Inactivation of prosurvival Bcl-2 proteins activates Bax/Bak through the outer mitochondrial membrane

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The mechanism of Bax/Bak activation remains a central question in mitochondria-dependent apoptotic signaling. While it is established that all proapoptotic Bcl-2 homology 3 (BH3)-only proteins bind and neutralize the anti-apoptotic Bcl-2 family proteins, how this neutralization leads to Bax/Bak activation has been actively debated. Here, genome editing was used to generate cells deficient for all eight proapoptotic BH3-only proteins (OctaKO) and those that lack the entire Bcl-2 family (Bcl-2 allKO). Although the OctaKO cells were resistant to most apoptotic stimuli tested, they underwent Bax/Bak-dependent and p53/Rb-independent apoptosis efficiently when both Bcl-xL and Mcl-1, two anti-apoptotic Bcl-2 proteins, were inactivated or eliminated. Strikingly, when expressed in the Bcl-2 allKO cells, both Bax and Bak spontaneously associated with the outer mitochondrial membrane (OMM) through their respective helix 9, and this association triggered their homo-oligomerization/activation. Together, these results strongly suggest that the OMM, not BH3-only proteins or p53/Rb, is the long-sought-after direct activator of Bax/Bak following BH3-only-mediated neutralization of anti-apoptotic Bcl-2 proteins.

Keywords: anti-apoptotic Bcl-2 proteins; BH3-only; Bax/Bak activation; outer mitochondrial membrane

Supplemental material is available for this article.

Received December 18, 2015; revised version accepted March 18, 2016.

Apoptosis, a major mode of programmed cell death, plays an essential role in development and maintenance of tissue homeostasis [Fuchs and Steller 2011]. Numerous extracellular and intracellular signals induce apoptosis by triggering mitochondrial outer membrane permeabilization (MOMP), commonly considered the point of no return, through the actions of two pore-forming Bcl-2 family proteins, Bax and Bak [Wei et al. 2001; Tait and Green 2010].

The Bcl-2 family proteins, containing one or more of the Bcl-2 homology [BH] domains [BH1–4], consist of both anti- and proapoptotic members, the latter of which are further divided into the multi-BH domain effector proteins. BH3-only proteins bind and sequester Bax/Bak. The disruption of this interaction is generally considered a prerequisite step prior to Bax/Bak activation [Fletcher et al. 2008; Llambi et al. 2011]. Second, the BH3-only proteins, through their BH3 domains, avidly bind to and inactivate the anti-apoptotic Bcl-2 family proteins. Interestingly, BH3-only proteins display vastly different affinities for various prosurvival Bcl-2 proteins [Chen et al. 2005; Willis and Adams 2005]. Third, many of the proapoptotic BH3-only proteins (most notably tBid, Bim, and Puma), through
their BH3 domains, also transiently engage Bax/Bak, causing their conformational transformation and activation (Letai et al. 2002; Gavathiotis et al. 2008; Czabotar et al. 2013; Moldoveanu et al. 2013).

Primarily based on in vitro studies, it has been proposed that, during apoptosis, activated BH3-only proteins bind and inactivate all anti-apoptotic Bcl-2 proteins, and this inactivation liberates direct activator BH3-only proteins, which in turn directly bind to and activate Bax/Bak (Kim et al. 2006; Llambi et al. 2011; Moldoveanu et al. 2014). However, whether such a direct BH3-only–Bax/Bak interaction is essential to BH3-only-mediated Bax/Bak activation remains unclear (Garcia-Saez 2012; Borner and Andrews 2014). Meanwhile, whether the BH3-only-mediated neutralization of anti-apoptotic Bcl-2 proteins is sufficient for Bax/Bak activation is not known (Chipuk and Green 2008). In this study, using a genetic system in conjunction with put-back experiments, we sought to address these two issues and identify the direct trigger for Bax/Bak activation following inactivation of the anti-apoptotic Bcl-2 proteins.

Results

Investigating how BH3-only proteins activate Bax/Bak is complicated by the fact that the direct activator BH3-only proteins have two BH3-mediated activities: direct activation of Bax/Bak and neutralization of the anti-apoptotic Bcl-2 proteins (Chipuk and Green 2008). To evaluate the distinct roles of these two activities in vivo, we used a two-part approach that combines genetic ablation of all of the potential direct activator BH3-only proteins with genetic or functional inactivation of the anti-apoptotic Bcl-2 proteins.

