RESEARCH ARTICLE

BH3-only proteins Noxa, Bik, Bmf, and Bid activate Bax and Bak indirectly when studied in yeast model

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Abstract

BH3-only proteins of the Bcl-2 family regulate programmed cell death in mammals through activation of multidomain proapoptotic proteins Bax and Bak in response to various proapoptotic stimuli by mechanism that remains under dispute. Here, we report that the cell death-promoting activity of BH3-only proteins Bik, Bmf, Noxa, and tBid can only be reconstituted in yeast when both multidomain proapoptotic and antiapoptotic Bcl-2 family proteins are present. Inability of these proteins to induce cell death in the absence of antiapoptotic proteins suggests that all tested BH3-only proteins likely activate Bax and Bak indirectly by inhibiting antiapoptotic proteins.

Introduction

The programmed cell death and its morphological manifestation, apoptosis, is an active process, by which multicellular organisms eliminate unwanted cells (Jacobson *et al.*, 1997; Vaux & Korsmeyer, 1999). These are either the cells that have completed their task and have to be removed (e.g. removal of tissues during development), or cells that have became damaged and their repair or maintenance would do more harm to the organism than their elimination. Precise regulation of this process is vital for organismal development and homeostasis, as defects in cell death processes result in serious diseases (Thompson, 1995). Two pathways that activate apoptosis have been described, extrinsic (receptor) pathway that responds to the external signals and intrinsic pathway (also called mitochondrial) that initiates apoptosis in response to cellular damage or internal signals. The crucial event leading to the activation of the cell death program, which is considered a commitment point of mitochondrial pathway, is the release of cytochrome *c* and several other proteins from mitochondria into the cytosol, and it is regulated by proteins of the Bcl-2 family.

The Bcl-2 family consists of both proapoptotic proteins that stimulate the release of cytochrome *c* and antiapoptotic proteins that inhibit the release. All members of the family share a sequence homology in one to four Bcl-2 homology domains (BH1-BH4). The mechanisms, by which proapoptotic members of the Bcl-2 family promote the release of mitochondrial factors like cytochrome *c*, or by which antiapoptotic family members block this release, are still not understood. In all models, proapoptotic family members Bax and Bak, which contain three BH domains (BH1-BH3), are essential for programmed cell death, as double knockout $bax^{-/-}bak^{-/-}$ mice exhibit severely impaired apoptosis during development (Lindsten *et al.*, 2000). These two proteins directly participate at the formation of the pore that permeabilizes outer mitochondrial membrane for cytochrome *c*, most likely by being a major component of the pore. Antiapoptotic family members (e.g. Bcl-X_L and Bcl-2), which contain all

four BH domains, on the other hand inhibit formation of the pore by Bax and Bak. In the absence of death signal, inactive Bax and Bak are localized in cytosol and outer mitochondrial membrane, respectively. Following death signal, Bax is translocated to mitochondria, and both proteins oligomerize in the outer mitochondrial membrane and form pores (Hsu *et al.*, 1997; Wolter *et al.*, 1997).

A third subset of proapoptotic proteins is characterized by containing only one of the conserved BH domains, BH3, and is therefore referred to as BH3-only proteins. These proteins control the activation of molecules like Bax and Bak in response to specific death/survival signal. Death-inducing activity of BH3-only proteins is regulated by mechanisms that vary widely and involve transcriptional control of their expression, as well as post-translational modifications. For example, BH3-only protein Noxa is regulated by p53-dependent control of transcription (Oda *et al.*, 2000). Bid is a BH3-only protein that links extrinsic and mitochondrial pathways of apoptosis as it transduces signals from 'death receptors' on the surface of the cell. After the ligand binding, the receptors recruit and activate caspase-8, which then activates Bid by a proteolytic cleavage to generate a truncated, active form, tBid (Korsmeyer *et al.*, 2000). Bik (Nbk) is a BH3-only protein, whose activity appears to depend on phosphorylation/dephosphorylation, although relevant details remain to be defined (Verma *et al.*, 2001). Two BH3-only proteins, Bim and Bmf, are bound to cytoskeletal elements (Bim to dynein complex associated with microtubules; Bmf to myosin complex bound to actin filaments) (Puthalakath *et al.*, 1999, 2001). Following activation, Bim and Bmf are released from the resting complexes and translocate to mitochondria. However, the precise signal that leads to the activation of these, and a number of other BH3-only proteins, has yet to be defined.

