Differential retrotranslocation of mitochondrial Bax and Bak

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Abstract

The Bcl-2 proteins Bax and Bak can permeabilize the outer mitochondrial membrane and commit cells to apoptosis. Pro-survival Bcl-2 proteins control Bax by constant retrotranslocation into the cytosol of healthy cells. The stabilization of cytosolic Bax raises the question whether the functionally redundant but largely mitochondrial Bak shares this level of regulation. Here we report that Bak is retrotranslocated from the mitochondria by pro-survival Bcl-2 proteins. Bak is present in the cytosol of human cells and tissues, but low shuttling rates cause predominant mitochondrial Bak localization. Interchanging the membrane anchors of Bax and Bak reverses their subcellular localization compared to the wild-type proteins. Strikingly, the reduction of Bax shuttling to the level of Bak retrotranslocation results in full Bax toxicity even in absence of apoptosis induction. Thus, fast Bax retrotranslocation is required to protect cells from commitment to programmed death.

Keywords apoptosis; Bcl-2 proteins; membrane association; tail anchor

Introduction

In response to stress, cells can initiate mitochondrial apoptosis signaling in multicellular animals. The intrinsic cell suicid program converges at the activation of the Bcl-2 proteins Bax and Bak that permeabilize the outer mitochondrial membrane (OMM). The release of cytochrome c (cyt c) and other intermembrane space proteins into the cytosol after OMM permeabilization (mitochondrial outer membrane permeabilization, MOMP) results in mitochondrial dysfunction and initiates the caspase cascade that efficiently dismantles the cell (Bratton & Cohen, 2001; Green & Kroemer, 2004). The activation of Bax or Bak commits the cell to apoptosis necessitating a tight control of pro-apoptotic Bcl-2 proteins (Lindsten et al, 2000). Thus, the Bcl-2 protein family contains two groups of structurally similar proteins involved in arbitration to apoptosis: pro-survival Bcl-2 proteins harboring four Bcl-2 homology domains (BH1-4, e.g. Bcl-2, Bcl-xL or Mcl-1) and pro-apoptotic Bcl-2 proteins with three BH domains (BH1-3, e.g. Bax or Bak). Proteins of both groups are regulated by a diverse group of proteins sharing only the BH3 domain with Bcl-2 proteins (BH3-only proteins). Pro-survival Bcl-2 proteins inhibit Bax and Bak via direct interactions or by sequestering ‘activator’ BH3-only proteins, thereby preventing their interaction with Bax and Bak (Letai et al, 2002; Kuwana et al, 2005; Willis et al, 2005, 2007; Kim et al, 2006; Liambi et al, 2011). Regulatory interactions between pro- and anti-apoptotic Bcl-2 proteins and Bax and Bak can only be observed in the presence of the OMM or liposomes and could result in membrane-integral protein complexes (Roucou et al, 2002), suggesting also mitochondrial apoptosis signaling via membrane-embedded proteins (Leber et al, 2007; Lovell et al, 2008; García-Sáez et al, 2009). Upon apoptosis induction, Bax and Bak oligomerize and at least partially insert into the OMM (Eskes et al, 1998; Antonsson et al, 2000; Wei et al, 2001).

The C-terminal Bax and Bak segments are sufficient for targeting protein fusions to the OMM, whereas deletion of the C-terminal segments abolishes mitochondrial localization and pro-apoptotic activity of Bax and Bak (Nechushtan et al, 1999; Schinzel et al, 2004; Setoguchi et al, 2006). C-terminal membrane anchors (MAs) direct proteins post-translationally to the target organelle and may insert into the membrane (Habib et al, 2003). Single amino acid substitutions in the C-terminal helix can either localize Bax almost
completely to the mitochondria or prevent OMM binding at all (Nechushtan et al., 1999). The potential binding site of Bax for pro-apoptotic BH3 motifs interacts with its MA in cytosolic Bax inhibiting MA exposure and binding to BH3 motifs (Suzuki et al., 2000). The exposure of the C-terminal Bax helix may involve prolyl isomerization in the loop preceding the MA or BH3-only protein binding to a low affinity site in the N-terminal part of Bax (Schinzel et al., 2004; Gavathiotis et al., 2008).