Generation of cells deficient for all eight proapoptotic BH3-only proteins (OctaKO)

Recent data from liposome experiments demonstrated that, in addition to tBid, Bim, and Puma, some other proapoptotic BH3-only proteins, including Noxa, Bik, Bmf, and Hrk, were able to directly activate Bax/Bak, albeit with weaker activities [Dai et al. 2011; Du et al. 2011]. To examine the requirement of direct activation by these proteins in vivo, we sought to generate cells deficient for all eight proapoptotic BH3-only proteins through genome editing.

HCT116 cells were first cotransfected with four CRISPR-expressing plasmids—separately targeting the genomic region upstream of the BH3 domains of Hrk, Bmf, Puma, and Bid—together with a RFP/GFP-HygR reporter plasmid [Fig. 1A]. Following a hygromycin selection, single clones displaying a loss of both Bid and Puma proteins were isolated. These clones were further screened for the frameshift mutations of both Hrk and Bmf genes by genomic sequencing around the target region of the CRISPRs, as antibodies recognizing endogenous Hrk and Bmf in HCT116 cells are unavailable. One clone with homogeneous and compound heterozygous frameshift mutations in the target regions [upstream of the BH3 domains] of Bmf and Hrk, respectively, was isolated and verified as Bid−/−Bmf−/−Hrk−/−Puma−/− by Western blot and genomic sequencing [Fig. 1B,C]. Cells from this clone were subsequently transfected with two CRISPR plasmids targeting Bim and Noxa separately. Following a flow cytometry sorting for RFP/GFP expression from the cotransfected reporter plasmid, single clones were isolated and screened for the additional loss of Bim and Noxa proteins. Two of these clones were mixed and subjected to a third round of transient transfection with two CRISPR plasmids targeting Bik and Bad separately followed by flow cytometry. Three single clones carrying the genotype of Bad−/−Bik−/−Bim−/−Bmf−/−Hrk−/−Noxa−/−Puma−/− (named OctaKO clones A, B, and C) were isolated and verified by Western blot and genomic sequencing [Fig. 1D; Supplemental Figs. S1,S2, Supplemental Table S1]. The loss of functional Bmf and Hrk proteins in these clones was further verified by sequencing the respective cDNAs amplified from these clones [Supplemental Fig. S3]. As a control, we also generated Bax−/−Bak−/−[DKO] HCT116 cells through CRISPRs [Fig. 1D]. Not surprisingly, the DKO cells and all three OctaKO clones were highly resistant to serum starvation-, thapsigargin-, and TRAIL-induced apoptosis, as evidenced by a complete inhibition of PARP cleavage [Fig. 1E]. In addition, Annexin V staining followed by flow cytometry analysis and a clonogenic survival assay were used to further confirm the resistance of OctaKO clone A to these treatments [Fig. 1F; Supplemental Fig. S4].

Efficient induction of apoptosis in the OctaKO cells following inactivation of Bcl-xL and Mcl-1

The direct activation model suggests that, following neutralization of the anti-apoptotic members, the direct activator BH3-only proteins are released from sequestration and in turn directly activate Bax/Bak [Kim et al. 2006, 2009; Llambi et al. 2011]. It predicts that at least one of the direct activators is necessary for Bax/Bak activation. We sought to test this hypothesis by inactivating the anti-apoptotic Bcl-2 proteins in the OctaKO cells. Through a combinatorial siRNA knockdown approach, our earlier study revealed that inactivation of Bcl-xL/Mcl-1 was sufficient to trigger Bax/Bak activation in HeLa cells [Loewith et al. 2010]. Using the same strategy, the wild-type HCT116, the DKO, and the three OctaKO clones were transfected with siRNAs against Bcl-xL and Mcl-1 individually or in combination. Remarkably, the wild-type and three OctaKO clones showed robust PARP cleavage following the simultaneous knockdown of Bcl-xL and Mcl-1 [Fig. 2A]. To examine the requirement of Bax/Bak in this activity, CRISPR was used to eliminate Bax and Bak from OctaKO clone A. Not surprisingly, PARP cleavage was completely abolished in Octa/Bax/Bak knockout cells, indicating that apoptosis induced by the simultaneous loss of Bcl-xL and Mcl-1 was Bax/Bak-dependent [Fig. 2A]. This result was also confirmed by Hoechst staining of the siRNA transfected cells [Supplemental Fig. S5].