For mechanism, by which BH3-only proteins activate Bax and Bak, two models have been suggested. In first, direct activation model, BH3-only proteins directly interact with Bax and Bak. In the indirect model, on the other hand, BH3-only proteins bind and inhibit the antiapoptotic proteins like Bcl-2 and Bcl-X_L. These two models are not mutually exclusive, and different BH3-only proteins may not necessarily employ the same one (Willis *et al.*, 2007).

Yeast *Saccharomyces cerevisiae* has been a model of choice for studying biochemical and biological phenomena in eukaryotic cells. Although programmed cell death pathways in yeast differ significantly from those in metazoans, yeast have been successfully used as model organism for studying the function of Bcl-2 proteins as an advantageous alternative to *in vitro* techniques. Expression of Bax or Bak in yeast induces permeabilization of mitochondrial membranes that is accompanied by the release of cytochrome *c* to the cytosol and that ultimately results in cell death (Manon *et al.*, 1997; Zha & Reed, 1997). While cytochrome *c* does not play an active role in this cell death scenario (Gross *et al.*, 2000), it is likely that cells die because the permeabilization of mitochondrial outer membrane results in impairment of mitochondrial biogenesis – a process essential in all eukaryotes – (Kissova *et al.*, 2000) as well as in the release of yeast-native proapoptotic molecules such as Aif1p and Endonuclease G from mitochondria (Wissing *et al.*, 2004; Buttner *et al.*, 2007). All of these effects can be counteracted by coexpression of antiapoptotic proteins, for example Bcl-X_L or Bcl-2, suggesting that mechanisms of their action in yeast do reflect those involved in permeabilization of the mitochondrial membrane in metazoans (Manon *et al.*, 1997). In this work, we expressed BH3-only proteins tBid, Bmf, Bik, and Noxa together with multidomain proapoptotic (Bax and Bak) and antiapoptotic (Bcl-X_L and Bcl-2) proteins in yeast *S. cerevisiae* to reconstruct the pathway responsible for the release of cytochrome *c* and to study the mechanisms by which BH3-only proteins act to activate Bax and Bak.

Materials and methods

Strains, plasmids, and growth conditions

The yeast strains used in this study were CML282 (*MATa ura3-1, ade2-1, leu2-3,112, his3-11,15, trp1-Δ2, can1-100, CMVp (tetR-SSN6)::LEU2*) (Belli *et al.*, 1998) and its derivatives, CML282/GAL-BAX (Polcic & Forte, 2003) and CML282/GAL-BAK (Juhasova *et al.*, 2011), containing coding sequence of murine Bax or Bak N-terminally tagged with haemagglutinin (HA) epitope, downstream from GAL1/10 promoter, integrated into genomic *HIS3* locus.

Cells were grown on minimal selective media (0.67% yeast nitrogen base, 0.055% complete supplement mixture) containing the indicated carbon sources and lacking the appropriate amino acids or nucleobases. Yeast were transformed by standard lithium acetate protocols (Schiestl & Gietz, 1989).

To modulate expression of Bax and Bak, cells were grown to the exponential phase in appropriate selective media containing 2% glucose, cells were then harvested by centrifugation, washed twice by resuspension in sterile water, and transferred to appropriate selective liquid or solid media containing 2% raffinose and the indicated concentration of galactose. To modulate the expression of Bcl-X_L/Bcl-2, cells were transformed with plasmids pCM252-HA-BCL-XL or pCM252-BCL2, grown in glucose-based media as described above, washed and transferred to selective liquid or solid media containing indicated concentration of doxycycline.

To express the BH3-only proteins, the following constructs were prepared.

Bik and HA-Bik

The BamHI-EcoRI fragment from plasmid pCMV-SPORT6-BIK (Open Biosystems, Huntsville; MMM1013-63239) containing full length of mouse BIK cDNA was subcloned into centromeric p416MET25 (Mumberg *et al.*, 1995) cleaved with BamHI/EcoRI. The *Kpn*I-*Sac*I fragment from resulting plasmid (p416MET-BIK) containing MET25-BIK sequences was then ligated with multicopy plasmid pRS426 (Christianson *et al.*, 1992) cleaved with the same enzymes, resulting in p426MET-BIK. The N-terminal HA tag was introduced by PCR using forward primer containing HA sequence and the BamHI restriction site and reverse primer with the EcoRI site (5′-CGCGGATCCATGTATCCATATGATGTTCCAGATTATGCTTCGGAGGCGAGACTTATGGCCAGAG-3′; 5′-CCGGAATTCTCACTGAAGCTGCAAATACC-3′) and p426MET-BIK as a template. The PCR product was cleaved with BamHI/EcoRI and ligated with p426MET25 cleaved with BamHI/EcoRI to produce p426MET-HA-BIK.

