Tyrosine dephosphorylation is required for Bak activation in apoptosis

Joanna L Fox¹, Ferina Ismail²,
Abul Azad¹, Nicola Ternet³,
Sabrina Leverrier⁴, Mariola J Edelmann³,
Benedikt M Kessler³, Irene M Leigh⁵,
Sarah Jackson¹ and Alan Storey^{1,*}

¹Department of Molecular Oncology, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, UK, ²Centre for Cutaneous Research, ICMS, London, UK, ³Henry Wellcome Building for Molecular Physiology, Nuffield Department of Medicine, University of Oxford, Oxford, UK, ⁴Department of Physiology and Biophysics, UC Irvine, Irvine, CA, USA and ⁵College of Medicine Dentistry and Nursing, University of Dundee, Dundee, UK

Activation of the cell-death mediator Bak commits a cell to mitochondrial apoptosis. The initial steps that govern Bak activation are poorly understood. To further clarify these pivotal events, we have investigated whether post-translational modifications of Bak impinge on its activation potential. In this study, we report that on apoptotic stimulation Bak undergoes dephosphorylation at tyrosine residue 108 (Y108), a critical event that is necessary but not sufficient for Bak activation, but is required both for early exposure of the occluded N-terminal domain and multimerisation. RNA interference (RNAi) screening identified non-receptor tyrosine phosphatases (PTPNs) required for Bak dephosphorylation and apoptotic induction through chemotherapeutic agents. Specifically, modulation of PTPN5 protein expression by siRNA and overexpression directly affected both Bak-Y108 phosphorylation and the initiation of Bak activation. We further show that MEK/ERK signalling directly affects Bak phosphorylation through inhibition of PTPN5 to promote cell survival. We propose a model of Bak activation in which the regulation of Bak dephosphorylation constitutes the initial step in the activation process, which reveals a previously unsuspected mechanism controlling the initiation of mitochondrial apoptosis.

The EMBO Journal (2010) 29, 3853–3868. doi:10.1038/emboj.2010.244; Published online 19 October 2010

Subject Categories: signal transduction; differentiation & death

Keywords: apoptosis; Bak; mitochondria; phosphatase

Introduction

Apoptosis or programmed cell death is a highly controlled cellular process essential for homeostasis. Evasion of apoptosis is a hallmark trait of cancer development and one of the

major obstacles to effective treatments (Hanahan and Weinberg, 2000). The decision making process as to whether a cell commits to cell death is based on the receipt of incoming signals emanating from cellular damage and micro-environmental factors. The mitochondrial apoptosis pathway is regulated by Bcl-2 family proteins, which regulate the commitment to apoptosis by their ability to govern mitochondrial outer membrane (MOM) potential and the resultant release of apoptogenic factors, including cytochrome *c*. The Bcl-2 family of proteins can be divided into three structurally and functionally distinct groups (Adams and Cory, 2007). The anti-apoptotic Bcl-2 family, which include Bcl-2, Bcl-xl and Mcl-1, contain up to four shared homology domain and are critical for cell survival. The pro-apoptotic BH3-only proteins, which include Bad, Bim, Bid, Noxa and Puma, contain a single BH3 domain and are required for the activation of the multi-domain effector proteins Bak and Bax (Green and Reed, 1998). Currently, two main models have been proposed for how Bax and Bak are controlled; first, indirect activation, in which BH3-only proteins bind to and neutralise Bcl-2 family members, which constitutively bind to Bax/Bak in healthy cells. BH3-only protein binding, therefore, unleashes Bak/Bax from sequestration by these pro-survival proteins, enabling them to be activated and facilitate MOM permeabilisation (Willis *et al*, 2005, 2007; Uren *et al*, 2007). Alternatively, the direct activation model classifies the BH3-only proteins as either ‘activating’, which are BID-like, or ‘sensitizing’, which are BAD-like (Letai *et al*, 2002). In this model ‘activator’ BH3-only proteins bind directly to Bak/Bax resulting in activation (Letai *et al*, 2002), and ‘sensitizer’ BH3-only proteins bind to Bcl-2 family members inhibiting their anti-apoptotic activity. In addition to these two models, p53 has been shown to bind directly to both Bak (Leu *et al*, 2004; Pietsch *et al*, 2008) and Bax (Chipuk and Green, 2004), and has been proposed to act in an analogous manner to the ‘activating’ BH3-only proteins (Leu *et al*, 2004), resulting in the initiation of apoptosis as measured by cytochrome *c* release.

The specific mechanism by which the two effector proteins, Bak and Bax, are activated has been widely studied and is known to require at least two key sequential steps; first, a conformational change that involves the expose of occluded N-terminal epitopes (Desagher *et al*, 1999; Griffiths *et al*, 1999) and second, the formation of homo-oligomeric complexes that permeabilise the MOM (Wei *et al*, 2000; Antonsson *et al*, 2001). The activation steps required for Bak and Bax activation, however, are different, as inactive Bax is found as an auto-inhibited monomer in the cytosol (Suzuki *et al*, 2000), whereas Bak is an integral mitochondrial membrane protein. Recent studies have established that Bax undergoes stepwise structural re-organisation that leads to its mitochondrial targeting and homo-oligomerisation (Kim *et al*, 2009). Bax activation was observed to require an N-terminal conformational change, which was triggered by BH3-only proteins tBid, BIM and PUMA, resulting in the

*Corresponding author. Department of Molecular Oncology, Weatherall Institute of Molecular Medicine, Oxford University, John Radcliffe Hospital, Oxford, OX3 9DS, UK. Tel.: +44 1865 222407; Fax: +44 1865 222431; E-mail: alan.storey@imm.ox.ac.uk

Received: 3 March 2010; accepted: 8 September 2010; published online: 19 October 2010

exposure of the $\alpha 1$ helix of Bax (Desagher *et al*, 1999; Nechushtan *et al*, 1999; Kim *et al*, 2009). The binding site for these BH3-only proteins involves helix $\alpha 1$ and helix $\alpha 6$ (Gavathiotis *et al*, 2008), which form an additional and distinct binding site. Even in this 'active' conformation Bax is still cytosolic, however, the transient binding of the BH3-only proteins to the $\alpha 1$ helix enables the C-terminal $\alpha 9$ helix to be exposed and target Bax to the mitochondria for insertion into the MOM (Kim *et al*, 2009). During the transition state after the initial conformational change, but before insertion in the mitochondrial membrane, BH3-only proteins remain stably bound to Bax (Kim *et al*, 2009). However, the interaction with the BH3-only proteins must be lost for higher order oligomers to form through interactions between the BH3-domain and the canonical dimerisation pocket of Bax (Sundararajan and White, 2001).

In contrast to Bax, Bak is an integral mitochondrial membrane protein and, therefore, does not require membrane insertion as part of its activation process. Bak has, however, been shown to interact with the minor VDAC isoform (VDAC2), an association that was proposed to restrain Bak activation in healthy cells, but was disrupted by binding of BH3-only proteins to Bak in response to death stimuli (Cheng *et al*, 2003). These findings were questioned by studies in MEFs, which indicated that all three VDAC isoforms were dispensable for mitochondrial-induced cell death driven by Bcl-2 family members (Baines *et al*, 2007). However, recent studies suggest that the role of the VDAC2–Bak interaction may be to serve to promote tBid-induced apoptosis by recruiting newly synthesised Bak protein to the mitochondria (Roy *et al*, 2009). Whether VDAC2 remains associated with Bak after mitochondrial targeting remains to be elucidated. Bak activation is also reported to require a series of conformational changes to enable it to form multimers, the first of which is the BH3-only-triggered exposure of the N-terminal-occluded epitope converting Bak into a 'primed' conformation (Griffiths *et al*, 1999). The subsequent transient exposure of the Bak BH3 domain allows for the insertion of one Bak molecule into the hydrophobic surface groove of another 'primed' Bak monomer in a reciprocal interaction to form symmetric homodimers (Dewson *et al*, 2008). These dimers further multimerise to form higher-order homo-oligomers through an $\alpha 6:\alpha 6$ interface that is distinct from, but dependent on, the BH3:groove interface, and is thought to be responsible for cytochrome *c* release (Dewson *et al*, 2009). Due to its mitochondrial localisation and lack of secondary binding site for BH3-only proteins, it has been proposed that Bak constitutively exposes the $\alpha 1$ helix thus 'bypassing' the first Bax activation step that may result in Bak having faster killing kinetics than Bax (Kim *et al*, 2009). However, analysis of the p53–Bak interaction revealed that p53 interacts with Bak through an electronegative region encompassing the N-terminal α -helix of Bak and can also trigger the initial N-terminal conformational change (Leu *et al*, 2004; Pietsch *et al*, 2008). Thus, discrete steps involved in the regulation of Bak activation have been postulated to occur as a cascade of events centred on a series of conformational changes driven by binding and release of apoptotic regulators.

In this study, we demonstrate, to the best of our knowledge, for the first time that Bak activation involves a tyrosine dephosphorylation event that is required to permit the triggering of the N-terminal conformational change by BH3-only

proteins and subsequent multimerisation. We identify specific Bak phosphatases and explore signalling pathways that control their activity and impinge on Bak activation. Our study reveals a tightly controlled dynamic equilibrium of Bak phosphorylation responsive to signalling pathways that determine cell fate.

Results

Bak is both serine/threonine and tyrosine dephosphorylated during activation

Bak is known to be a phospho-protein (Griffiths *et al*, 1999), which is stabilized after UV treatment (Jackson *et al*, 2000); however, so far no phosphorylation sites have been mapped nor the potential role of phosphorylation on Bak activity been explored. We hypothesised that diverse apoptotic stimuli may trigger changes in Bak phosphorylation that may be required for its activation. We initially used 2D gel analysis to monitor global changes in Bak phosphorylation. The theoretical pI value of Bak is about 5.6; however, Bak in unstimulated HT1080 cells had an observed pI value of ~ 3.1 – 3.3 , suggesting multiple phosphorylations (Figure 1A). Treatment of cells with CPT or UV resulted in the appearance of new species with higher pI values, suggesting loss of phosphatase moieties, but complete Bak dephosphorylation was only achieved after λ -phosphatase treatment that shifted Bak migration close to the theoretical pI value of 5.6 (Figure 1A). This suggests that only partial Bak dephosphorylation occurs in response to apoptotic stimuli. Further analysis using either a purified serine/threonine phosphatase (PP1) or tyrosine phosphatase (YOP) to treat mitochondrial extracts resulted in the appearance of new Bak species with higher pI values, indicating that in undamaged cells Bak has phosphorylations on both serine/threonine and tyrosine residues (Figure 1B).