Bak could be controlled by mechanisms similar to Bax regulation, considering the dependence of activation and oligomerization of both proteins on major conformational changes, exposing the BH3 motifs (Wang et al., 1998; Dewson et al., 2008; Edlich et al., 2011; Moldoveanu et al., 2013). The conversion of inactive into active Bak seems to depend exclusively on transient BH3-only protein interactions with the hydrophobic cleft of Bak (Dai et al., 2011). In healthy cells, Bax primarily resides in the cytosol (Wolter et al., 1997), contrasting with the predominant mitochondrial Bak localization. Although Bak is constantly translocating to the OMM, it is stabilized in the cytosol by interactions with pro-survival Bcl-2 protein interactions with the hydrophobic cleft of Bak (Dai et al., 2011). In healthy cells, Bax primarily resides in the cytosol (Wolter et al., 1997), contrasting with the predominant mitochondrial Bak localization. Although Bak is constantly translocating to the OMM, it is stabilized in the cytosol by interactions with pro-survival Bcl-2 activities on the OMM, establishing an equilibrium between cytosolic and mitochondrial Bak (Edlich et al., 2011; Schellenberg et al., 2013). Bak retrotranslocation from the mitochondria requires recognition of its exposed BH3 motif by the hydrophobic groove of Bcl-xL and interaction between the C-terminal Bcl-xL helix and Bak (Edlich et al., 2011; Todt et al., 2013). Bak shuttling could also involve Bcl-xL-independent mechanisms (Schellenberg et al., 2013). When Bak retrotranslocation is compromised, Bak accumulates on the OMM, but requires further stimulation to become active dependent on the size of the mitochondrial Bax pool prior to apoptosis stimulation (Todt et al., 2013). Reversible mitochondrial Bak accumulation can be observed during anoikis (Valentijn et al., 2003).

The functional redundancy between Bax and Bak raises the question, why Bak is predominantly found on the mitochondria. Are Bax and Bak controlled by different mechanisms or does differential regulation of the same mechanism cause different localizations of these pro-apoptotic Bcl-2 proteins? Considering that resistance to apoptosis is one hallmark of cancer (Hanahan & Weinberg, 2011), understanding the regulation of Bax and Bak is imperative for targeting the mitochondrial apoptosis pathway in cancer therapy.

Results

Bak is present in the cytosol

The importance of the subcellular localization for the regulation of Bax raises the question whether Bak is also regulated by shuttling between mitochondria and cytosol in spite of its predominant mitochondrial localization. Thus, the presence of Bak in the cytosol (C) and the mitochondria-containing heavy membrane fraction (HM) from human tissues was analyzed with specific antibodies (Fig 1A and B). Different levels of Bak are present on the mitochondria of all tissue samples (Fig 1C). Strikingly, in heart, kidney and lung tissue, Bak is also present in the cytosolic fraction (Fig 1D; Supplementary Fig S1).

Interestingly, human lung tissue not only shows cytosolic Bak but the HM fraction also contains membrane-associated Bak in addition to membrane-integral protein (Fig 1E). The presence of Bak in the cytosol of human tissues raises the question, whether Bak is shuttled into the cytosol by a mechanism similar to Bax retrotranslocation (Edlich et al., 2011; Todt et al., 2013).

Bcl-xL retrotranslocates Bak

We tested the possibility of Bak shuttling by Fluorescence Loss in Photobleaching (FLIP) measurements of mitochondrial Bak with and without ectopically expressed Bcl-xL. The experiments were performed with transient protein expression in HCT116 Bak/Bak DKO cells to avoid high protein levels resulting from stable protein expression (Supplementary Fig S2A–D). FLIP experiments target cytosolic Bak fluorescence by repeated cycles of bleaching within a defined region, while changes in mitochondrial fluorescence are monitored by confocal imaging between bleaching events (Ishikawa-Ankerhold et al., 2012).