Bmf and HA-Bmf

DNA containing open reading frame of BMF obtained by PCR using mouse cDNA (a gift from Dr Eva Varečková, Slovak Academy of Sciences, Bratislava, Slovak Republic) as a template and primers containing the BamHI and EcoRI restriction sites (5′-ATGCGGATCCATGGAGCCACCCTCAGTGTGT-3′; 5′-TACGGAATTCTGCAGTCACCAGGGCCCCACCCCTT-3′) was cloned into BamHI/EcoRI sites of p416MET25. The product of second PCR using primers containing HA sequence and the BamHI restriction site (5′-CGCGGATCCATGTATCCATATGATGTTCCAGATTATGCTGAGCCACCTCAGTGTGTGGAGGAGCTA-3′) and the EcoRI site (5′-CCGGAATTCTCACCAGGGCCCCACCCCTT-3′) and p416MET-BMF as a template was digested with BamHI/EcoRI and ligated with p426MET25 cleaved with BamHI/EcoRI, producing p426MET-HA-BMF.

Noxa

To prepare p416MET-NOXA, the DNA fragment containing full-length mouse NOXA cDNA obtained by PCR using plasmid pCMV-SPORT6.1-NOXA (Open Biosystems; EMM1002-6901985) as a template and oligonucleotide primers containing the BamHI (5′-ATGCGGATCCATGCCCGGGAGAAAGGCGCG-3′) and EcoRI (5′-TACGGAATTCTGCAGCTGGGAGGTCCCTTCTTGCA-3′) restriction sites was subcloned into BamHI/EcoRI sites of p416MET25. The *Kpn*I-*Sac*I fragment from resulting plasmid (p416MET-NOXA) containing MET25-NOXA sequences was then ligated with multicopy plasmid pRS426 cleaved with same enzymes, resulting in p426MET-NOXA. The product of second PCR using primers that contain HA sequence and the BamHI restriction site (5′-CGCGGATCCATGTATCCATATGATGTTCCAGATTATGCTCCCGGGAG-AAAGGCGCGTCGGAAGCGC-3′) and the EcoRI site (5′-CCGGAATTCTCAGGTTACTAAATTGAAGAGC-3′) and p416MET-NOXA as a template was digested with BamHI/EcoRI and ligated with p426MET25 cleaved with BamHI/EcoRI to produce p426MET-HA-NOXA.

tBid

To prepare p416MET-tBID, a plasmid pYES2-tBid (kindly provided by Dr Atan Gross, Weizmann Institute, Rehovot, Israel) was cleaved with EcoRI/XhoI, and obtained DNA fragment containing coding sequence of murine tBid gene was ligated with p416MET25 cleaved with same enzymes.

All constructs were verified by DNA sequencing. To induce the expression of BH3-only proteins, cells were grown in glucose-based media, washed twice, and transferred to the indicated selective liquid or solid media without methionine.

To assess the growth potential of individual strains, cells were grown overnight in media containing 2% glucose, diluted to $A_{600} = 0.5$, and 10 μL aliquots of serial fivefold dilutions were spotted on to test plates. Growth was evaluated following incubation at 30 °C for 2–3 days.

Preparation of yeast extracts, immunoblotting, and cell fractionation

Protein extracts were prepared by alkaline lysis and trichloroacetic acid precipitation (Yaffe & Schatz, 1984), separated by electrophoresis on SDS-polyacrylamide gels (12 or 15%), and transferred to nitrocellulose membrane by semi-dry or wet electroblotting. Antibodies used for protein detection were a mouse monoclonal antibody directed against the HA epitope (1 : 1000; Santa Cruz Biotechnology, sc-7392), a mouse monoclonal anti-yeast cytochrome *c* oxidase subunit II (1 : 5000; Molecular Probes, #A6407), and a rabbit polyclonal anti-Bid (1 : 1000; BD Pharmingen, #559681). Immunoblots were subsequently incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1 : 5000; Promega, #W401(2)B), and binding was visualized using a chemiluminescent substrate (Pierce, #34080). The chemiluminescence was detected by scanning with a Kodak Digital Image station 4000MM.