In addition, a UV/ABT-737 combination treatment of the cells [with ABT-737 added immediately following
UV treatment) was used as an alternative way to eliminate/inactivate both Mcl-1 and Bcl-xL. While UV is known to induce a rapid, proteasome-mediated elimination of Mcl-1 (Nijhawan et al. 2003), ABT-737 is a highly specific and potent small molecule inhibitor of Bcl-xL/Bcl-2/Bcl-w (Oltersdorf et al. 2005). Indeed, 5 h after UV treatment, Mcl-1 was essentially eliminated from wild-type, DKO, and OctaKO cells (Supplemental Fig. S6). While both single treatments caused a modest level of apoptosis in wild-type cells but not in OctaKO cells, the combined treatment caused robust apoptosis in both wild-type and OctaKO cells but not in DKO or Octa/Bax/Bak knockout cells (Fig. 2B,C). Together, these results demonstrate that inactivation of both Bcl-xL and Mcl-1 is sufficient for Bax/Bak activation in the absence of the proapoptotic BH3-only proteins.

However, the above conclusion does not exclude the possibility that, once released from sequestration by the anti-apoptotic family proteins, the direct activator BH3-only proteins may accelerate Bax/Bak activation by engaging Bax/Bak. We therefore sought to compare the kinetics of Bax/Bak activation or apoptosis in the wild-type and OctaKO cells following the inactivation/elimination of Bcl-xL and Mcl-1. Due to its slow kinetics, siRNA knockdown was not suitable for this effort. To circumvent this difficulty, we resorted to inactivating the Mcl-1 gene in both the wild-type and OctaKO cells and assessing the kinetics of apoptosis following the addition of the fast-acting Bcl-xL/Bcl-2/Bcl-w inhibitor ABT-737. We therefore generated Mcl-1 knockout and Octa/Mcl-1 knockout HCT116 cells by transfecting the wild-type HCT116 cells and OctaKO clone A with a CRISPR plasmid against Mcl-1 (Fig. 2D,E). Two Mcl-1 knockout clones and two Octa/Mcl-1 knockout clones were isolated and verified by Western blot and genomic sequencing (Fig. 2F; Supplemental Fig. S7). As expected, TRAIL or thapsigargin treatments...
induced strong apoptosis in the wild-type and the Mcl-1 knockout cells but not in the OctaKO or Octa/Mcl-1 knockout cells [Fig. 2G]. Strikingly, while the addition of ABT-737 induced a very low level of apoptosis in the wild-type cells and no apoptosis in the OctaKO cells, robust apoptosis was observed in both the Mcl-1 knockout cells and the Octa/Mcl-1 knockout cells within 2 h [Fig. 2G; Supplemental Fig. S8]. To examine the kinetics of apoptosis, cells were harvested at different time points following the addition of ABT-737 and analyzed for PARP cleavage and a caspase 3/7 substrate assay. The two clones of Mcl-1 knockout and two clones of Octa/Mcl-1 knockout cells displayed similar kinetics of caspase activation [Fig. 2H; Supplemental Fig. S9], suggesting that loss of the proapoptotic BH3-only proteins does not significantly affect Bax/Bak activation following inactivation of both Bcl-xL and Mcl-1.

Apoptosis in the absence of the proapoptotic BH3-only proteins, p53, and Rb

The above results suggested that the neutralization function of the BH3-only proteins is sufficient to trigger Bax/Bak activation. How does inactivation of the anti-apoptotic Bcl-2 proteins then lead to the activation of Bax and Bak? As both tumor suppressors p53 and Rb have been shown to engage and activate Bax/Bak in vitro studies [Chipuk et al. 2004; Hilgendorf et al. 2013], it is possible that they directly activate Bax and Bak in the OctaKO cells following Bcl-xL/Mcl-1 inactivation. To test this possibility, two pairs of nickase-expressing plasmids [Mali et al. 2013; Ran et al. 2013a] targeting the p53 gene and the Rb1 gene separately were constructed and cotransfected into the OctaKO cells together with the RFP/GFP-HygR reporter plasmid [Fig. 3A]. Following a
GFP/RFP sorting, a single clone of Octa/p53/Rb knockout cells was isolated and validated by Western blot and genomic sequencing (Fig. 3B; Supplemental Fig. S10). While siRNA knockdown of Bcl-xL or Mcl-1 individually had little effect on the Octa/p53/Rb knockout cells, the double siRNA transfection induced robust apoptosis (Fig. 3C; Supplemental Fig. S11). In addition, while the Octa/p53/Rb knockout cells were completely resistant to TRAIL, thapsigargin, UV, or ABT-737 treatments, the UV/ABT-737 combination treatment induced robust apoptosis (Fig. 3D,E; Supplemental Fig. S12), indicating that endogenous p53 and Rb are not involved in Bax/Bak activation in the OctaKO cells when both Bcl-xL and Mcl-1 are inactivated.