GFP-Bak localizes to the mitochondria but cytosolic Bak is apparent when Bcl-xL is overexpressed (Fig 2A). After the cytosolic fluorescence was bleached within the initial cycles, mitochondrial GFP-Bak fluorescence diminished during the FLIP measurement indicating Bak shuttling (Fig 2B and C). Retrotranslocation of mitochondrial GFP-Bak is increased by Bcl-xL, accelerating the loss of mitochondrial fluorescence during the measurements (Fig 2B and C, arrows). Therefore, Bcl-xL retrotranslocates mitochondrial Bak into the cytosol. Low Bak shuttling rates could explain the differential localization of Bak and Bak in cells (Table 1).

The same retrotranslocation process shuttles Bax and Bak

Bcl-xL increases Bak shuttling. Therefore, the effect of wild-type Bcl-xL or Bcl-xL G138A on Bak localization was analyzed in HCT116 Bak/Bak DKO cells. The G138A substitution prevents Bax BH3 binding to the hydrophobic groove of Bcl-xL and Bak retrotranslocation (Sedlak et al., 1995; Desagher et al., 1999; Edlich et al., 2011). While Bak is robustly found in the HM fraction, an additional Bak pool is also present in the cytosol (Fig 3A and B; Supplementary Fig S3A), corroborating the Bak localization in human tissues (Fig 1). Elevated levels of wild-type Bcl-xL, but not Bcl-xL G138A, increase the cytosolic Bak pool (Fig 3A and C; Supplementary Fig S3A and B). Increased levels of Mcl-1 accelerate Bak retrotranslocation similar to Bak shuttling (Fig 3C; Edlich et al., 2011). On the other hand, the BH3 mimetic ABT-737 decreases Bak retrotranslocation in the presence of ectopically expressed Bcl-xL (Supplementary Fig S3C). In contrast to previously observed Bak shuttling (Edlich et al., 2011), ectopic Bcl-2 expression does not accelerate Bak retrotranslocation.

The D83R substitution prevents Bak BH3 interactions with pro-survival Bcl-2 proteins (Kvansakul et al., 2007). Consistent with the effects of ABT-737 and Bcl-xL G138A, the Bak D83R substitution inhibits pro-survival Bcl-2 protein-mediated Bak shuttling (Fig 3C). Therefore, interactions between the BH3 motif and hydrophobic groove of Bcl-xL are required for Bak/Bax retrotranslocation. In parallel to ectopically expressed Bak, endogenous Bak is significantly increased in the cytosol of HeLa cells when Bcl-xL levels are elevated (Fig 3D; Supplementary Fig S3D and E). Cytosolic Bak results from constant shuttling and is not diminished when protein synthesis is blocked (Supplementary Fig S3F). Thus, different Bak shuttling rates can account for the differential localization of Bak in human tissues (Fig 1).
Bax and Bak shuttling is determined by the membrane anchor

Single amino acid substitutions in the C-terminal MA of Bax can shift the protein to the mitochondria and decelerate its retrotranslocation (Nechushtan et al., 1999; Edlich et al., 2011; Schellenberg et al., 2013). Therefore, the impact of MA substitutions on the subcellular localization and retrotranslocation of Bax and Bak was analyzed (Fig 4A). Both chimeras (BaxTBak and BakTBax) were expressed to levels comparable to the wild-type proteins in the presence or the absence of ectopically expressed Bcl-xL (Supplementary Fig S4A and B). While Bax resides in most cells primarily in the cytosol, BaxTBak is largely localized to the mitochondria, similar to the localization of wild-type Bak (Fig 4B and C). Interestingly, HCT116 Bax/Bak DKO cells expressing BaxTBak often show a punctate fluorescence pattern (Fig 4C), as is characteristic for active Bax (Karbowski et al., 2002). BakTBax, on the other hand, exhibits a large pool of cells with significant amounts of cytosolic protein, corroborating the central role of the Bak MA in protein localization.
However, the different localization observed for BakTBax chimeras (Fig 4C and Ferrer et al., 2012) could be caused by differences in MA composition, protein expression and the resulting cell stress. Despite increased mitochondrial BakTBax levels compared to wild-type Bax, cell fractionations substantiated greater similarity between BakTBax and wild-type Bax localization compared to Bak (Fig 4B–D).