During the transition of Bak from 'inactive' to the 'primed' state an occluded N-terminal epitope is exposed in intact cells (Griffiths *et al*, 1999), the exposure of which is associated with the N-terminal conformational change. X-ray structures of the non-activated Bak conformer (Moldoveanu *et al*, 2006; Wang *et al*, 2009), show the protein fold in all three structures (2IMS, 2JCN and 2YV6) closely resembles the non-activated cytosolic form of Bax (Suzuki *et al*, 2000). Therefore, they provide no clear insights regarding Bak activation. It is important to note that in these structures the N-terminal residues of Bak were removed to facilitate crystal formation (Moldoveanu *et al*, 2006), this maybe because these residues constitute a flexible region, similar to 12 N-terminal residues of Bax (Suzuki *et al*, 2000). The 'primed' form of Bak, however, can be detected in cells using a conformation-specific antibody (Ab-1). The FACS analysis using this antibody enabled us to determine whether phosphatase activity was required for the early exposure of the N-terminus of Bak. The Bak-specific fluorescence detected here was similar to that observed by others using different cell lines (Kepp *et al*, 2007; Kutuk *et al*, 2009); furthermore, the shift in the fluorescence peak was eliminated in cells in which Bak expression has been knocked down using siRNA (Supplementary Figure 1). The UV-damaged HT1080 cells showed an increase in Bak-specific fluorescence associated with conformational change that was insensitive to serine/threonine phosphatase inhibitors Calyculin A and

Figure 1 Bak undergoes tyrosine dephosphorylation during the initiation of apoptosis. **(A)** 2D gel analysis of Bak from HT1080 cells using narrow-range linear pI gradient IPG-focusing strips and 15% Tris-glycine SDS-PAGE showed partial dephosphorylation after UV treatment, complete dephosphorylation was achieved by λ -phosphatase treatment. **(B)** 2D gel analysis of Bak of mitochondrial extracts from HT1080 cells treated either with ser/thr (PP1) or tyr (YOP) phosphatase, which resulted in new Bak species (arrowed). **(C)** Histograms of FACS analysis of Bak Ab-1-specific fluorescence in control untreated HT1080 cells, HT1080 cells $\pm$ pre-treatment for 30 min before UV treatment with phosphatase inhibitors sodium stibogluconate (SS; 110 μ M), phenylarsine oxide (PAO; 5 μ M), Cyclosporin A (75 μ M) and Calcyulin A (2 nM). Samples were analysed 4 h after UV damage. **(D)** Quantification of the increase in Bak Ab-1-specific fluorescence $\pm$ 4 h apoptotic stimuli camptothecin (CPT; 6 μ M), Etoposide (EP; 10 μ M), Staurosporine (STS; 100 nM) or 5 mJ/cm² UV in the presence and absence of tyrosine phosphatase inhibitors 110 μ M SS or 5 μ M PAO with the y-axis showing levels of Bak-specific fluorescence and the x-axis showing treatment conditions.

Cyclosporin A (Figure 1C). In contrast, the protein tyrosine phosphatase (PTP) inhibitors, sodium stibogluconate (SS) and phenylarsine oxide (PAO), were each able to prevent the increase in Bak-specific fluorescence after UV damage (Figure 1C), indicating that a tyrosine phosphatase activity is required for Bak to undergo the conformational change that results in the exposure of the N-terminal epitope. Furthermore, these two inhibitors were able to prevent the conformational change occurring when HT1080 cells were treated with other chemotherapeutic agents, including Camptothecin (CPT), Etoposide, Staurosporine (STS) and UV as apoptotic inducers (Figure 1D). In the 'primed' conformation Bak is more susceptible to proteolytic cleavage than the 'inactive' conformer (Ruffolo and Shore, 2003); therefore, we used this as an independent method to further confirm our FACS analysis. Mitochondria isolated from cells treated using the same UV and phosphatase inhibitor treatment conditions, as in the FACS assay (Figure 1D), were subjected to limited tryptic digestion. The

failure of Bak to be cleaved after SS+UV treatment confirmed that only tyrosine phosphatase inhibition affected the Bak N-terminal conformational change (Supplementary Figure 2). This requirement for PTP activity for Bak conformational change was also observed in multiple other cell lines when treated with the above agents (Supplementary Figure 3). Furthermore, in HT1080 cells pre-treatment with SS, which resulted in a decrease in Bak activation, was also associated with a reduction in the percentage of Annexin V-positive cells after CPT treatment (Supplementary Figure 4). As PTP inhibitors were used in these experiments, and PTPs are known to be involved in multiple signalling pathways, the effect on Bak conformational change could have been a result of either a PTP acting directly on Bak, or alternatively indirectly through signalling pathways upstream of Bak activation. These results, however, provide strong evidence for a previously undefined mechanism involving a PTP signalling pathway that directs those early events of Bak activation.

Bak is phosphorylated in undamaged cells

As our results indicated that in undamaged cells Bak was tyrosine-phosphorylated, we next sought to determine which sites were involved and whether tyrosine dephosphorylation of Bak itself was required for the initial steps of Bak activation to proceed. Analysis of the Bak protein sequence using

NetPhos software (www.cbs.dtu.dk/services/NetPhos/) predicted that only one tyrosine (residue 108; Supplementary Figure 5) was likely to be phosphorylated. The X-ray crystal structure of Bak shows Y108 to be in helix $\alpha 4$ and is solvent exposed (Supplementary Figure 6; Moldoveanu *et al*, 2006), and is highly conserved between species (Figure 2A). This

Figure 2 Bak is phosphorylated at tyrosine residue 108. (A) Residue Y108 is conserved in the Bak protein sequence in chimpanzee, dog, cow, mouse, rat and chicken. (B) An isogenic pair of cell lines was created in MEF^{Bak-/-} cells expressing either the wild-type (WT) or Y108A form of Bak. 2D gel analysis of MEFs expressing WT or Y108A Bak using narrow-range linear pI gradient IGF-focusing strips and 15% Tris-glycine SDS-PAGE after 4-h 6 μ M CPT treatment $\pm$ 30 min 110 μ M SS pre-treatment. (C) Immunoprecipitation of pY108 Bak from WT and Y108A MEFs using anti-pan-phosphotyrosine antibody pY100 or an affinity-purified serum raised against phospho-Bak peptide (TAENApYEYFTK). Samples were western blotted with anti-mouse Ab-1 Bak antibody. (D) Immunoprecipitation of pY108 Bak from WT-expressing MEFs treated $\pm$ 6 μ M CPT treatment $\pm$ 30-min 110 μ M SS pre-treatment using anti-Phospho-Y108Bak antibody. Samples were western blotted with Ab-1 anti-mouse Bak antibody. (E) MS/MS spectrum of the tryptic peptide fragment 104–127 AENAYEYFTKIATSLFESGINWGR corresponding to human Bak (Sprot acc. Nr Q16611); observed mass: $[M+4H]^{4+}$ 776.06 Da; theoretical mass 3100.21 Da (delta mass 0.01 Da). The phosphorylated residues are indicated with a 'p' and the identified b and y fragment ions are indicated; ++, $[M+2H]^{2+}$ ions; *, NH_3 ; o, $-H_2O$; -98, neutral loss of phosphoric acid. + indicates an additional modification (+14 Da) that is present in one of the fragment ions indicated by the dotted lane.

predicted phosphorylation site was mutated to alanine and either the wild type (WT) or the Y108A mutant expressed in MEFs^{bak-/-}. The MEFs were selected for these studies, as they are routinely used to study Bak activation. The 2D gel analysis of the phosphorylation status of the WT Bak protein revealed two species, ~pI 3.1 and 3.3, similar to the profile we observed in HT1080 cells, with the appearance of new species with higher pI values when the cells were treated with CPT (Figure 2B). The appearance of these new dephosphorylated species could be blocked by pre-treating the cells with the PTP inhibitor SS. The Y108A mutant lacked the lowest pI species compared with the WT Bak, indicating that the protein was differently charged. Comparing the 2D gel profiles, we noted that this lowest pI spot was also absent when mitochondrial extracts from HT1080 cells that were treated with the tyrosine phosphatase YOP (Figure 1B). However, the Y108A Bak mutation did not affect the generation of further dephosphorylated forms when cells were treated with CPT (Figure 2B), suggesting that further dephosphorylation was still able to occur after DNA damage. We therefore raised and affinity purified a Bak-Y108-phosphospecific antiserum, the binding of which was dependent on the presence of the phosphate moiety on Y108 (see Supplementary Figure 7 for a detailed characterisation). Immunoprecipitation using the pan anti-p-Tyr antibody p-Y100 or the affinity-purified serum raised against a phospho-Y108Bak peptide showed that while the anti-pY-100 antibody was only capable of pulling down a small fraction of phospho-Bak, the anti-pY108 serum clearly showed that tyrosine-phosphorylated Bak was detectable only in MEFs expressing WT, but not the Y108A-expressing or MEFs^{bak-/-}, cells, suggesting Y108 is phosphorylated in Bak (Figure 2C). The difference in intensity of the pulldown bands with the different antibodies may be due to the contextual environment of the phosphorylated residue or greater specificity of the phospho-Y108 Bak sera. Furthermore, the amount of phospho-Bak pulled down decreased when cells were treated with CPT before immunoprecipitation; however, pre-treatment with SS prevented CPT-induced dephosphorylation (Figure 2D). While the 2D gel analysis coupled with the phospho-specific IP western blots provide strong evidence for Y108 phosphorylation, we further confirmed phosphorylation at this residue using direct analysis by tandem mass spectrometry. As indicated in Figure 2E, and consistent with the presence of multiple forms of Bak containing both S/T and Y phosphorylations that could be separated by their isoelectric point (Figure 1A), there seem to be multiple phosphorylations present on the Bak protein in this region. In addition to a phosphorylation at residue Y108, the mass spectrometry analysis also detected an additional phosphorylation at Y110, which interestingly is the only tyrosine residue in the Bak protein that is not conserved among species (Figure 2A). Further S/T phosphorylations were also detected in this region on the α 4 helix, specifically at T116 and S117; however, our inhibitor studies (Figure 1C) indicated that these phosphorylations were not involved in early Bak activation, therefore, only tyrosine phosphorylations were further investigated in this study. Overall, the mutational analysis together with the specific phospho-anti-serum and mass spectrometry led us to conclude that Bak is phosphorylated in cells at the conserved tyrosine 108 residue and this phosphorylation is lost after an apoptotic stimulus.