Suppression of Bcl-xL and Mcl-1 activates Bax in the absence of potential direct activator BH3-only proteins

Although normally existing as a monomeric, inactive, and cytosolic protein, Bax moves to the mitochondria and forms homo-oligomers on the OMM following apoptotic stimulation (Hsu et al. 1997b; Walensky and Gavathiotis 2011). The mechanism of this translocation remains not well understood. To examine whether suppression of the anti-apoptotic Bcl-2 family proteins can induce the mitochondrial translocation of Bax, we sought to carry out a “put-back” experiment in which GFP-Bax is expressed in cells without Bax/Bak and the proapoptotic BH3-only proteins. Bax and Bak proteins were eliminated from the Octa/Mcl-1 knockout cells by transiently transfecting the Octa/Mcl-1 knockout cells with two CRISPR-expressing plasmids against Bax and Bak separately followed by flow cytometry sorting and single-clone screening. A single clone of Octa/Mcl-1/Bax/Bak knockout cells was isolated [Supplemental Fig. S13], retrovirally infected, and subsequently sorted by flow cytometry for the stable expression of either GFP or GFP-Bax [Fig. 4A]. The level of GFP-Bax expression in the resulting pool was comparable with the level of endogenous Bax in wild-type HCT116 cells [Fig. 4B]. The addition of ABT-737 resulted in robust apoptosis in Octa/Mcl-1/Bax/Bak knockout cells expressing GFP-Bax but not in those expressing GFP, indicating that the GFP-Bax in these cells was fully functional [Fig. 4C; Supplemental Fig. S14]. Not surprisingly, while GFP-Bax is predominantly cytosolic in the Octa/Mcl-1/Bax/Bak knockout cells under normal conditions, it efficiently moved to the mitochondria within 3 h following the addition of ABT-737 [Fig. 4D], indicating that suppression of anti-apoptotic Bcl-2 family proteins is sufficient to cause Bax translocation in the absence of the proapoptotic BH3-only proteins.

Among the eight proapoptotic BH3-only proteins, Bad is the only widely accepted sensitizer due to its selective binding to Bcl-xL, Bcl-2, and Bcl-w but not Mcl-1, A1, or Bax/Bak (Chen et al. 2005; Llambi et al. 2011). If suppression of Bcl-xL and Mcl-1 by the BH3-only proteins is sufficient to induce Bax/Bak activation, forced expression of Bad in cells with inactivated Mcl-1 should induce apoptosis without the need for other BH3-only proteins. To test this possibility, a plasmid expressing GFP-Bad (human) or its BH3 mutant, BadL114E, was transfected into either the OctaKO or the Octa/Mcl-1 knockout cells. Indeed, expression of G-Bad induced apoptosis in Octa/Mcl-1 knockout cells but not in OctaKO cells [Fig. 4E]. Not surprisingly, the BH3 mutant G-BadL114E failed to induce apoptosis in the Octa/Mcl-1 knockout cells.