In parallel to localization, the shuttling of BaxTBak and BakTBax is altered compared to their wild-type counterparts. Bax retrotranslocation is slowed down to the level of Bak shuttling by the Bak MA, while Bak shuttling is significantly accelerated by the Bax MA (Supplementary Fig S4C and D; Table 1). These measurements show that the C-terminal membrane anchor determines retrotranslocation rates, and thus, localization of Bax and Bak.

Increased retrotranslocation is required to protect cells from Bax activation

The size of the mitochondrial Bax pool determines cellular commitment to apoptosis (Todt et al., 2013). The pronounced influence of shuttling rates on the subcellular localization of Bax and Bak suggests that both proteins are regulated by the rate of retrotranslocation.

Table 1. Retrotranslocation rates of Bax/Bak variants.

<table>
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<tr>
<th></th>
<th>$K_{abs} (10^{-3} \text{s}^{-1})$-Bcl-xL</th>
<th>$K_{abs} (10^{-3} \text{s}^{-1}) + \text{Bcl-xL}$</th>
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<tr>
<td>Bax</td>
<td>4.68 ± 0.11 (23)</td>
<td>8.61 ± 0.37 (23)</td>
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<tr>
<td>Bax S184V</td>
<td>3.48 ± 0.20 (23)</td>
<td>5.90 ± 0.13 (23)</td>
</tr>
<tr>
<td>BaxTBak</td>
<td>0.43 ± 0.10</td>
<td>2.33 ± 0.67</td>
</tr>
<tr>
<td>BaxTBakSS</td>
<td>3.91 ± 0.25</td>
<td>6.32 ± 0.65</td>
</tr>
<tr>
<td>Bak</td>
<td>0.14 ± 0.02</td>
<td>1.94 ± 0.09</td>
</tr>
<tr>
<td>BaxTBax</td>
<td>3.68 ± 0.06</td>
<td>5.71 ± 0.19</td>
</tr>
<tr>
<td>BaxTBakS184V</td>
<td>3.25 ± 0.22</td>
<td>5.30 ± 0.37</td>
</tr>
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Retrotranslocation rates determined by FLIP measurements of different Bax/Bak variants ± SD as depicted in Figs 2B and 6A, Supplementary Figs S3C and D and S6B compared to rates of wild-type Bax and Bax S184V (Edlich et al., 2011).
mitochondrial-cytosolic shuttling. Thus, the apoptotic activities of Bax, BaxTBak, Bak, or BakTBax ectopically expressed in HCT116 Bax/Bak DKO cells were analyzed by measuring caspase-3/7 activity and the cleavage of the caspase substrate poly (ADP-ribose) polymerase (PARP). In the absence of staurosporine (STS), Bax, Bak and BakTBak expression results in low caspase activity that can be inhibited by Bcl-xL overexpression (Fig 5A; Supplementary Fig S5C). Both wild-type proteins and BakTBax can release despite robust binding to the Bax variant (Fig 5C and D; Supplementary Fig S5A). Strikingly, BaxTBak gains full activity in the absence of apoptotic stimuli that is not completely inhibited by Bcl-xL, contrasting the regulation of both wild-type proteins.