Dephosphorylation of Y108 is an initial step in Bak-mediated mitochondrial apoptosis

Bak activation occurs in a series of sequential steps. We have established that tyrosine phosphatase inhibitors, SS and PAO, are able to block the early N-terminal conformational change after apoptotic stimuli. In addition, we have shown that Bak Y108 phosphorylation is lost after CPT or UV treatment. We therefore next wanted to establish whether this dephosphorylation of Bak is required for the early N-terminal conformational change and subsequent activation steps, such as multimerisation and cytochrome *c* release. Using WT Bak and the Y108A mutant expressed in MEFs (Supplementary Figure 8), we determined the effect of Y108 phosphorylation on Bak activation status. Expression of Y108A Bak in cells did not have an adverse affect on cell viability, alter Bak mitochondrial localisation or significantly increase the amount of Bak in the 'primed' conformation in the absence of apoptotic stimuli as determined using the Ab-1 FACS assay. However, treatment of Y108A Bak-expressing cells with CPT (Figure 3A) or UV (Supplementary Figure 9) resulted in a dramatic increase in the amount of 'primed' Bak that was far in excess of that seen in the WT Bak cells as determined by Ab-1 FACS analysis, which is displayed both as frequency histograms and the quantification of the change in Bak-specific fluorescence after damage (Figure 3A). The increase can be seen both by the greater percentage of cells that shift to the right in the histogram, which indicates an increase in Bak-specific fluorescence, in the Y108A Bak-expressing cells compared with those expressing WT Bak and the decrease in Ab-1-negative peak after 4-h CPT treatment. Conversely, the introduction of a negatively charged residue at this site in the Y108E mutant, although not strictly a tyrosine phospho mimetic, prevented the N-terminal conformational change after CPT treatment (Supplementary Figure 10). The additional non-conserved Y110 phosphorylation identified by mass spectrometry analysis of Bak (Figure 2E) in human Bak was also investigated to determine whether it also functioned in the early steps of Bak activation, specifically in the N-terminal conformational change as determined by Ab-1-specific FACS. Mutational analysis of this residue to mimic either the phosphorylated or dephosphorylated states by generating Y110E and Y110F mutants, respectively, did not alter the ability of Bak to undergo the N-terminal conformational change after CPT treatment (Supplementary Figure 11). This suggests that the presence of a negative charge at this residue does not affect the initiation of Bak activation in marked contrast to the Y108E mutant that prevented Bak activation. Taken together the tyrosine mutational analysis suggests first that Y108, but not Y110, dephosphorylation is a limiting step that determines the amount of Bak that can undergo the conformational change to the 'primed' state, and second that only a fraction of WT Bak in the cell is dephosphorylated after apoptotic stimuli that then goes on to undergo the N-terminal conformational change. As previously shown in the HT1080 cells, the conformational change to the 'primed' state could be blocked by the phosphatase inhibitor SS in MEFs expressing WT Bak (Figure 3A). However, the phosphatase inhibitor had no effect on the ability of cells expressing Y108A Bak to undergo the N-terminal conformational change (Figure 3A), providing further supporting evidence that Y108 is the critical residue that is being dephosphorylated after CPT treatment.

Furthermore, the fact that the phosphatase inhibitors cannot block the activation of Y108A suggests that they are acting on enzymes that are directly dephosphorylating Bak, and not through an alternative indirect mechanism. To further confirm these FACS data, we assessed the trypsin sensitivity of WT and Y108A Bak in the presence and absence of apoptotic stimuli to further analyse the affects of Y108 phosphorylation on the ability of Bak to undergo the conformational change.

In mitochondrial preparations, full-length Y108A Bak levels were reduced to a greater extent than WT Bak after CPT treatment, indicating there was more Bak in the primed conformation (Figure 3B) in agreement with our previous FACS analysis. In the absence of CPT, both forms of Bak were equally resistant to proteolytic cleavage, indicating that the phosphorylation status of Bak did not directly affect the transition between the two states with exposure of the

N-terminal epitope and Bak remained in the WT conformation. For conversion of Bak to the 'primed' state, an additional stimulus such as CPT was required.

After the N-terminal conformational change, Bak transiently exposes its BH3 domain (Dewson *et al*, 2008) and multimerises to form pores in the mitochondrial membrane, which coincides with the release of cytochrome *c* and other apoptotic factors from the mitochondria (Wei *et al*, 2000). To determine the effect of Y108 phosphorylation status on these later steps, we investigated the ability of Bak to form multimers with and without Y108 phosphorylation. Mitochondria from cells expressing WT Bak or Y108A Bak were isolated and Bak protein crosslinked using BMH to determine the multimeric state of Bak as previously described (Jiang *et al*, 2006). In untreated cells, irrespective of the form of Bak being expressed, Bak was observed mainly as monomers (Figure 3C). After CPT treatment, higher-order Bak multimers were observed in both WT and Y108A-expressing cells (Figure 3C). However, pre-treatment of cells with SS before CPT treatment, which we have shown kept Bak in its Y108-phosphorylated form (Figure 2D), prevented WT Bak forming multimers, whereas the Y108A Bak mutation renders Bak refractory to SS inhibition and still-formed multimers, thereby confirming that this is the critical phospho-tyrosine residue involved (Figure 3C). Further supporting our multimerization studies, we found in experiments in which the addition of peptides comprising of BH3 domain sequences, such as that of tBid, which have previously been shown by others to be able to activate Bak resulting in the release of cytochrome *c* from isolated mitochondria (Wei *et al*, 2000; Chipuk *et al*, 2008), that mitochondria isolated from cells pre-treated with SS were unable to release cytochrome *c*. Whereas, mitochondria from untreated cells after the addition of a peptide comprised of the tBid BH3 sequence were still able to release cytochrome *c* (Supplementary Figure 12). As Bak activation is believed to be linear process (Kim *et al*, 2009), these data further confirm that Y108 dephosphorylation is an early step in this process, which if blocked, prevents subsequent activation steps from occurring. After Bak multimerisation, cytochrome *c* release can be detected in cells by immunofluorescence staining and FACS analysis. Early time points after CPT treatment were analysed by immunocytochemistry. The MEFs expressing WT Bak revealed that cytochrome *c* remained in a punctate mitochondrial pattern at 4 h after CPT treatment. In contrast, Y108A-expressing MEFs began to display altered cytochrome *c* staining patterns as early as 2 h after CPT treatment. Further analysis of cytochrome *c* released by the mitochondria over a longer time course of CPT treatment enabled the

extent of cytochrome *c* release to be quantified using FACS. This revealed that release of cytochrome *c* occurred earlier in the Y108A Bak-expressing cells compared with those expressing WT Bak, with the majority of cells having released their cytochrome *c* by 16 h in Y108A Bak-expressing cells, a time at which only ~20% of the cells expressing WT Bak had released their cytochrome *c* (Figure 3E). Furthermore, analysis of cell viability revealed that at early time points cell expressing Y108A Bak were more susceptible to cell death compared with those expressing WT Bak (Figure 3F). We therefore conclude that in the absence of Y108 phosphorylation, Bak bypasses an initial activation step, which results in more Bak undergoing N-terminal conformational activation, leading to greater multimerisation and cytochrome *c* release, which are responsible for the observed increased susceptibility to apoptotic cell death. It is important, however, to note that in the absence of Bak phosphorylation (Y108A), Bak does not undergo a spontaneous conformational change (Figure 3A); full activation requires a death stimuli such as CPT. These data, therefore, support a model in which Y108 dephosphorylation of Bak is an early event in the multi-step activation process and results in the formation of what we now term an 'activation competent' form of Bak, which must be achieved before the exposure of the N-terminal epitope and conversion to the 'primed' state, multimerisation and cytochrome *c* release.

Involvement of non-receptor protein tyrosine phosphatase in Bak activation

Taken together, our data suggests a model whereby direct dephosphorylation of Bak by PTPs occurred after apoptotic stimuli, rather than an indirect effect of tyrosine phosphatase inhibitor SS on other pathways, (Figure 1C) which subsequently modulated Bak Y108 phosphorylation status. We therefore sought to gain insight into which PTPs were involved in the dephosphorylation of Bak and conversion into the 'activation competent' form. Initial RNAi screening focused on the non-receptor tyrosine phosphatases (NRPTs; Supplementary Table 1), as some are known targets of the PTP inhibitors used earlier in this study. Using pools of siRNA duplexes, the expression of each member of the NRPT family was knocked down in HT1080 cells as these cells had been used in the initial experiments, and the siRNA oligonucleotides were designed against human not mouse phosphatases. All siRNA pools effectively decreased the mRNA levels of their target phosphatase (Supplementary Figure 13). Decreasing the expression of three of the phosphatases screened, *PTPN2*, *PTPN5* and *PTPN23*, prevented Bak undergoing the conformational change when cells were

Figure 3 Dephosphorylation of Y108 is required for Bak activation. (A) Histograms of FACS analysis of Bak Ab-1-specific fluorescence and quantification of the mean Bak Ab-1-specific fluorescence in control (grey bars), 6 μ M CPT (black bars) or 6 μ M CPT + 110 μ M SS pre-treated (white bars) MEFs expressing WT or Y108A forms of Bak ($N = 3$, $\pm$ s.e.m.). (B) Trypsin proteolysis of Bak. Mitochondria extracted from MEFs expressing either WT or Y108A Bak treated $\pm$ CPT for 4 h, were subjected to limited trypsin proteolysis, and cleavage products were detected by immunoblotting using an antibody recognising the Bak BH1 domain. Data are representative of three independent experiments. (C) Mitochondria were extracted from MEFs expressing WT or Y108A Bak treated $\pm$ 8-h UV $\pm$ 30-min SS (110 μ M) pre-treatment and treated with cysteine crosslinker BMH to induce intramolecularly linked monomers (M_x) and intermolecularly linked dimers (D) and trimers (T). Bak in which the cysteines are not cross-linked runs as a monomer (M). Samples were separated by SDS-PAGE and immunoblotted for Bak. Approximately, 5% of input mitochondria from each sample were run as a loading control for Bak levels. (D) Two-colour fluorescence microscopy after 0-, 2- and 4-h 6 μ M CPT treatment. Green indicates cytochrome *c* and blue is DAPI nuclear staining. (E) WT and Y108A MEF cell lines were treated with 6 μ M CPT over 24-h time course and mean percentage cells with cytochrome *c* release was determined by FACS analysis ($N = 3$, $\pm$ s.e.m.). (F) WT and Y108A MEF cell lines were treated with 6 μ M CPT and cell viability was quantified by flow cytometric detection of Annexin V/PI double-negative cells at indicated time points ($N = 3$, $\pm$ s.e.m.).