Mitochondria were prepared as described previously (Zinser & Daum, 1995; Gross *et al.*, 2000). Briefly, cells

were converted into spheroplasts by enzymatic digestion with Zymolyase 20T (MP Biomedicals) and homogenized with a glass homogenizer. The homogenate was centrifuged at 3000 ***g***, and the resulting supernatant was centrifuged at 10 000 ***g*** to pellet heavy membranes enriched in mitochondria. The supernatant was recentrifuged at 10 000 ***g***, and subsequent supernatant containing the cytosol (proteins were precipitated by addition of trichloroacetic acid to 7%, centrifuged and washed with acetone) was loaded on to SDS-PAGE gels. The heavy membrane fraction was washed twice with homogenization buffer prior to separation on SDS-PAGE gels.

Results and discussion

Both models of activation of Bax and Bak by BH3-only proteins did gain a significant experimental support. We attempted to address the question of which model is correct by characterizing phenotypes that selected combinations of Bcl-2 family members induce when expressed in yeast. To test the activity of selected BH3-only proteins in *S. cerevisiae* model system, we constructed yeast expression vectors containing coding sequences of murine genes encoding for BH3-only proteins Bik, Bmf, Noxa, and Bid, downstream of promoter repressible by methionine (pMET25). As in mammalian cells full-length Bid is inactive and has to be activated by proteolytic cleavage, to express active Bid, we used a gene construct encoding for truncated form of Bid (tBid). To be able to immunodetect expressed proteins, the versions encoding for proteins tagged with HA epitope were also constructed.

As expected, expression of either of these proteins did not induce any growth phenotype in yeast when no other members of Bcl-2 family were expressed (Fig. 1), reflecting the fact that their cell death-inducing activity in mammalian cell is strictly dependent on the presence of proapoptotic proteins Bax or Bak, both absent in yeast cells.

On the other hand, all of the tested BH3-only proteins did induce cell death in cells coexpressing both proapoptotic Bax or Bak and antiapoptotic Bcl-X_L or Bcl-2 (Figs 2a and Supporting Information, S1). As reported earlier, the level of expression of antiapoptotic proteins using pCM252-based plasmids is proportional to the concentration of doxycycline in the growth media (Polcic & Forte, 2003). When concentration of doxycycline is higher than 0.1 μg mL^{-1}, the amount of expressed antiapoptotic protein is sufficient to completely inhibit cell death-promoting activity of Bax or Bak and to maintain the viability of Bax or Bak expressing strain indistinguishable from the viability of the control yeast strain not expressing any of Bcl-2 family proteins. Cell death-inducing effect of BH3-only proteins was strongest on media containing 0.05–0.1 μg mL^{-1} doxycycline (Fig. 2) and decreased progressively with increasing concentration of doxycycline (not shown). As indicated by Western blot (Fig. 2b), the expression of BH3-only proteins did not interfere with the expression of antiapoptotic proteins. The growth phenotype can thus be solely attributed to cell death-promoting activity of BH3-only proteins. Virtually no difference in effects of BH3-only proteins on cell viability was observed between strains coexpressing Bax with Bcl-X_L and Bax with Bcl-2 (Fig. 2a). The same results were obtained in strains coexpressing Bak with Bcl-X_L and Bak with Bcl-2 (Fig. S1). In this case, the only BH3-only protein displaying limited selectivity for Bcl-X_L was Noxa.

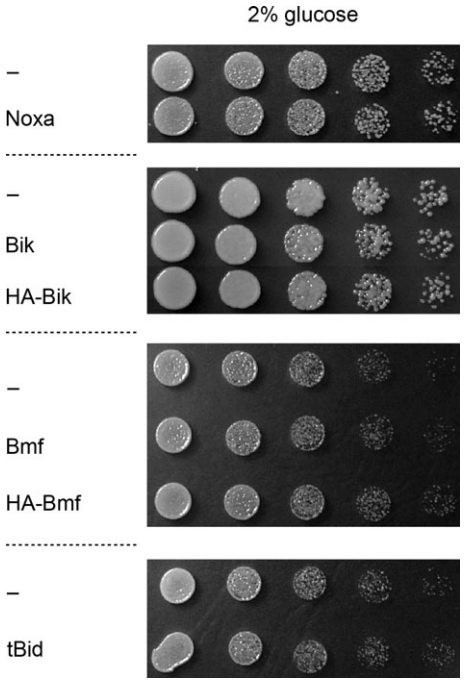

Fig. 1. Ectopic expression of BH3-only family members alone does not affect the viability of yeast cells. CML282 cells were transformed with plasmids supporting expression of Noxa (p426MET-NOXA), HA-Bik (p426MET-HA-BIK), HA-Bmf (p426MET-HA-BMF), tBid (p416MET-tBID), or with control plasmid (respective empty vector), cultivated in selective glucose media, and the viability was assessed by spotting serial fivefold dilutions on synthetic complete (SC) media containing 2% glucose and no methionine.