In response to STS-induced apoptosis, BaxTBak activity in the presence of Bcl-xL overexpression is even higher than Bax, Bak, or BakTBax activities in the absence of overexpressed Bcl-xL (Fig 5B; Supplementary Fig S5B). Bcl-xL fails to prevent the transition towards the active conformation of BakTBak and cytochrome c release despite robust binding to the Bak variant (Fig 5C and D; Supplementary Fig S5C). Both wild-type proteins and BakTBax can be inhibited to basal apoptotic activity by Bcl-xL overexpression.

The Bak activity is reduced when containing the Bax MA. These results establish the link between increased retrotranslocation rates and reduced pro-apoptotic activity of Bax and Bak but show also the requirement for accelerated Bax shuttling.

Ectopic Bcl-xL expression reduces the pool of Annexin V-positive cells expressing Bax, Bak or BakTBax but not that of cells expressing BaxTBak (Fig 5E). Colony formation after apoptosis stimulation in the presence of Bax, BaxTBak, Bak or BakTBak reflects the results of caspase-3/7 activity measurements and PARP cleavage (Fig 5F and G). In the presence of BaxTBak the predominant fate is cell death even when Bcl-xL was overexpressed. Bcl-xL also fails to protect cells from BaxTBak activity in the absence of apoptotic stimuli (Fig 5H). Thus, slow Bak retrotranslocation commits cells to apoptosis.

**Bax and Bak are shuttled depending on the MA hydrophobicity**

Apoptosis activity measurements revealed a striking difference between BaxTBak and Bak despite similar localization and shuttling
Figure 4. The C-terminal membrane anchor determines the localization of Bax and Bak.

A The influence of the C-terminal membrane anchor (MA) on the differential localization and function of Bax and Bak has been analyzed using the wild-type proteins of Bax (blue) and Bak (green) and MA substitutions of both proteins resulting in the chimeras BaxTBak and BakTBax.

B Quantification of HCT116 Bax/Bak DKO cells expressing Bax, BaxTBak, BakTBax or Bak with the expressed protein being present largely cytosolic (white bars), in a mixed distribution between cytosol and mitochondria (grey bars) or largely mitochondrial (black bars). Data represent averages ± SEM from seven independent experiments with n ≥ 100 cells.

C Confocal images of HCT116 Bax/Bak DKO cells expressing Bax, BaxTBak, BakTBax or Bak. The GFP/YFP fluorescence of the expressed protein variants is depicted in the top panels and in green in the merged image on the bottom. The mitochondria were stained by MitoTracker far red depicted in red in the merged images (bottom row). Scale bar, 10 μm. n ≥ 5.

D Western blot analysis of Bax, BaxTBak, Bak and BakTBax localization expressed in HCT116 Bax/Bak DKO cells. Cytosol (C) and heavy membrane fraction (HM) of HCT116 Bax/Bak DKO cells are displayed. GAPDH and VDAC serve as fractionation controls. n = 3.

Source data are available online for this figure.
rates. Bax is regulated by conformational changes (Hsu & Youle, 1997; Edlich et al., 2011). Therefore, the presence of the Bak MA might induce conformational changes and pre-activate Bax. We tested whether BaxTBak activity was likely based on pre-activation by conformational changes or increased MA hydrophobicity by comparing BaxTBak and its V197/198S variant (BaxTBakSS, Supplementary Fig S6A). Similar hydrophobicity of the MA of BaxTBakSS and Bax S184V suggests similar shuttling rates of both Bax variants, if MA hydrophobicity determines Bax/Bak retrotranslocation. Bax S184V is shifted to the mitochondria compared to wild-type Bax due to the exposure and the hydrophobicity of the MA (Nechushtan et al., 1999; Suzuki et al., 2000). In
Figure 5. Mitochondrial Bax is activated apoptosis stimulus-independently.

A Caspase-3/7 activity measured in HCT116 Bax/Bak DKO cells overexpressing Bax, BaxTBak, Bak or BakTBak with or without Bcl-xL overexpression in the absence of apoptosis stimuli. Caspase activity is displayed in relative fluorescence units (RFU). pcDNA3.1-transfected cells served as a control. Data represent averages ± SEM. n ≥ 3. P-values according to one-way ANOVA are displayed.