Figure 4 Specific non-receptor tyrosine phosphatases are required for Bak activation. (A) siRNA screen showing of effect of knocking down individual nonreceptor tyrosine phosphatases on Bak conformational change. A panel of nonreceptor tyrosine phosphatases was knocked down in HT1080 cells using 30 nM siRNA oligonucleotide pools. Graph shows mean quantification of the increase in Bak Ab-1-specific fluorescence in untreated (grey bars) or 4-h camptothecin (CPT) treated (black bars) HT1080 cells 48 h after RNAi ($N = 2$). (B) Mean percentage cells with cytochrome *c* release ($\pm$ s.e.m.) in HT1080 cells with PTPNs knocked down by siRNA followed by 4-h CPT treatment as determined by FACS analysis ($N = 3$). (C) Immunoprecipitation of pY108 Bak from empty vector and PTPN5 shRNA-expressing HCT116 cells retrovirally transduced with WT Bak $\pm$ CPT treatment using anti-Phospho-Y108Bak antibody. Samples were western blotted with Ab-1 anti-mouse Bak antibody. (D) Empty vector or PTPN5 shRNA-expressing HCT116 cells retrovirally transduced with WT Bak were treated with CPT (6 μ M) or etoposide (10 μ M). Upper panel shows quantification of cell death by flow cytometric detection of Annexin-V staining over a 36- or 48-h time course ($N = 3$, $\pm$ s.e.m., $*P \leq 0.05$), lower panel shows extent of caspase 3 cleavage by immunoblotting using an antibody recognising the full-length caspase 3 protein. GAPDH was included as a loading control. (E) Quantification of the effect of transient overexpression of pEFIRE5 (empty vector), HA-PTPN5 and HA-PTPN5 mutant (S245A/C496S) on Bak Ab-1-specific fluorescence in transfection alone (grey bars) or transfection + 4-h CPT (black bars) HCT116 WT Bak cells ($N = 3$, $\pm$ s.e.m.). Constructs were co-transfected into cells with EGFP as a transfection marker enabling only the Bak Ab-1-specific fluorescence of transfected cells to be analysed.

treated with CPT (Figure 4A), with PTPN5 knockdown being the most effective in our assay. The hits from the screen were re-confirmed by repeating the FACS analysis with individual

oligonucleotides from each siRNA pool (Supplementary Figure 14). Initiation of cytochrome *c* release was observed even at this early time point (4 h) in mock-transfected and

PTPN11 siRNA-transfected cells in which Bak priming had occurred after CPT treatment. However, knocking down expression of *PTPN2*, *PTPN5*, and to a limited extent *PTPN23*, resulted in a marked attenuation of the initiation of cytochrome *c* release in response to CPT treatment (Figure 4B). Taken together our results implicate strongly these PTPNs in Bak activation. Interestingly, the expression of a number of phosphatases (*PTPN4*, *PTPN14* and *PTPN20*) seemed to amplify the affect of CPT on Bak 'priming' through currently uncharacterized indirect mechanisms.

***PTPN5* directly affects Bak Y108 phosphorylation and N-terminal conformational change**

To determine whether the phosphatases identified in our screen were influencing Bak conformational change by directly modulating Bak dephosphorylation after CPT treatment, we focused on *PTPN5*, which had the greatest effect in our assays. For these experiments, we used HCT116 cells in which we expressed WT Bak to establish more clearly the effects of modulating Bak Y108 phosphorylation on mitochondrial apoptosis in the absence of Bax. First, we analysed the phosphorylation status of Y108 in Bak-expressing HCT116 cells in which *PTPN5* levels were stably knocked down by shRNA (Supplementary Figure 15). This revealed that pY108-

Bak levels were slightly elevated in *PTPN5* knock down cells compared with the empty vector controls (Figure 4C), indicating *PTPN5* silencing resulted in increased pY108-Bak levels in undamaged cells. After CPT treatment, however, levels of pY108-Bak were markedly decreased in empty vector (pRS) cells but only marginally reduced in *PTPN5* RNAi-silenced cells. The consequence of silencing *PTPN5* and reducing the extent of Bak Y108 dephosphorylation was that these cells were less sensitive to both CPT and etoposide treatment, with markedly decreased surface exposure of phosphatidylserine (Figure 4D, upper panels) together with a reduced cleavage of full-length caspase 3 (Figure 4D, lower panels). Conversely, overexpression of *PTPN5* enhanced Bak activation. Either WT *PTPN5* or as a control, a *PTPN5* double mutant in which the phosphatase active site cysteine was mutated to serine (C496S), which has been shown by others to abolish phosphatase activity (Snyder *et al*, 2005), together with a second mutation in the kinase interaction motif (KIM), which is involved in *PTPN5* regulation (S245A; Paul *et al*, 2000; Snyder *et al*, 2005), were transiently overexpressed in HCT116 cells expressing WT Bak (Supplementary Figure 16). The effect of the elevated levels of the WT and mutant *PTPN5* proteins on Bak conformational change was measured only in the transfected

Figure 5 Downregulation of MEK/ERK signalling pathway initiated Bak activation. (A) HCT116 cells retrovirally transduced with WT Bak treated with CPT (6 μ M) over 6-h time course were assessed for ERK1/2 phosphorylation by immunoblot. The observed decrease was quantified by densitometry. (B) Immunoprecipitation of pPTPN5 and pY108 Bak from HCT116 cells retrovirally transduced with WT Bak $\pm$ CPT treatment with an immobilised anti-pan-phospho-serine antibody and our affinity-purified serum raised against phospho-Bak peptide (TAENApYEFYTK). Equal loading of protein into the immunoprecipitation was determined by analysis of GAPDH and Bak levels in the starting lysate (input). (C) Immunoprecipitation of pY108 Bak from HCT116 cells retrovirally transduced with WT Bak $\pm$ MEK inhibition by U0126 (10 μ M) using anti-phospho-Y108 Bak antibody. Efficiency of MEK inhibition was determined by immunoblot of the input lysates for phosphorylation of ERK1/2, Bak was included as a loading control. (D) HCT116 cells retrovirally transduced with WT Bak were treated with U0126 (10 μ M), CPT (6 μ M) or both agents for 16 h. Cell death was quantitated by flow cytometric detection of Annexin-V staining ($N=3$, $\pm$ s.e.m.).

cells, as determined by EGPF positivity, by Ab-1 FACS analysis, as described previously, after CPT treatment. Increased expression of the WT PTPN5 protein, but not mutant PTPN5, resulted in increased Bak activation when compared with empty vector-transfected control (Figure 4E).

ERK1/2 controls PTPN5 activity that impinges on Bak dephosphorylation

To investigate whether there is a direct link between the signalling pathways that control activation of these phosphatases and formation of the 'activation competent' state of Bak, we have again focused on signalling to PTPN5. The activity of PTPN5 is reported to be phospho-regulated by members of the MAP kinase family, whereby PTPN5 is inactivated by phosphorylation of its KIM by ERK1/2 (Pulido *et al*, 1998). The HCT116 cells that were used for these studies have an activating K-RAS mutation and as a result a constitutively activated MAPK pathway (Hoshino *et al*, 1999). After treatment with CPT, we and others (Hetman *et al*, 1999) have observed an early transient decrease in ERK1/2 phosphorylation before the well-characterized increase in ERK1/2 activity (Figure 5A). We hypothesised that this decrease would be sufficient to increase PTPN5 activity and result in Y108 dephosphorylation of Bak and formation of the 'activation competent' state. Immunoprecipitation of pPTPN5 using an agarose-conjugated anti-pSer or our anti-pY108-Bak antibody showed that after 4 h of CPT treatment the phosphorylation levels of both PTPN5 and pY108-Bak had been decreased (Figure 5B). These data indicated that modulation of the activity of the MEK/ERK signalling pathway can affect directly the Y108 phosphorylation status of Bak in this system. To probe this further, we used the small molecule MEK inhibitor U0126 to inhibit specifically the ERK1/2 signalling pathway (Figure 5C). U0126 inhibition resulted in a decrease in the amount of pY108 Bak that could be immunoprecipitated from cells (Figure 5C). Interestingly, treatment of cells with U0126 alone did not result in an increase in Annexin-V-positive cells

(Figure 5D); however, the combination of the MEK inhibitor with CPT resulted in a greater increase in Annexin-V-positive cells compared with CPT alone (Figure 5D). These data are in agreement with our previous observations that Bak Y108 dephosphorylation is not sufficient to commit a cell to apoptosis and further stimuli that have been shown to induce BH3-only proteins and/or p53 are also required to initiate the N-terminal conformational change.

We therefore propose that the Bak phosphorylation/dephosphorylation equilibrium is tightly controlled by the signalling pathways that determine cell fate. These findings lead us to propose a new model for the events early in Bak activation in which Bak is Y108-phosphorylated in undamaged cells and held in its 'inactive' conformation. The transition from the 'inactive' to 'primed' states requires dual signals from both PTPNs that dephosphorylated Y108 converting Bak to the 'activation competent' form and from pro-apoptotic proteins that trigger the N-terminal conformational change required for the 'primed' conformation (Figure 6). Neither one of these signals alone is sufficient to convert Bak from the 'inactive' to the primed conformation. Once in the 'primed' state Bak is able to form dimers (Dewson *et al*, 2008) and subsequently multimers (Dewson *et al*, 2009), which enables cytochrome *c* to be released from the mitochondria.