To test whether the expression of BH3-only proteins can directly potentiate the Bax- or Bak-induced killing in the absence of an antiapoptotic protein, we have expressed BH3-only proteins in strains coexpressing Bax or Bak. As shown in Fig. 3, expression of either BH3-only protein did not induce any growth phenotype when Bax was expressed to sublethal doses. The same results were obtained in cells coexpressing BH3-only proteins and Bak (Fig. S2), suggesting that tested BH3-only proteins are

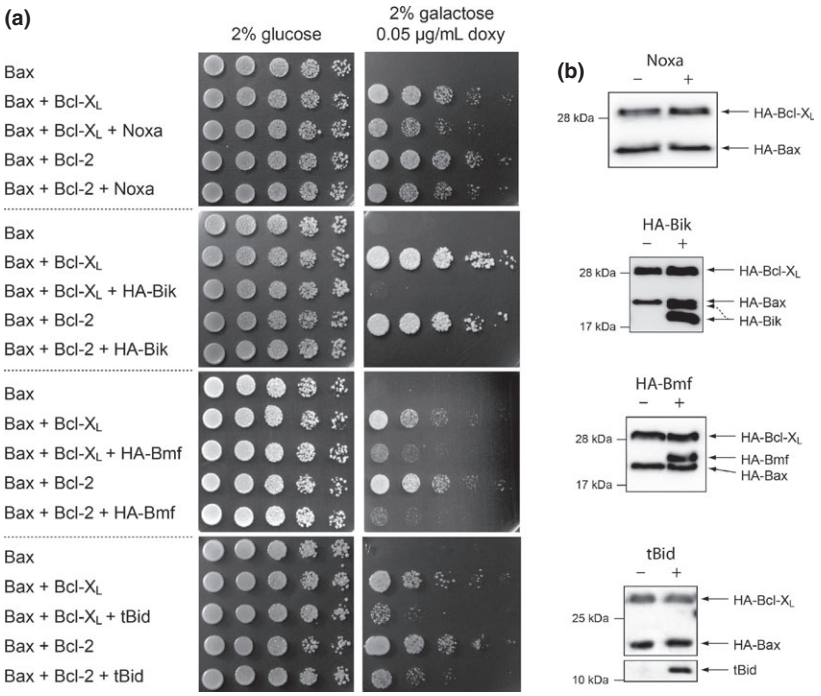

Fig. 2. Expression of BH3-only members of Bcl-2 family inhibits prosurvival activity of Bcl-X_L and Bcl-2. (a) CML282/GAL-BAX (Bax) cells transformed with TET-BCL-XL (Bcl-X_L) or TET-BCL2 (Bcl-2) plasmid together with either p426MET-NOXA (Noxa), p426MET-HA-BIK (HA-Bik), p426MET-HA-BMF (HA-Bmf), p416MET-tBID (tBid), or corresponding empty vector were cultivated in glucose media, and cell suspensions were spotted onto SC plates containing indicated carbon source and concentration of doxycycline. (b) Expression of Bax and Bcl-XL in cells, either not containing or containing BH3-only proteins, under relevant cultivation conditions was detected by immunoblotting.

