity was determined using the PP2A immunoprecipitation phosphatase assay kit (Millipore). Briefly, whole-cell lysates in 50 nM Tris/HCl pH7.4, 150 mM NaCl, 1 mM EDTA, and 1% NP40 were incubated for 2 h at 4 °C with 4 mg of an antibody against PP2A-C and protein A-agarose. After three washes, immunoprecipitates were used in a phosphoatase reaction according to the manufacturer's instructions. As an internal control, the specificity of the reaction was assessed by inhibiting PP2A activity with 1 nM OA before titration. The percentage of phosphatase activity was determined by dividing the free phosphate of the test cells by that of the untreated cells.

Establishment of melanoma cell lines carrying an inducible Bim or CHOP expression system. A lentivirus-based inducible gene expression system described previously was used to express Bim and CHOP conditionally in melanoma cells.[18] Briefly, the system involves coinfection of two lentiviral particles; one encoding the inducible transcriptional activator Gal4 1-147 ER^{T2}VP16 (GEV16) cloned into pFU-GEV16-PGK-Hygro containing a bygromycin-B-resistance gene, and another, Bim or CHOP cDNA cloned into pF-5xUAS-SV40-puro containing a puromycin-resistance gene. Dual antibiotic selection was applied deriving a cell population carrying both GEV16 and Bim or CHOP. Application of low nM concentrations of (4-OHT) drives the expression of Bim or CHOP. The melanoma cell line Mel-RM was used to establish sublines carrying inducible exogenous Bim (Mel-RM.Bim) or CHOP (Mel-RM.CHOP).

Plasmid vectors and transfection. The pCMV6-XL4-Bim_{EL} (SC306048), pCMV6-AC-CHOP (SC324377-20), and pCMV6-AC-PP2A-C (SC321401) were purchased from Origene (Rockville, MD, USA). Cells were transfected with 2 μg plasmid as well as the empty vector in Opti-MEM medium (Invitrogen, Carlsbad, CA, USA) with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

Quantitative reverse transcription and real-time PCR. Total RNA was isolated using RNeasy mini kit (Qiagen, Doncaster, VIC, Australia) following the manufacturer's instructions. RNA was reverse transcribed into cDNA using Superscript III (Invitrogen) following the manufacturer's instructions. Quantitative PCR (qPCR) was performed using the ABI Prism 7900 sequence detection system (Applied Biosystems, Mulgrave, VIC, Australia) with specific-gene primers: Bim forward, 5′-TGCAGACATTTTGCTTGTTCAA-3′; Bim reverse, 5′-GAACCGCT GGCTGCATAATAAT-3′; β-actin forward, 5′-GGCACCCAGCACAATGAAG-3′; β-actin reverse, 5′-GCCGATCCACACGGAGTACT-3′). The following PCR conditions were used: standard fast cycle 95°C for 20 s, 40 cycles of 95°C for 1 s and 60°C for 20 s using Fast SYBR Green mastermix (Applied Biosystems). Cycle threshold (C_T) values for specific genes were normalized to the C_T value for the house-keeping gene, β-actin. The fold changes of mRNA expressed were determined by comparison with β-actin, where the control sample was arbitrarily designated as 1, and the values of the relative abundance of mRNA of other samples were calculated accordingly. The specificity of the qPCR was controlled using non-template control.

siRNA knockdown. The siRNA constructs used were obtained as the siGENOME SMARTpool reagents (Dharmacon, Lafayette, CO, USA). The siRNA constructs used are: MEK1 siGENOME SMARTpool (M-003571-01-0010), ERK1 siGENOME SMARTpool (M-003592-03-0005), ERK2 siGENOME SMARTpool (M-003555-04-0005), CHOP siGENOME SMARTpool (M-004819-03-0005), and non-targeting siRNA pool (D-001206-13-20) as control. Transfection of siRNA pools was carried out as described previously.[40]

Detection of XBP1 mRNA splicing. Reverse transcription-PCR (RT-PCR) products of XBP1 mRNA were obtained from total RNA extracted using primers 5′-CGGTGCGCGGTGCGTAGTCTGGA-3′ (sense) and 5′-TGAGGG GCTGAGAGGTGCTTCCT-3′ (antisense). As a 26-bp fragment containing an ApaLI site is spliced on activation of XBP1 mRNA, the RT-PCR products were digested with *Apa*LI to distinguish the active spliced form from the inactive unspliced form. Subsequent electrophoresis revealed the inactive form as two cleaved fragments and the active form as a noncleaved fragment.

Conflict of Interest

The authors declare no conflict of interest.

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