B Staurosporine (STS, 1 µM)-induced caspase-3/7 activity of Bax/Bak DKO cells overexpressing Bax, BaxTBak, Bak or BakTBak with or without Bcl-xL overexpression is displayed in relative fluorescence units (RFU). Data represent averages ± SEM. n ≥ 3. P-values according to one-way ANOVA BaxTBak activities with and without Bcl-xL expression revealed no significant difference (n.s.) in the absence of Bcl-xL, overexpression BaxTBak activity is significantly higher than Bax, Bak or BakTBak activities (P < 0.001).

C Analysis of the active Bax conformation in HCT116 Bax/Bak DKO cells expressing wild-type Bax or BaxTBak with (dark grey bars) or without Bcl-xL overexpression (light grey bars) by the monoclonal antibody 66A7 (Sigma) detecting the active Bax protein fold by fluorescence imaging. Cells were analyzed prior to or after treatment with 1 µM STS in the presence of the pan-caspase inhibitor qVD. Data are represented as % of the expressing cell population ± SEM. n = 4.

D HCT116 Bax/Bak DKO cells ectopically expressing wild-type Bax or BaxTBak with (dark grey bars) or without Bcl-xL (light grey bars) were analyzed in the presence or the absence of 1 µM STS and qVD for retained mitochondrial cyt.c. Data are represented as % of the expressing cell population ± SEM. n = 4.

E Flow cytometry analysis of Annexin V staining of Bax/Bak DKO cells expressing Bax, BaxTBak, Bak or BakTBax in the absence (red line) or the presence of Bcl-xL overexpression (black line), following STS treatment. The percentage of gated cells is displayed in the color of the corresponding graph. Data represent averages ± SD. n = 4.

F Colony formation of Bax/Bak DKO cells transfected with pcDNA, Bax, BaxTBak, Bak or BakTBak with or without Bcl-xL overexpression. STS (1 µM) was added for 24 h before cells were replated and colonies were stained with methylene blue typically 14 days after treatment.

G Quantification of colony formation (F) of Bax/Bak DKO cells expressing Bax, BaxTBak, Bak or BakTBak with or without Bcl-xL overexpression after STS treatment. Data represent averages ± SEM. n = 4. P-values according to one-way ANOVA. BaxTBak-expressing cells with or without Bcl-xL expression showed no significant difference (n.s.).

H Quantification of the colony formation of Bax/Bak DKO cells expressing Bax, BaxTBak, Bak or BakTBax in presence of Bcl-xL overexpression without apoptosis stimulation. Data represent averages ± SEM. n ≥ 5. P-values according to one-way ANOVA.
Figure 6.
Discussion

Bax and Bak share a common regulation of their localization and activity despite differences in their distribution between cytosol and mitochondria. Both pro-apoptotic Bcl-2 proteins are retrotranslocated by pro-survival Bcl-2 proteins from the mitochondria into the cytosol of healthy cells, dependent on the interaction with their BH3 motif. While Bax is retrotranslocated by Bcl-xL, Bcl-2 and Mcl-1, Bcl-2 fails to accelerate Bak shuttling. This difference between Bax and Bak emphasizes the central role of retrotranslocation in Bax/Bak regulation, as Bcl-2 seems to be not involved in Bak regulation (Oltersdorf et al., 2005). The hydrophobicity of the C-terminal MAs of Bax or Bak determines different localization pattern and differential shuttling of both Bcl-2 proteins by the same retrotranslocation process (Figs 6H and 7G). Bax and Bak share similar apoptotic activity and subcellular localization at high shuttling rates, when the same MA is exposed, emphasizing the major role of the C-terminal MA in the regulation of Bax and Bak. Interestingly, retrotranslocation shuttles OMM-integral forms of Bax, Bak and Bcl-xL that either are in equilibrium with OMM-associated protein or are directly shuttled into the cytosol by the retrotranslocation machinery. However, active Bax is not retrotranslocated, as Bax activation blocks shuttling into the cytosol (Edlisch et al., 2011).