Figure 3. BH3-only and p53/Rb-independent apoptosis following inactivation of Bcl-xL and Mcl-1. (A) Diagram for the generation of Octa/p53/Rb1 knockout (KO) cells. (B) Western blot of the indicated cell lines. An asterisk indicates a nonspecific protein. (C) Cells were harvested following siRNA transfection for Western blot with anti-PARP antibody. (D) Cells were treated with 25 ng/mL TRAIL for 5 h, 3 µM thapsigargin (TG) for 24 h, 2.5 µM ABT-737 for 16 h, 500 J/M2 UV, or a combination of 500 J/M2 UV and 2.5 µM ABT-737 for 16 h. Following the indicated treatments, cell lysates were generated for Western blot with anti-PARP antibody. (E) Cells were treated by TRAIL, thapsigargin, or the combination of UV and ABT-737 as described in D and were stained with Annexin V followed by flow cytometry analysis. The results are the mean ± SEM of at least three independent experiments.
To examine the ability of Bad to induce Bax activation, the stable pools of Octa/Mcl-1/Bax/Bak knockout/GFP-Bax cells were transfected with a plasmid expressing Flag-Bad or its BH3 mutant, Flag-BadL114E, together with a RFP-expressing plasmid (pDsRed) as a marker for transfected cells. Not surprisingly, the transient expression of wild-type Bad, but not BadL114E, resulted in apoptosis in these cells (Fig. 4F; Supplemental Fig. S15). In the presence of the pan-caspase inhibitor z-VAD, expression of F-Bad, but not F-BadL114E, caused a mitochondrial translocation of G-Bax [Fig. 4G]. Quantification showed that while >30% of cells transfected with F-Bad-expressing plasmid displayed punctate GFP staining (an indication of the mitochondrial translocation of GFP-Bax), none was observed in cells transfected with either vector or the plasmid expressing F-BadL114E, suggesting that Bad expression is sufficient to activate GFP-Bax in these cells [Fig. 4H]. Together, these results establish that inactivation of the anti-apoptotic Bcl-2 proteins causes the activation of Bax/Bak independently of the proapoptotic BH3-only proteins, p53, and Rb. What, then, is the direct trigger for Bax/Bak activation?

**Generation of Bcl-2 allKO cells (cells that lack the entire Bcl-2 family) and the constitutive activities of Bax/Bak**

The challenge in elucidating the mechanism of Bax/Bak activation largely stems from the complex interactions among the Bcl-2 family members and the uncertainty of whether Bax/Bak exist as inactive molecules without
the interference from the BH3-only proteins and the anti-apoptotic Bcl-2 proteins. We therefore sought to generate cells deficient for all Bcl-2 family proteins and then examine the behavior of Bax/Bak in such a cellular environment.

Using the OctaKO cells as a starting point, CRISPR plasmids against the remaining Bcl-2 family proteins (Bcl-2, Bcl-xL, Mcl-1, Bcl-w, A1, Bnip3, Nix, Bax, and Bak) were constructed and transiently transfected into OctaKO clone B in three successive rounds [each round involving three CRISPR plasmids] followed by a flow cytometry sorting for GFP/RFP signals from the cotransfected reporter plasmid and the isolation of single clones of the desired loss of the target proteins (Fig. 5A). Following the last round of transfection/sorting, single clones were isolated and screened for the disruption of the A1 gene in the CRISPR target site and the loss of the Bcl-xL and Nix proteins. Finally, two clones of HCT116 cells deficient for the 10 BH3-only proteins (Bad, Bik, Puma, Hrk, Bmf, Bid, Bim, Noxa, Bnip3, and Nix), five anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and A1), and Bax/Bak were isolated and named the Bcl-2 allKO cells (Fig. 5A). The loss of 14 of the target proteins was confirmed by Western blot (Fig. 5B). The genetic alterations in the CRISPR targeted region for each gene of the Bcl-2 family were confirmed by DNA sequencing (Fig. 5C; Supplemental Fig. S16; Supplemental Table S1). The Bcl-2 allKO cells do not undergo spontaneous apoptosis and show distribution of cell cycle stages and proliferation curves similar to those of the wild-type HCT116 cells, indicating that the entire Bcl-2 network is not necessary for survival and proliferation (Fig. 5D; Supplemental Fig. S17). As expected, these cells did not undergo apoptosis following treatment by TRAIL or the UV/ABT-737 combination (Fig. 5D; Supplemental Fig. S18).

To examine the behavior of Bax and Bak in the Bcl-2 allKO cells, GFP-Bax or GFP-Bak was individually expressed at increasing amounts by transient transfection of both clone A and clone B. Although G-Bax or G-Bak was hardly detected across the range of DNA amounts used in the transfections, both proteins caused robust apoptosis, which was mostly inhibited by the addition of the pan-caspase inhibitor z-VAD (Fig. 5E,F; Supplemental Figs. S19, S20). As a comparison, a similar transfection was carried out in Bax/Bak DKO cells. Unlike in Bcl-2 allKO cells, despite modest apoptosis after transfection, both G-Bax and G-Bak were readily detected without the addition of z-VAD, indicating a significant accumulation of these proteins in the DKO cells before the onset of apoptosis [Supplemental Fig. S21]. As expected, mutations in Bax or Bak in the respective BH3 domains of G-Bax or G-Bak completely abolished the killing in Bcl-2 allKO cells (Fig. 5E,F; Supplemental Figs. S19, 20). Together, these results indicate that, when expressed in the Bcl-2 allKO cells, both Bax and Bak are constitutively active.