Discussion

Regulation of the cellular life-death switch is essential in healthy cells as aberrant regulation of cell death has been implicated in numerous human diseases, including cancer and neurodegenerative disorders such as Alzheimer's Disease. We provide evidence in this study for an important additional regulatory system controlling the initiation of the Bak-mediated mitochondrial apoptotic pathway. Bak activation has been widely studied and BH3-only proteins have been observed to be required to trigger the two critical events in Bak activation: conformational change and homo-oligomerisation (Griffiths *et al*, 1999; Korsmeyer *et al*, 2000). The

Figure 6 Working model of Bak activation after apoptotic stimuli.

induction of conformational changes in Bak by BH3-only proteins has been intensively studied (Letai *et al*, 2002; Willis *et al*, 2007). In this study, we now show that post-translational modification events are also important early in Bak activation. We find that phosphorylation of Bak in undamaged cells holds Bak in an inactive conformation and that Bak dephosphorylation must occur in response to apoptotic signals for Bak to be further activated. Our analysis revealed that there was a clear shift in the species of Bak detected by 2D analysis of cells treated with either UV or CPT (Figure 1A). This indicates that the changes in Bak observed were conserved between the different sources of apoptotic stimuli and suggests for the first time that there is an additional level of regulation involved in the activation of Bak.

The identification of both serine/threonine and tyrosine dephosphorylation events after apoptotic stimuli suggests that there may be multiple levels of phospho-regulation (Figure 1B). Our study has established that only the tyrosine dephosphorylation event seemed to be involved in the early steps of Bak activation. Analysis of the location in the Bak structure of both the S/T and Y phosphorylations identified in this study reveals that they all reside on the $\alpha 4$ helix, suggesting that this region of the protein may be important for regulating Bak function. Interestingly, the Y108 residue is in a different phase of the helical turn to Y110, resulting in the Y108 phosphate moiety being orientated to a different face of the Bak molecule compared with Y110 (Supplementary Figure 6). Although multiple phosphorylated residues were detected in this domain the mutational analysis coupled with inhibitor studies, specific anti-serum and mass spectrometry enabled us to identify Y108 as the critical tyrosine phosphorylated residue in Bak with respect to the initiation of Bak activation (Figure 2). It is, however, worth noting that there are also likely to be additional phosphorylations in other regions of the molecule that may contribute to the low pI value of Bak in undamaged cells (Figure 1A). Interestingly, the Y108 dephosphorylation event alone did not trigger the N-terminal conformational change or Bak homo-oligomerisation (Figure 3A–C). Furthermore, in undamaged cells expressing Y108A Bak the protein localised to the mitochondria, which had the same filamentous mitochondrial morphology as cells expressing WT Bak (Figure 3D), indicating that the tyrosine dephosphorylation event *per se* is not involved in the recently described role of Bak in mitochondrial fragmentation (Brooks *et al*, 2007). However, inhibition of CPT-induced Bak dephosphorylation by tyrosine phosphatase inhibitors did result in inhibition of Bak N-terminal conformational change (Figure 1C and D). Analysis of cells expressing Bak with residue 108 mutated to alanine showed that they were able to grow in culture in a manner comparable to cells expressing WT Bak, having no increase in basal levels of cell death in the absence of apoptotic stimuli. However, after apoptotic stimuli, the Y108A Bak-expressing cells were sensitised both to release cytochrome *c* and cell death when compared with cells expressing WT Bak (Figure 3A–C). We further noted that whereas Y108A Bak could still be activated, presumably by BH3-only proteins, after treatment with DNA damaging agents in the presence of PTP inhibitors, activation of WT Bak was blocked (Figure 3). This suggests that although other events are required to generate ‘primed’ Bak, dephosphorylation of Y108 seems to be the first step

in the Bak activation process. Together these findings indicate that Bak Y108 dephosphorylation is necessary but not sufficient for Bak activation, and we have therefore termed this tyrosine 108-dephosphorylated form of Bak ‘activation competent’. The observed increase in cell death in cells expressing Y108A Bak compared with those expressing WT Bak, indicates that only a proportion of the total WT Bak becomes Y108-dephosphorylated in response to apoptotic stimuli that would then be available to undergo the N-terminal conformational change triggered by binding of BH3-only proteins or p53. Our findings, therefore, support a new model in which Bak activation is not only driven by a series of conformational changes induced by the binding and release of BH3-only proteins, but is also subject to additional levels of regulation. It is, therefore, possible that the other phosphorylations identified in this study may be involved in the regulation of other steps in the Bak activation process. However, further detailed analysis will be required to elucidate any role these phosphorylations may have in Bak activation. The requirement of multiple input signals may also act to prevent inappropriate Bak activation and cell death that may result from fluctuations in mitogenic signalling or transient exposure to apoptotic stimuli. During Bak activation BH3-only proteins engage the new $\alpha 1:\alpha 6$ binding site causing the N-terminal conformational change and triggering the exposure of the $\alpha 9$ helix, which facilitates membrane insertion. As Bak is MOM-associated, it was proposed that the faster killing kinetics of Bak could be explained by the bypass of this step (Kim *et al*, 2009). The data from this study, however, suggests that Bak is required to undergo Y108 dephosphorylation, which is consistent with rapid activation and cell killing.

The siRNA screening enabled us to identify three PTPs (PTPN2, PTPN5 and PTPN23), each reported to be capable of responding to different growth/DNA damage signals that are required for Bak conformational change and cytochrome *c* release from mitochondria in our assays (Figure 4A and B). PTPs are already recognised to function in many pathways that determine the balance between cell death and cell survival; however, this is still an emerging field. Various PTPs, including PTPN1 (PTP-1B), PTPN6 (SHP-1) and PTPN11 (SHP-2), which are members of the non-receptor family of PTPs, have been identified as enzymes that modulate apoptosis (Halle *et al*, 2007b), both by negatively regulating pro-survival signalling (e.g. SHP-1; Yousefi and Simon, 2003) or by directly participating in apoptosis pathways (e.g. PTP-PEST; Halle *et al*, 2007a). PTPN5 and PTPN23 have also both been previously reported to be linked to apoptotic induction (Mariotti *et al*, 2006; Unschuld *et al*, 2006). Furthermore, PTPN5 is reported to be mutated in primary colorectal carcinomas (Korff *et al*, 2008), and the locus containing the *PTPN23* gene is frequently deleted during the formation of many solid tumours (Hesson *et al*, 2007). Our results now also implicate PTPN2 in these apoptotic networks, PTPN2 is also reported to be downregulated in hepatocellular carcinoma (Lee *et al*, 2009) and STI-571-resistant CML cells (Shimizu *et al*, 2004). These reports provide strong supporting evidence that these phosphatases have a key function in the regulation of apoptosis and the development of cancer. Focusing on PTPN5, our knockdown studies established that this phosphatase acts directly on Bak by modulating the Y108 phosphorylation status (Figure 4C),

which limited severely the ability of Bak to be activated and decreased the cellular sensitivity to chemotherapeutic agents (Figure 4D). Conversely, overexpression of WT PTPN5 greatly sensitised cells to Bak activation, whereas the PTPN5 protein carrying mutations in both the phosphatase active site and KIM had only minor effects on Bak activation (Figure 4E). We further noted that the exposure of cells to the transfection reagent provided sufficient stress to activate Bak in the WT PTPN5-transfected cells, with only a modest enhancement of Bak activation being seen after treatment with CPT. These cells proved to be exquisitely sensitive to PTPN5 overexpression and resulted in rapid cell killing (JL Fox and A Storey, personal observations), such that it was, therefore, not possible to derive lines that stably over-expressed PTPN5. Further studies using different overexpression strategies for PTPN5 will need to be undertaken to investigate the effects of increased cellular levels of the phosphatase on Bak-mediated cell death. Taken together these knockdown and overexpression models provide insight into the mechanisms by which this phosphatase is able to affect apoptosis. Other PTPs identified in our screen enhanced the amount of Bak activation. It is likely, however that these phosphatases act indirectly on Bak N-terminal conformational change, either by modulating the activity of the, to date, unidentified Bak kinase(s) or they may be involved in pathways that have yet to be linked to apoptosis signalling. Overexpression of the Bak kinase(s) or perturbation of the signalling pathways that are able to modulate Bak phosphorylation status could, therefore, constitute mechanisms by which cells evolve to evade apoptosis. Whatever their mode of action investigation into the role of phosphatase in Bak activation and identification of the kinase(s) responsible for Bak phosphorylation represents one of the future challenges in the continued investigation into the initiation and regulation of Bak activity.

Several questions are raised by our results, such as how diverse apoptotic signals in different cell types are integrated and transduced to culminate in Bak dephosphorylation and through which signalling networks? PTPN5, which was the most affective in our assay system, is reported to be held in an inactivated form by phosphorylation of its KIM by ERK1/2 (Pulido *et al*, 1998). It is important to note that the HCT116 colon cells used in these studies have constitutively active MAPK signalling due to a K-RAS mutation. This therefore raises the possibility that in different genetic or physiological backgrounds, and in response to different genotoxic insults, cells may use different phosphatases and signalling pathways to regulate Bak phosphorylation status. However, the mechanism outlined in this study, in which Bak Y108 dephosphorylation is required for Bak activation to proceed, is applicable irrespective of genetic background of the cell line tested (Supplementary Figure 3). Furthermore, the inhibition of this dephosphorylation event using tyrosine phosphatase inhibitors before treatment of cells with DNA-damaging drugs, such as CPT, was able to block/delay cell death (Supplementary Figure 4), which further highlights the importance of this post-translation modification in the regulation of apoptosis. The transient decrease in ERK1/2 phosphorylation, which we and others (Hetman *et al*, 1999) have observed, was sufficient to release the inhibitory phosphorylation of PTPN5 in our model system (Figure 5A and B). Furthermore, direct inhibition of MEK1 by U0126 and

subsequently ERK1/2 phosphorylation directly affected, we propose through PTPN5, Bak Y108 phosphorylation. This, therefore, provides a link between the mitogenic growth signalling in the cell and the Bak-mediated apoptotic pathway. The MAPK survival pathway has previously been reported to promote cell survival by altering the stability and binding properties of multiple members of the Bcl-2 family, including Bim, Bcl-2 and Mcl-1 (Balmanno and Cook, 2009). Our study now suggests a direct role for MEK1/ERK1/2 signalling in the prevention of apoptosis induction. Interestingly, inhibition of MEK1 was sufficient to cause Bak dephosphorylation and convert Bak to the 'activation competent' form, but this alone did not cause an increase in cell death, a further apoptotic stimulus, such as CPT, was required (Figure 5D). MEK inhibitors, in most instances, have been reported to have a cytostatic rather than cytotoxic effect (Milella *et al*, 2005), which may be due to the requirement for multiple additional signals to initiate Bak activation and subsequent cell death as we suggest here. Our study, therefore, provides a potential mechanism for the striking pro-apoptotic synergism observed in MEK inhibitor combination studies in which the MEK inhibitor alone has a predominantly cytostatic phenotype (Bertrand *et al*, 2005; Haass *et al*, 2008; Balko *et al*, 2009).