The analysis of Bax/Bak chimeras and variants revealed a potential requirement for increased Bax retrotranslocation in the absence of apoptosis signaling. Strikingly, the reduction of Bax retrotranslocation to the level of Bak shuttling initiates full Bax toxicity in the absence of apoptotic stimuli (Figs 5 and 7G). If the Bax shuttling rate is reduced sufficiently, mitochondrial Bax commits the cell to apoptosis in the absence of an apoptotic stress. Bcl-xL overexpression does not prevent Bax activation at low shuttling rates. Therefore, the survival of the cell requires fast Bax retrotranslocation from the mitochondria. Bak, however, commits the cell to apoptosis only in the presence of apoptotic stimuli despite its predominant mitochondrial localization. Mitochondrial Bax activation adds to the differential regulation of both redundant proteins (Sarosiek et al., 2013). Bax activation probably only occurs with wild-type proteins when Bax shuttling is decreased, for instance, by BH3-only protein signaling (Edlisch et al., 2011). However, the underlying mechanism of Bax activation at low shuttling rates remains to be solved. Mitochondrial Bax activation might explain cellular ‘priming’ to death by BH3-only proteins regardless of the presence of Bid, Bim and Puma (Ni Chonghaile et al., 2011; Vo et al., 2012). Depending on individual retrotranslocation rates, Bax activation could differ among different mitochondria, resulting in different organelle fates under stress conditions (Tait et al., 2010). On the other hand, mitochondrial Bax accumulation does not per se lead to Bax activation (Todt et al., 2013). Accordingly, similar levels of wild-type Bax and BaxTBak on the OMM result in BaxTBak but not Bax activity (Fig 7E). Under these conditions, both Bax variants most likely differ only in their translocation and retrotranslocation rates, thus resulting in different organelle individual molecules spend on the mitochondria. Therefore, healthy cells inhibit Bax by accelerated shuttling into the cytosol and minimizing the time Bax molecules spend on the OMM in order to prevent Bax activation in the absence of apoptosis signaling.

Materials and Methods

Constructs

Bax and Bak constructs were cloned in pEGFP-C1 or pEYFP-C1 expression vectors. The chimeras BaxTBak and BakTBak were obtained by overlap-extension-PCR using the following primers: Bax–BakTail for (5’–GCGGGAATTCTCATGATTTGAAGAATCTTCGTACCACAAAC–3’); Bak–BaxTail for (5’–GCGGAAATGGTCCACGGTGACCATCTTTG–3’); Xhol–Bak for (5’–GCTACTCCGAGCTATGGCCTCCACCGGACGAGG–3’); Bak–EcoRI rev (5’–GCGGCAATGGTCCCACCGTGACCATCTTTG–3’); Bak–BaxTail rev (5’–CCACGGTCAAGTGCACCGTGACCATCTTTG–3’); Bax–BakTail rev (5’–GCCTCCGAGCTATGGCCTCCACCGGACGAGG–3’); Bax/EcoRI rev (5’–GCCTCCGAGCTATGGCCTCCACCGGACGAGG–3’); Xhol–Bak for (5’–ATATCTCGAGCTATGGCCTCCACCGGACGAGG–3’); Bak/EcoRI rev (5’–GCCTCCGAGCTATGGCCTCCACCGGACGAGG–3’); Bax–BakTail rev (5’–GCCTCCGAGCTATGGCCTCCACCGGACGAGG–3’); Xhol–Bak for (5’–ATATCTCGAGCTATGGCCTCCACCGGACGAGG–3’).
Figure 7.
Amphotropic Phoenix cells (0.5 McCoy’s 5A medium supplemented with 10% heat-inactivated fetal HCT116 cells) and HCT116 Bax/Bak DKO cells were cultured in Cell culture and transfection selected by growth in zeocin (200 μg/ml). Two days post-infection, stably expressing cells were harvested, filtered, seeded in 6-well plates and transfected with the same amount of plasmids containing different Bax or Bak variants in the presence or absence of Bcl-xL. Then, cells were treated with 1 μM STS or left untreated. After 12 h incubation, whole-cell lysates were isolated and fluorogenic caspase-3 substrate N-acetyl-DEVD-AMC (BD Pharmingen) was added to each sample according to manufacturer’s protocol. Samples were incubated for 1 h at 37°C and then fluorescence signal was measured 30 times at 2-min intervals with an excitation wavelength of 355 nm and an emission wavelength of 460 nm in a plate reader. The same samples were also subjected to analysis of PARP cleavage by Western blot.