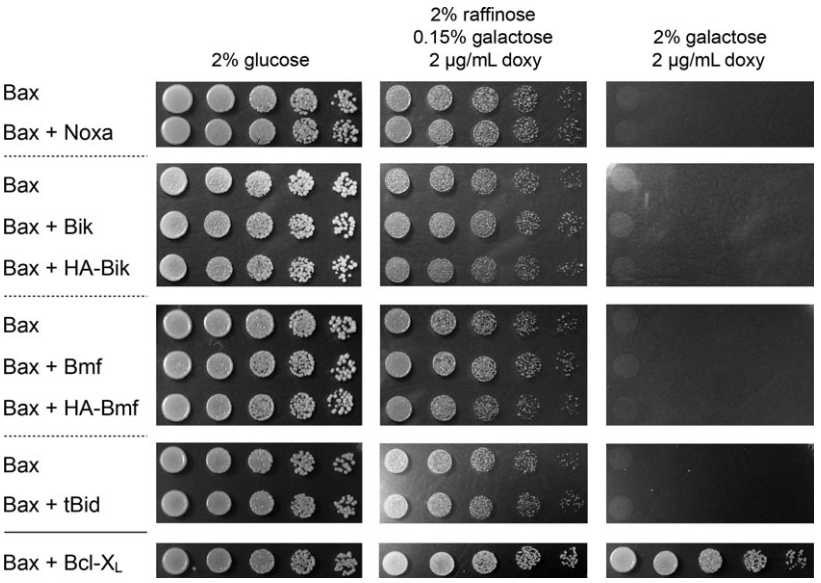

Fig. 3. Mammalian BH3-only proteins expressed in yeast do not potentiate proapoptotic effect of Bax. CML282/GAL-BAX cells transformed with plasmids supporting expression of Noxa (p426MET-NOXA), HA-Bik (p426MET-HA-BIK), HA-Bmf (p426MET-HA-BMF), tBid (p416MET-tBID), or with control plasmid (respective empty vector) were cultivated in glucose media, and cell suspensions were spotted on SC plates containing indicated carbon source and no methionine. Medium was supplemented with 2 μg mL^{-1} doxycycline to support viability of control strain – CML282/GAL-BAX + TET-BCL-XL (Bax + Bcl-X_L).

not able to directly potentiate cell-killing activity of Bax or Bak.

All of the BH3-only proteins tested did induce cell death in yeast when they were expressed together with both antiapoptotic and proapoptotic proteins, while no phenotype of BH3-only protein expression could be observed when the BH3-only proteins were coexpressed with proapoptotic proteins Bax or Bak in the absence of antiapoptotic proteins. This observation would favor the indirect model, in which BH3-only proteins do inhibit the antiapoptotic activity of Bcl-X_L and Bcl-2 that would lead to activation of Bax and Bak. One of the weaknesses of the indirect model is the fact that prior to death signal, Bax and Bak do not appear to be bound to antiapoptotic proteins. This can be explained by the multistep activation of Bax and Bak, in which the inhibition of antiapoptotic proteins would be one of the steps (Letai *et al.*, 2002). However, our previous analyses of the activity of Bax and Bcl-X_L in yeast have shown that mechanisms involved in the inhibition of proapoptotic activity of Bax

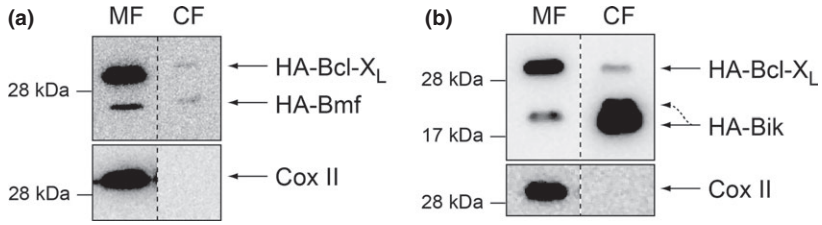

Fig. 4. Cellular localization of BH3-only proteins HA-Bmf and HA-Bik in yeast. Heavy membrane fractions enriched in mitochondria (MF) and cytosolic fractions (CF) were prepared from cells containing HA-tagged murine Bmf (a) or Bik (b) together with HA-Bcl-X_L and HA-Bax. Proteins in subcellular fractions were separated by SDS-PAGE and analyzed by Western blotting. Both BH3-only proteins as well as coexpressed HA-Bcl-X_L were detected by anti-HA antibody. No expression of HA-Bax is observed under conditions of cultivation used (SC + raffinose + doxycycline, no methionine). Mitochondrial cytochrome *c* oxidase subunit II protein (Cox II) is shown as mitochondrial marker.

by Bcl-X_L may depend on mechanisms other than heterodimerization of these proteins (Polcic & Forte, 2003), and this also reflects observed ability of Bcl-X_L mutant unable to bind to Bax to inhibit Bax in both yeast and mammalian cells (Minn *et al.*, 1999). In the model proposed to describe these observations, for their death-inducing activity Bax or Bak needs to associate with not yet identified target site in outer mitochondrial membrane. Antiapoptotic proteins (e.g. Bcl-X_L or Bcl-2) would associate with these sites preferentially, preventing the binding of Bax or Bak and thus inhibiting their activity (Polcic & Forte, 2003). Inhibition of antiapoptotic proteins by BH3-only proteins would then indirectly activate Bax or Bak, without binding of Bax and Bak to any Bcl-2 proteins.