Many, and possibly all cancers, show defects in apoptosis due to selective pressures operating during tumourigenesis. We show here, to the best of our knowledge, for the first time that insight into an important regulatory system controlling the initiation of the mitochondrial apoptotic pathway that integrates signalling pathways that act directly on the effector protein Bak (Figure 6). Our new model, therefore, supports the idea that at the molecular level that life, not death, is the cellular default setting (Green, 2005), and that Bak activation requires multiple positive signals for apoptosis to proceed without inhibition. Furthermore, our findings have important potential implications for interventional approaches as modulating the activation of Bak through its phosphorylation status may represent a new therapeutic strategy for enhancing cancer therapy. At present much effort has been placed on developing BH3 mimetics to reactivate mitochondrial apoptosis for the treatment of cancer (Labi *et al*, 2008; Letai, 2008). However, our data implies that the ability to modulate the phosphorylation status of Bak, and hence its activation, provides wider opportunities to either unleash the pro-apoptotic activity of the protein to enhance cell killing in cancer cells and auto-immune disorders, or alternatively to promote cell survival in other conditions, such as Alzheimer's and ischaemic heart disease.

Materials and methods

Cell culture

HT1080 (human fibrosarcoma) cells, MEFs^{bak-/-} (a gift from C Thompson), HCT116^{bax-/-} (human colorectal carcinoma that express very low levels of endogenous Bak were a gift from B Vogestein) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and glutamine. Retroviruses were produced in PT67 cells and transduced as previously described (Akgul *et al*, 2007). For retroviral infection, the MEFs^{bak-/-} or HCT116 culture was infected with pLXSN or pLXSN-WT Bak or pLXSN-Y108A Bak recombinant viruses. For transient protein expression plasmids were introduced to cells by transfection using Turbofect (Fermentas) according to the manufacturers instructions. Where required, cells were irradiated

with UVB using a UVP CL-1000 ultra-violet cross-linker with F8T5 bulbs giving a spectral peak at 312 nm.

Plasmid construction

Bak expression plasmid was a gift from T Chittenden. This was PCR amplified and cloned into the pLSXN construct. A full-length sequence verified IMAGE clone (IMAGE ID: 5726473 containing the PTPN5 sequence was obtained from Source BioScience (Cambridge, UK)). The PTPN5-coding sequence was PCR amplified with an HA-tag and inserted into pEFIRE construct (a gift from S Hobbs; Hobbs *et al*, 1998). The pLSXN-Y108A, pLSXN-Y108E, pLSXN-Y108F, pLSXN-Y110E and pLSXN-Y110F constructs and pEFIRE-HA PTPN5-S245A/C496S construct were generated using QuickChange II Site-Directed Mutagenesis Kit (Stratagene). All constructs were sequence verified. In the transient overexpression experiments in HCT116 expressing WT Bak, the pEFIRE, pEFIRE-HA PTPN5 and pEFIRE-HA PTPN5-S245A/C496S constructs were co-transfected with EGFP N3 expression vector (Invitrogen) so transfected cells could be identified.

Chemicals and drug treatments

All chemicals, except where specified, were purchased from Calbiochem and used at the final concentrations indicated. Treatments with CPT (6 μ M), EP (10 μ M), STS (100 nM) and U0126 (10 μ M) were carried out for 4 h. Pre-treatment of cells with phosphatase inhibitors SS (110 μ M), PAO (5 μ M), Cyclosporin A (75 μ M) and Calyculin A (2 nM) was for 30 min before the drug treatment and these compounds remained on the cells for the duration of the drug treatment.

Flow cytometric analysis

To analyse Bak-specific immunofluorescence after drug treatment or UV damage, cells were fixed in paraformaldehyde (0.25% PFA/PBS) for 10 min at room temperature. Cells were collected by scrapping and washed with PBS. Cells were permeabilised with 0.01% saponin/PBS and incubated with Bak Ab-1 primary antibody (AM03; Calbiochem), Bak Ab-2 primary antibody (AM04; Calbiochem) or mouse IgG1 (Pharmingen) for 30 min at 4°C. Cells were then washed and incubated with rabbit anti-mouse phycoerythrin or with goat anti-mouse Allophycocyanin secondary antibody for 30 min at 4°C. Cells were washed and resuspended in PBS for analysis with a CyAn ADP Analyser (Beckman). Approximately, 10 000 cells were analysed per sample.

To quantify the flow cytometric results using Ab-1, the data were then manipulated as previously described (Griffiths *et al*, 1999). Briefly, cells exhibiting a light scatter profile associated with apoptotic cells were gated out and the median Bak-associated fluorescence was determined by subtracting the median fluorescence of the parallel IgG control from each test sample. The median value was then multiplied by the percentage of Bak-positive cells as determined by the IgG control to give the Ab-1 Bak-specific fluorescence of each sample. In samples in which EGFP had been used as a transfection marker the GFP-positive cells were gated and only the Ab-1 fluorescence of these cells quantified as described above.

Flow cytometric analysis of cytochrome *c* release was performed as described previously (Waterhouse and Trapani, 2003). After CPT treatment, adherent cells and non-adherent cells were pooled and permeabilised with 0.05% digitonin in PBS/KCl before fixation with 4% PFA/PBS. Cells were washed three times with PBS then incubated with anti-cytochrome *c* antibody (clone 6H2.B4; BD Biosciences) in 3% BSA/PBS with 0.05% saponin overnight at 4°C. Cells were then washed and incubated with rabbit anti-mouse phycoerythrin secondary antibody for 30 min at 4°C. Cells were washed and resuspended in PBS for analysis with a CyAn ADP Analyser (Beckman). Approximately, 10 000 cells were analysed per sample.

To quantify levels of cell death cells were collected using trypsin and were stained with Annexin V Alexa Fluor 647 conjugate (Cambridge BioScience) and propidium iodide (Sigma) and their profile were analysed by flow cytometry. Detection of Annexin V was used as a marker for early apoptotic cells, double labelled Annexin V/propidium iodide cells represented late apoptotic cells and double negative cells represented viable cells.

Western blotting

Cells were washed with PBS, collected by scrapping and lysed at 4°C for 1 h. Lysis buffer contained 50 mM Tris-HCl, 150 mM NaCl (pH 7.5), 2 mM EDTA (pH 8.0), 1% CHAPS, Complete mini protease inhibitor tablet (Roche), 1% phosphatase inhibitor cocktail 1 (Sigma) and 1% phosphatase inhibitor 2 (Sigma). Lysates were centrifuged (15 000 r.p.m. for 15 min at 4°C) and protein quantification was carried out using Bradford protein assay (Perbio Science UK). The resulting protein extracts were then separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. Antibodies used were as follows: anti-Bak (ab32371; Abcam), anti-Bak Ab-1 (AM03; Calbiochem), anti-Bak BH1 domain (#3814, Cell Signaling Technology), anti-PTPN5 (ab77123, Abcam), anti-pERK1/2 (#9101; Cell Signaling Technology), total anti-ERK1/2 (#9102, Cell Signaling Technology), anti-COX IV (ab14744, Abcam), anti-GAPDH (#MAB-374, Millipore), anti-HA (MMS-101P, Covance) and anti-Caspase 3 (#9662, Cell Signaling Technology). Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit (used at a dilution of 1:2000; Dako UK). Reactive proteins were visualised by chemiluminescence with ECL plus (Amersham).

Mitochondrial fractionation

Mitochondria were prepared from treated cells using a method that preserves mitochondrial integrity (Yamaguchi *et al*, 2007). Briefly, cells were collected and washed with ice-cold PBS, then mitochondria were isolated by Dounce homogenisation in AT buffer (300 mM trehalose, 10 mM HEPES-KOH (pH 7.7), 10 mM KCl, 1 mM EGTA, 1 mM EDTA and 0.1% BSA). The homogenate was centrifuged at 600 g for 5 min. The supernatant was removed and centrifuged at 3500 g for 20 min, and the resulting mitochondrial pellet was resuspended in ice-cold AT buffer. The protein content of each sample was quantified by Bradford assay and equal amounts run on SDS-PAGE gels for western blotting. Mitochondria extracts were analysed by western blotting to ensure they were free of cytoplasmic contamination (Supplementary Figure 10).

Conformational change determined by trypsin proteolysis

To measure Bak cleavage by limited trypsin proteolysis mitochondria were prepared from cells treated with or without CPT (6 μ M) for 4 h. Mitochondria were incubated with trypsin (100 μ g/ml) on ice for 20 min. Samples were then analysed by western blotting using anti-Bak antibodies against the BH1 domain (#3814; Cell Signaling Technology).

Chemical cross-linking and oligomerisation assay

Isolated mitochondria were incubated with the cross-linker BMH (10 mM) in HIM buffer (20 mM HEPES-KOH, 10 mM KCl, 1.5 mM MgCl₂, 250 mM sucrose, 1 mM EGTA (pH 7.4); Jiang *et al*, 2006) at 37°C for 1 h before being subjected to SDS-PAGE and immunoblotting using anti-Bak antibody. COX IV (ab14744, Abcam) was used as mitochondrial loading control.