Apoptosis activity assays

For caspase-3/7 measurements, HCT116 Bax/Bak DKO cells were seeded in 6-well plates and transfected with the same amount of plasmids containing different Bax or Bak variants in the presence or absence of Bcl-xL. Then, cells were treated with 1 μM STS or left untreated. After 12 h incubation, whole-cell lysates were isolated and fluorogenic caspase-3 substrate N-acetyl-DEVD-AMC (BD Pharmingen) was added to each sample according to manufacturer’s protocol. Samples were incubated for 1 h at 37°C and then fluorescence signal was measured 30 times at 2-min intervals with an excitation wavelength of 355 nm and an emission wavelength of 460 nm in a plate reader. The same samples were also subjected to analysis of PARP cleavage by Western blot.

Whole-cell lysis and subcellular fractionation

Cells were harvested and incubated in cell lysis buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, protease inhibitor cocktail) for 15 min on ice. Whole-cell extracts were obtained by centrifugation at 15,000 × g for 10 min at 4°C. Protein concentrations were determined using a Bradford Assay (RotiQuant, Roth). Samples were boiled in SDS sample buffer for 10 min at 95°C and subsequently subjected to SDS–PAGE and Western Blot analysis. Subcellular fractions were performed as previously described (Todt et al., 2013).
Carbonite extraction

Mitochondrial pellets were prepared as described above and resuspended in 100 mM Na2CO3 at pH 11.5. The samples were incubated on ice for 20 min to disrupt protein–protein interactions of peripheral proteins while interactions protected by the lipid bilayer like lipid–protein interactions remain intact. Then, the membranes were pelleted at 150,000 × g for 30 min at 4°C. The supernatant, containing membrane-associated proteins, was subjected to protein precipitation by acetone. The pellet was resuspended once more in 100 mM Na2CO3 at pH 11.5 and incubated on ice for 20 min. Finally, the resuspended samples were centrifuged at 155,000 × g for 30 min at 4°C to obtain carbonite inextractable proteins. Both fractions were assayed by Western blot.

Clonogenic survival assay

For the evaluation of clonogenic survival, HCT116 Bax/Bak DKO cells were seeded in 6-well plates. After the transfection with different Bax or Bak variants in the presence or the absence of Bcl-xL, 1 μM STS was applied to each sample for 24 h or cells were left untreated. After 12–16 days, surviving colonies were fixed and stained with 1% methylene blue.

FACS analysis

Cells were seeded in 6-well plates and transfected with equal DNA amounts of Bax or Bak and pcDNA or Bcl-xL. Cells were harvested and washed with PBS. Cells were incubated in Annexin V binding buffer substituted with Annexin V–PE (eBioscience) for 10 min at room temperature. Finally, cells were fixed with 1% paraformaldehyde (neoLab) and measured using a BD FACS Calibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo Version 10.0.7.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

FT, ZC and FR designed and performed experiments, analyzed data and wrote the paper. FE, JH, GI and AK performed experiments and analyzed data. SWGT performed experiments, analyzed data and edited the paper. SF and HFL analyzed data and helped to conceptualize the project. FEd conceptualized the project, designed experiments, analyzed data and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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