Other model, which seems to be supported by patterns of behavior of these proteins in yeast, is the model, in which Bax may induce the formation of lipid channels in the outer mitochondrial membrane (Colombini, 2010). These proposed channels assembled from molecules of ceramide are disassembled by Bcl-X_L (Siskind *et al.*, 2008; Ganesan *et al.*, 2010). Inhibition of Bcl-X_L by BH3-only proteins would then in the presence of Bax result in the formation of pores and release of cytochrome *c*.

As mentioned earlier, in mammalian cell, Bmf is localized in the cytosol bound to actin filaments and only after death signal, Bmf translocates to mitochondria. Bik is believed to be localized in the membrane of the endoplasmic reticulum, and its translocation to mitochondria was not observed. We assessed the intracellular localization of these proteins in yeast. Bmf was detected in the fraction enriched in mitochondria (Fig. 4a), indicating that it does not bind to actin cytoskeleton in yeast and is rather targeted directly to the mitochondria. This is indeed consistent with constitutive cell-killing activity of Bmf in yeast and no need for activation of Bmf as it is in mammalian cells. In contrast to mitochondrial localization of Bmf, most of the Bik was detected in the cytosolic fraction (Fig. 4b). This reflects the localization of Bik observed in mammalian cells as cytosolic fraction also contains microsomes, however, makes the question of mechanisms by which Bik inhibits antiapoptotic proteins more complicated. Possibly, Bik does not translocate to mitochondria, but the protein present in the fraction of endoplasmic reticulum localized adjacent to mitochondria is able to bind antiapoptotic molecules in the outer mitochondrial membrane.

Although yeast have been used as a model system devoid of native Bcl-2 proteins, recently a motif resembling the BH3 domain has been identified in a protein previously described as yeast homologue of Bax inhibitor-1 (Chae *et al.*, 2003; Buttner *et al.*, 2011). The activity of this protein, referred to either as Bxi1p (Bax inhibitor-1) or Ybh3p (Yeast BH3-only protein), has been discrepantly described either as an inhibitor or inducer of yeast cell death (Buttner *et al.*, 2011; Cebulski *et al.*, 2011). As all strains used in this work contained intact *BXI1/YBH3,* differences in the growth phenotypes induced by the expression of murine BH3-only proteins are unlikely to be affected by Bxi1p/Ybh3p. If the expression of native *BXI1/YBH3* does influence the activity of pro- (e.g. Bax, Bak) or antiapoptotic proteins (e.g. Bcl-X_L, Bc-2) as suggested in Chae *et al.* (2003) or Buttner *et al.* (2011), then it would be reasonable only to expect that the baseline sensitivity toward the expression of Bcl-2 proteins would be equally affected in all strains used in this work as compared to the strains devoid of this putative BH3-only protein.

To conclude, all of the studied BH3-only proteins did activate Bax and Bak indirectly by inhibition of antiapoptotic activity of Bcl-2 and Bcl-X_L when expressed in yeast *S. cerevisiae*. Even though no support for direct activation of Bax and Bak was observed in our experimental setting, this alternative cannot be ruled out for other BH3-only proteins of Bcl-2 family.

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Authors' contribution

P.P. and M.M. contributed equally to this work.

References

Belli G, Gari E, Piedrafita L, Aldea M & Herrero E (1998) An activator/repressor dual system allows tight tetracycline-regulated gene expression in budding yeast. *Nucleic Acids Res* **26**: 942–947.

Buttner S, Eisenberg T, Carmona-Gutierrez D *et al.* (2007) Endonuclease G regulates budding yeast life and death. *Mol Cell* **25**: 233–246.

Buttner S, Ruli D, Vogtle FN *et al.* (2011) A yeast BH3-only protein mediates the mitochondrial pathway of apoptosis. *EMBO J* **30**: 2779–2792.

Cebulski J, Malouin J, Pinches N, Cascio V & Austriaco N (2011) Yeast Bax inhibitor, Bxi1p, is an ER-localized protein that links the unfolded protein response and programmed cell death in *Saccharomyces cerevisiae*. *PLoS One* **6**: e20882.

Chae HJ, Ke N, Kim HR, Chen S, Godzik A, Dickman M & Reed JC (2003) Evolutionarily conserved cytoprotection provided by Bax Inhibitor-1 homologs from animals, plants, and yeast. *Gene* **323**: 101–113.

Christianson TW, Sikorski RS, Dante M, Shero JH & Hieter P (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**: 119–122.