Immunoprecipitation

Cells were washed with PBS, collected by scrapping and lysed using the western blotting lysis buffer. Lysate containing 1.5 mg total protein was incubated at 4°C overnight with either immobilised anti-pY100 antibody (Cell Signalling Technology), immobilised pSer antibody (PSR-45; Ab49665, Abcam) or pre-washed protein G beads and 1 μ g affinity-purified serum raised against a phospho-tyrosine Bak peptide (TAENApYEYFTK). Serum was purified using both phospho and non-phospho peptide columns (BioGenex, Berlin, Germany). Beads were pelleted and washed three times in lysis buffer. Precipitated proteins were eluted from the beads in 2 $\times$ SDS-PAGE loading buffer. Resultant samples were analysed by western blotting.

Two-dimensional gel electrophoresis

Cells, or where indicated mitochondria $\pm$ phosphatase PP1 or YOP treatment (performed according to the manufacturers instructions), were solubilised in urea sample buffer of 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris base, 65 mM DTT, 1% IPG buffer pI 3–10 (GE Healthcare). Samples were diluted and loaded in rehydration buffer (8 M urea, 2% CHAPS, 5% glycerol and Bromophenol Blue) onto ReadyStrip IPG focusing strips (pH3–6; BioRad). Gels were incubated with equilibration buffer (50 mM Tris, 6 M Urea, 30% glycerol, 2% SDS and Bromophenol blue) for 2 $\times$ 15 min before

overlaying onto 12% polyacrylamide gels and electrophoresis carried out as described by western blotting (for scans of full 2D gels see Supplementary Figure 17).

Determination of Bak phosphorylation sites by mass spectrometry

Full-length Bak protein was epitope-tagged with 6 × His by PCR amplification using a primer containing the 6 × His sequence. The amplified fragment was re-inserted into pLXSN and transfected into PT67 packaging cells for retroviral production. His-Bak recombinant virus was used to infect HCT116 cells and transduced cells selected using G418 as before. All buffers and procedures used in the purification protocol were supplemented with PhosSTOP (Roche) to prevent non-specific dephosphorylation of proteins due to release of phosphatases from cellular compartments during cell lysis. Bak-expressing cells ($\sim 5 \times 10^7$ per isolation) were washed in ice-cold PBS, collected, then resuspended in lysis buffer (50 mM sodium phosphate (pH 8.0), 0.3 M NaCl, 2% CHAPS and 0.01% Tergitol). After incubation on ice for 30 min, lysates were cleared by centrifugation at 13000g. Cleared supernatant was incubated with Dynabeads[®] TALON[™] (Invitrogen) to isolate the His-tagged Bak protein. Bound proteins were collected and washed three times in lysis buffer and once in 50 mM sodium phosphate (pH 8.0), 7 M urea, 2 M thiourea, 2% CHAPS, then once more in lysis buffer. Bound proteins were eluted in denaturing SDS buffer and electrophoresed on 4–12% NuPAGE bis-Tris gels (Invitrogen). Fractionated proteins were stained with FOCUS-Fast silver (G Biosciences) before being excised for analysis. The purification and migration position of Bak in the gel system was confirmed by western blotting. Sample preparation of isolated Bak protein material and analysis by tandem mass spectrometry was performed essentially as described previously (Batycka *et al*, 2006; Sobott *et al*, 2009). In brief, the excised silver-stained gel bands containing Bak protein were digested with trypsin (Kinter and Sherman, 2000). Digested protein material was kept at 4°C until analysis. Sample analysis was performed by LC-MS/MS using an Ultimate[™] (LC-Packings, Dionex, Amsterdam, The Netherlands) HPLC system coupled on-line to a 3D high-capacity ion trap (HCTplus[™], Bruker Daltonics, Bremen, Germany) mass spectrometer through a pneumatically assisted nano-electrospray source (Batycka *et al*, 2006; Sobott *et al*, 2009). MS/MS spectra (peak lists) were searched against the SwissProt (UniProtKB/Swiss-Prot Release 2010_05 of April 20, 2010 contains 516 603 sequence entries) using Mascot version 2.2 (Matrixscience, London, UK) and the following parameters: peptide tolerance: 1Da, ¹³C=1, fragment tolerance: 1.2 Da, missed cleavages: 3, instrument type: ESI-TRAP. The interpretation and presentation of MS/MS data was performed according to published guidelines (Taylor and Goodlett, 2005) and the MS/MS spectra containing phosphorylated residues were inspected and interpreted manually for additional verification.

References

- Adams JM, Cory S (2007) The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* **26**: 1324–1337
- Akgul B, Ghali L, Davies D, Pfister H, Leigh IM, Storey A (2007) HPV8 early genes modulate differentiation and cell cycle of primary human adult keratinocytes. *Exp Dermatol* **16**: 590–599
- Antonsson B, Montessuit S, Sanchez B, Martinou JC (2001) Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. *J Biol Chem* **276**: 11615–11623
- Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD (2007) Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol* **9**: 550–555
- Balko JM, Jones BR, Coakley VL, Black EP (2009) Combined MEK and EGFR inhibition demonstrates synergistic activity in EGFR-dependent NSCLC. *Cancer Biol Ther* **8**: 522–530
- Balmano K, Cook SJ (2009) Tumour cell survival signalling by the ERK1/2 pathway. *Cell Death Differ* **16**: 368–377
- Batycka M, Inglis NF, Cook K, Adam A, Fraser-Pitt D, Smith DG, Main L, Lubben A, Kessler BM (2006) Ultra-fast tandem mass spectrometry scanning combined with monolithic column liquid

RNA interference

The siGenome Smartpools were synthesised by Dharmacon Research. The siRNA oligonucleotides targeted non-receptor tyrosine phosphatases listed in Supplementary Table 1. Oligonucleotides were transfected into HT1080 human fibrosarcoma cells, seeded to be 50–60% confluent on the day of transfection, using DharmaFECT 4 reagent (Dharmacon Research) according to the manufacturer's instructions. The oligonucleotide pools were used at a final concentration of 30 nM and incubated with the cells for 48 h before drug treatment and analysis by FACS. The screen was performed in duplicate with a correlation co-efficient = 0.75, indicating that the screen was robust. OriGene HuSH shRNA plasmids were transfected into HCT116 cells expressing pLXSN-WT Bak and selected using 0.4 µg/ml puromycin to generate cell lines with stable knockdown of PTPN5 (TR310053).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank A Barr, WH Lee and S. Knapp from The Structural Genome Consortium, Oxford, UK for discussion on the candidate phosphatases and structure; D Frith from CRUK London Research Institute for discussions on 2D gel methodology and analysis; B Vogestein and C Thompson for the kind gift of cell lines; members of the WIMM FACS core facility. FI was the recipient of an MRC clinical training fellowship. This study was funded by Cancer Research UK.

Authors Contributions: JLF constructed the MEF and HCT116 cells lines expressing different Bak mutants, performed immunoprecipitations, conducted the siRNA screen, constructed stable shRNA cell lines, performed transient overexpression studies, tryptic digests and qRT-PCR, performed 2D gel analysis, performed mitochondrial preparations, designed the study, analysed the data and wrote the paper; AS constructed the MEF and HCT116 cells lines expressing different Bak mutants, performed 2D gel analysis, designed the study, analysed the data and wrote the paper; FI performed and analysed FACS and performed 2D gel analysis; SJ performed 2D gel analysis; AA performed mitochondrial preparations and performed chemical cross-linking, oligomerisation assay and *in vitro* cytochrome release assay; SL performed initial phosphatase inhibitor studies; NT, MJE and BMK performed and analysed mass spectrometry; all authors discussed the results and commented on the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

- chromatography increases throughput in proteomic analysis. *Rapid Commun Mass Spectrom* **20**: 2074–2080
- Bertrand FE, Spengeman JD, Shelton JG, McCubrey JA (2005) Inhibition of PI3K, mTOR and MEK signaling pathways promotes rapid apoptosis in B-lineage ALL in the presence of stromal cell support. *Leukemia* **19**: 98–102
- Brooks C, Wei Q, Feng L, Dong G, Tao Y, Mei L, Xie ZJ, Dong Z (2007) Bak regulates mitochondrial morphology and pathology during apoptosis by interacting with mitofusins. *Proc Natl Acad Sci USA* **104**: 11649–11654
- Cheng EH, Sheiko TV, Fisher JK, Craigen WJ, Korsmeyer SJ (2003) VDAC2 inhibits BAK activation and mitochondrial apoptosis. *Science* **301**: 513–517
- Chipuk JE, Fisher JC, Dillon CP, Kriwacki RW, Kuwana T, Green DR (2008) Mechanism of apoptosis induction by inhibition of the anti-apoptotic BCL-2 proteins. *Proc Natl Acad Sci USA* **105**: 20327–20332
- Chipuk JE, Green DR (2004) Cytoplasmic p53: bax and forward. *Cell Cycle* **3**: 429–431
- Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, Maundrell K, Antonsson B, Martinou JC (1999) Bid-induced