Colombini M (2010) Ceramide channels and their role in mitochondria-mediated apoptosis. *Biochim Biophys Acta* **1797**: 1239–1244.

Ganesan V, Perera MN, Colombini D, Datskovskiy D, Chadha K & Colombini M (2010) Ceramide and activated Bax act synergistically to permeabilize the mitochondrial outer membrane. *Apoptosis* **15**: 553–562.

Gross A, Pilcher K, Blachly-Dyson E, Basso E, Jockel J, Bassik MC, Korsmeyer SJ & Forte M (2000) Biochemical and genetic analysis of the mitochondrial response of yeast to BAX and BCL-X(L). *Mol Cell Biol* **20**: 3125–3136.

Hsu YT, Wolter KG & Youle RJ (1997) Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. *P Natl Acad Sci USA* **94**: 3668–3672.

Jacobson MD, Weil M & Raff MC (1997) Programmed cell death in animal development. *Cell* **88**: 347–354.

Juhasova B, Bhatia-Kissova I, Polcicova K, Mentel M, Forte M & Polcic P (2011) Reconstitution of interactions of Murine gammaherpesvirus 68 M11 with Bcl-2 family proteins in yeast. *Biochem Biophys Res Commun* **407**: 783–787.

Kissova I, Polcic P, Kempna P, Zeman I, Sabova L & Kolarov J (2000) The cytotoxic action of Bax on yeast cells does not require mitochondrial ADP/ATP carrier but may be related to its import to the mitochondria. *FEBS Lett* **471**: 113–118.

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.

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.

Lindsten T, Ross AJ, King A *et al.* (2000) The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell* **6**: 1389–1399.

Manon S, Chaudhuri B & Guerin M (1997) Release of cytochrome c and decrease of cytochrome c oxidase in Bax-expressing yeast cells, and prevention of these effects by coexpression of Bcl-xL. *FEBS Lett* **415**: 29–32.

Minn AJ, Kettlun CS, Liang H, Kelekar A, Vander Heiden MG, Chang BS, Fesik SW, Fill M & Thompson CB (1999) Bcl-xL regulates apoptosis by heterodimerization-dependent and -independent mechanisms. *EMBO J* **18**: 632–643.

Mumberg D, Muller R & Funk M (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **156**: 119–122.

Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T & Tanaka N (2000) Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288**: 1053–1058.

Polcic P & Forte M (2003) Response of yeast to the regulated expression of proteins in the Bcl-2 family. *Biochem J* **374**: 393–402.

Puthalakath H, Huang DC, O'Reilly LA, King SM & Strasser A (1999) The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* **3**: 287–296.

Puthalakath H, Villunger A, O'Reilly LA, Beaumont JG, Coultas L, Cheney RE, Huang DC & Strasser A (2001) Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science* **293**: 1829–1832.

Schiestl RH & Gietz RD (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr Genet* **16**: 339–346.

Siskind LJ, Feinstein L, Yu T *et al.* (2008) Anti-apoptotic Bcl-2 family proteins disassemble ceramide channels. *J Biol Chem* **283**: 6622–6630.

Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* **267**: 1456–1462.

Vaux DL & Korsmeyer SJ (1999) Cell death in development. *Cell* **96**: 245–254.

Verma S, Zhao LJ & Chinnadurai G (2001) Phosphorylation of the pro-apoptotic protein BIK: mapping of phosphorylation sites and effect on apoptosis. *J Biol Chem* **276**: 4671–4676.

Willis SN, Fletcher JI, Kaufmann T *et al.* (2007) Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science* **315**: 856–859.

Wissing S, Ludovico P, Herker E *et al.* (2004) An AIF orthologue regulates apoptosis in yeast. *J Cell Biol* **166**: 969–974.

Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG & Youle RJ (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* **139**: 1281–1292.

Yaffe MP & Schatz G (1984) Two nuclear mutations that block mitochondrial protein import in yeast. *P Natl Acad Sci USA* **81**: 4819–4823.

Zha H & Reed JC (1997) Heterodimerization-independent functions of cell death regulatory proteins Bax and Bcl-2 in yeast and mammalian cells. *J Biol Chem* **272**: 31482–31488.

Zinser E & Daum G (1995) Isolation and biochemical characterization of organelles from the yeast, *Saccharomyces cerevisiae*. *Yeast* **11**: 493–536.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Expression of BH3-only members of Bcl-2 family inhibits prosurvival activity of Bcl-XL and Bcl-2.

Fig. S2. BH3-only proteins expressed in yeast do not potentiate proapoptotic effect of Bak.