- conformational change of Bax is responsible for mitochondrial cytochrome *c* release during apoptosis. *J Cell Biol* **144**: 891–901
- Dewson G, Kratina T, Czabotar P, Day CL, Adams JM, Kluck RM (2009) Bak activation for apoptosis involves oligomerization of dimers via their alpha6 helices. *Mol Cell* **36**: 696–703
- Dewson G, Kratina T, Sim HW, Puthalakath H, Adams JM, Colman PM, Kluck RM (2008) To trigger apoptosis, Bak exposes its BH3 domain and homodimerizes via BH3:groove interactions. *Mol Cell* **30**: 369–380
- Gavathiotis E, Suzuki M, Davis ML, Pitter K, Bird GH, Katz SG, Tu HC, Kim H, Cheng EH, Tjandra N, Walensky LD (2008) BAX activation is initiated at a novel interaction site. *Nature* **455**: 1076–1081
- Green DR (2005) Apoptotic pathways: ten minutes to dead. *Cell* **121**: 671–674
- Green DR, Reed JC (1998) Mitochondria and apoptosis. *Science* **281**: 1309–1312
- Griffiths GJ, Dubrez L, Morgan CP, Jones NA, Whitehouse J, Corfe BM, Dive C, Hickman JA (1999) Cell damage-induced conformational changes of the pro-apoptotic protein Bak *in vivo* precede the onset of apoptosis. *J Cell Biol* **144**: 903–914
- Haass NK, Sproesser K, Nguyen TK, Contractor R, Medina CA, Nathanson KL, Herlyn M, Smalley KS (2008) The mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitor AZD6244 (ARRY-142886) induces growth arrest in melanoma cells and tumor regression when combined with docetaxel. *Clin Cancer Res* **14**: 230–239
- Halle M, Liu YC, Hardy S, Theberge JF, Blanchetot C, Bourdeau A, Meng TC, Tremblay ML (2007a) Caspase-3 regulates catalytic activity and scaffolding functions of the protein tyrosine phosphatase PEST, a novel modulator of the apoptotic response. *Mol Cell Biol* **27**: 1172–1190
- Halle M, Tremblay ML, Meng TC (2007b) Protein tyrosine phosphatases: emerging regulators of apoptosis. *Cell Cycle* **6**: 2773–2781
- Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* **100**: 57–70
- Hesson LB, Cooper WN, Latif F (2007) Evaluation of the 3p21.3 tumour-suppressor gene cluster. *Oncogene* **26**: 7283–7301
- Hetman M, Kanning K, Cavanaugh JE, Xia Z (1999) Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *J Biol Chem* **274**: 22569–22580
- Hobbs S, Jitrapakdee S, Wallace JC (1998) Development of a bicistronic vector driven by the human polypeptide chain elongation factor 1alpha promoter for creation of stable mammalian cell lines that express very high levels of recombinant proteins. *Biochem Biophys Res Commun* **252**: 368–372
- Hoshino R, Chatani Y, Yamori T, Tsuruo T, Oka H, Yoshida O, Shimada Y, Ari-i S, Wada H, Fujimoto J, Kohno M (1999) Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene* **18**: 813–822
- Jackson S, Harwood C, Thomas M, Banks L, Storey A (2000) Role of Bak in UV-induced apoptosis in skin cancer and abrogation by HPV E6 proteins. *Genes Dev* **14**: 3065–3073
- Jiang P, Du W, Heese K, Wu M (2006) The Bad guy cooperates with good cop p53: Bad is transcriptionally up-regulated by p53 and forms a Bad/p53 complex at the mitochondria to induce apoptosis. *Mol Cell Biol* **26**: 9071–9082
- Kepp O, Rajalingam K, Kimmig S, Rudel T (2007) Bak and Bax are non-redundant during infection- and DNA damage-induced apoptosis. *EMBO J* **26**: 825–834
- Kim H, Tu HC, Ren D, Takeuchi O, Jeffers JR, Zambetti GP, Hsieh JJ, Cheng EH (2009) Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis. *Mol Cell* **36**: 487–499
- Kinter M, Sherman NE (2000) *Protein Sequencing and Identification Using Tandem Mass Spectrometry*. New York: John Wiley and Sons
- Korff S, Woerner SM, Yuan YP, Bork P, von Knebel Doeberitz M, Gebert J (2008) Frameshift mutations in coding repeats of protein tyrosine phosphatase genes in colorectal tumors with microsatellite instability. *BMC Cancer* **8**: 329
- Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, Schlesinger PH (2000) Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome *c*. *Cell Death Differ* **7**: 1166–1173
- Kutuk O, Arisan ED, Tezil T, Shoshan MC, Basaga H (2009) Cisplatin overcomes Bcl-2-mediated resistance to apoptosis via preferential engagement of Bak: critical role of Noxa-mediated lipid peroxidation. *Carcinogenesis* **30**: 1517–1527
- Labi V, Grespi F, Baumgartner F, Villunger A (2008) Targeting the Bcl-2-regulated apoptosis pathway by BH3 mimetics: a breakthrough in anticancer therapy? *Cell Death Differ* **15**: 977–987
- Lee CF, Ling ZQ, Zhao T, Fang SH, Chang WC, Lee SC, Lee KR (2009) Genomic-wide analysis of lymphatic metastasis-associated genes in human hepatocellular carcinoma. *World J Gastroenterol* **15**: 356–365
- Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ (2002) Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* **2**: 183–192
- Letai AG (2008) Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nat Rev Cancer* **8**: 121–132
- Leu JI, Dumont P, Hafey M, Murphy ME, George DL (2004) Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. *Nat Cell Biol* **6**: 443–450
- Mariotti M, Castiglioni S, Beguinot L, Maier JA (2006) The tyrosine phosphatase HD-PTP is regulated by FGF-2 through proteasome degradation. *Front Biosci* **11**: 2138–2143
- Milella M, Precupanu CM, Gregorj C, Ricciardi MR, Petrucci MT, Kornblau SM, Tafuri A, Andreeff M (2005) Beyond single pathway inhibition: MEK inhibitors as a platform for the development of pharmacological combinations with synergistic anti-leukemic effects. *Curr Pharm Des* **11**: 2779–2795
- Moldoveanu T, Liu Q, Tocilj A, Watson M, Shore G, Gehring K (2006) The X-ray structure of a BAK homodimer reveals an inhibitory zinc binding site. *Mol Cell* **24**: 677–688
- Nechushtan A, Smith CL, Hsu YT, Youle RJ (1999) Conformation of the Bax C-terminus regulates subcellular location and cell death. *EMBO J* **18**: 2330–2341
- Paul S, Snyder GL, Yokakura H, Picciotto MR, Nairn AC, Lombroso PJ (2000) The dopamine/D1 receptor mediates the phosphorylation and inactivation of the protein tyrosine phosphatase STEP via a PKA-dependent pathway. *J Neurosci* **20**: 5630–5638
- Pietsch EC, Perchiniak E, Canutescu AA, Wang G, Dunbrack RL, Murphy ME (2008) Oligomerization of BAK by p53 utilizes conserved residues of the p53 DNA binding domain. *J Biol Chem* **283**: 21294–21304
- Pulido R, Zuniga A, Ullrich A (1998) PTP-SL and STEP protein tyrosine phosphatases regulate the activation of the extracellular signal-regulated kinases ERK1 and ERK2 by association through a kinase interaction motif. *EMBO J* **17**: 7337–7350
- Roy SS, Ehrlich AM, Craigen WJ, Hajnoczky G (2009) VDAC2 is required for truncated BID-induced mitochondrial apoptosis by recruiting BAK to the mitochondria. *EMBO Rep* **10**: 1341–1347
- Ruffolo SC, Shore GC (2003) BCL-2 selectively interacts with the BID-induced open conformer of BAK, inhibiting BAK auto-oligomerization. *J Biol Chem* **278**: 25039–25045
- Shimizu T, Miyakawa Y, Iwata S, Kuribara A, Tiganis T, Morimoto C, Ikeda Y, Kizaki M (2004) A novel mechanism for imatinib mesylate (STI571) resistance in CML cell line KT-1: role of TC-PTP in modulating signals downstream from the BCR-ABL fusion protein. *Exp Hematol* **32**: 1057–1063
- Snyder EM, Nong Y, Almeida CG, Paul S, Moran T, Choi EY, Nairn AC, Salter MW, Lombroso PJ, Gouras GK, Greengard P (2005) Regulation of NMDA receptor trafficking by amyloid-beta. *Nat Neurosci* **8**: 1051–1058
- Sobott F, Watt SJ, Smith J, Edelmann MJ, Kramer HB, Kessler BM (2009) Comparison of CID versus ETD based MS/MS fragmentation for the analysis of protein ubiquitination. *J Am Soc Mass Spectrom* **20**: 1652–1659
- Sundararajan R, White E (2001) E1B 19K blocks Bax oligomerization and tumor necrosis factor alpha-mediated apoptosis. *J Virol* **75**: 7506–7516
- Suzuki M, Youle RJ, Tjandra N (2000) Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell* **103**: 645–654
- Taylor GK, Goodlett DR (2005) Rules governing protein identification by mass spectrometry. *Rapid Commun Mass Spectrom* **19**: 3420
- Unschuld PG, Dachsel J, Darios F, Kohlmann A, Casademunt E, Lehmann-Horn K, Dichgans M, Ruberg M, Brice A, Gasser T,

- Lucking CB (2006) Parkin modulates gene expression in control and ceramide-treated PC12 cells. *Mol Biol Rep* **33**: 13–32
- Uren RT, Dewson G, Chen L, Coyne SC, Huang DC, Adams JM, Kluck RM (2007) Mitochondrial permeabilization relies on BH3 ligands engaging multiple prosurvival Bcl-2 relatives, not Bak. *J Cell Biol* **177**: 277–287
- Wang H, Takemoto C, Akasaka R, Uchikubo-Kamo T, Kishishita S, Murayama K, Terada T, Chen L, Liu ZJ, Wang BC, Sugano S, Tanaka A, Inoue M, Kigawa T, Shirouzu M, Yokoyama S (2009) Novel dimerization mode of the human Bcl-2 family protein Bak, a mitochondrial apoptosis regulator. *J Struct Biol* **166**: 32–37
- Waterhouse NJ, Trapani JA (2003) A new quantitative assay for cytochrome *c* release in apoptotic cells. *Cell Death Differ* **10**: 853–855
- Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson CB, Korsmeyer SJ (2000) tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome *c*. *Genes Dev* **14**: 2060–2071
- Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, Adams JM, Huang DC (2005) Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* **19**: 1294–1305
- Willis SN, Fletcher JI, Kaufmann T, van Delft MF, Chen L, Czabotar PE, Ierino H, Lee EF, Fairlie WD, Bouillet P, Strasser A, Kluck RM, Adams JM, Huang DC (2007) Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science* **315**: 856–859
- Yamaguchi R, Andreyev A, Murphy AN, Perkins GA, Ellisman MH, Newmeyer DD (2007) Mitochondria frozen with trehalose retain a number of biological functions and preserve outer membrane integrity. *Cell Death Differ* **14**: 616–624
- Yousefi S, Simon HU (2003) SHP-1: a regulator of neutrophil apoptosis. *Semin Immunol* **15**: 195–199