To examine the constitutive activities of the expressed Bax/Bak in Bcl-2 allKO cells in an inducible fashion, we established a tetracycline [Tet]-inducible system [pRetro-Tet-on] for the expression of GFP-Bax or GFP-Bak in both the DKO and Bcl-2 allKO cells. Not surprisingly, 6 h after doxycycline [Dox]-induced expression of G-Bax or G-Bak, robust apoptosis was detected in Bcl-2 allKO cells when G-Bax and G-Bak were barely detectable [Fig. 5G]. In contrast, little apoptosis was observed at 6 h after the addition of Dox when significant amounts of G-Bax and G-Bak had accumulated in the DKO cells. To ascertain that the spontaneous activation of Bax or Bak in these cells was not due to the GFP tag, we established Dox-inducible Bcl-2 allKO cell lines that express untagged Bax or Bak. The Dox-inducible expression of Bax or Bak caused robust apoptosis even when these proteins were expressed at extremely low levels [Fig. 5H,I]. Together, these results strongly suggest that, upon neutralization of the anti-apoptotic Bcl-2 proteins, Bax and Bak become activated spontaneously without the help from any BH3-only proteins.

When expressed in the Bcl-2 allKO cells, both Bax and Bak spontaneously associate with the OMM, and this association triggers their homo-oligomerization

To monitor the subcellular localization of G-Bax and G-Bak in the Bcl-2 allKO cells, we induced the expression of G-Bax and G-Bak by Dox in the presence of z-VAD and observed cells through fluorescence microscopy. At all time points, all GFP-Bax- or GFP-Bak-expressing cells displayed a punctate staining that colocalized with TOM20, an OMM marker [Fig. 6A]. It is worth noting that the mitochondrial staining for both G-Bax and G-Bak coalesced, suggesting that Bax/Bak formed homooligomers on the mitochondria. The spontaneous mitochondrial localization of both Bax and Bak strongly suggests that Bax, similar to Bak, is inherently a membrane protein in the absence of other Bcl-2 family proteins. The differential localization of Bax and Bak under normal circumstances is therefore likely due to the presence of the anti-apoptotic Bcl-2 proteins. Indeed, in DKO cells carrying the G-Bax/Bak tet-inducible system, G-Bak localized to the mitochondria, whereas G-Bax is predominantly cytosolic following the addition of Dox [Fig. 6A].

To monitor the homo-oligomerization of G-Bax and G-Bak in the Bcl-2 allKO cells, gel filtration analysis was carried out in the presence of 2% CHAPS. While both G-Bax and G-Bak formed high-order homo-oligomers after Dox-induced expression, homo-oligomer formation was essentially abolished when an untagged Bcl-xL was constitutively expressed in these cells [Fig. 6B]. These results suggest that Bax and Bak spontaneously form homo-oligomers in the absence of other Bcl-2 family members and that the anti-apoptotic Bcl-2 proteins actively suppress this tendency.

The spontaneous mitochondrial association and homo-oligomerization of Bax and Bak in the Bcl-2 allKO cells also suggests that the OMM may serve as the direct activator of Bax/Bak. To test this hypothesis, we generated truncation mutants of Bax and Bak that lack the C-terminal transmembrane domains [TM; helix 9] and tested their mitochondrial association and homo-oligomerization in Bcl-2 allKO cells [Fig. 6C]. As expected, when expressed in Bcl-2 allKO cells following induction by Dox, unlike the wild-type proteins, both G-BaxΔTM and G-BakΔTM
displayed apparent cytosolic staining and were present as monomers or dimers in gel filtration in CHAPS, indicating that helix 9 is required for homo-oligomerization, a hallmark and a critical step in Bax/Bak activation (Fig. 6D,E).

To further examine the causal relationship between mitochondrial association and homo-oligomerization of Bax/Bak, we generated chimeric proteins that attached the helix 9 of Bcl-xL (amino acids 214–233), a well-defined mitochondrial targeting sequence, to the BaxΔTM and BakΔTM mutants (Kaufmann et al. 2003). Similar to wild-type Bax and Bak, both chimeric proteins spontaneously localized to the OMM and formed homo-oligomers in the Bcl-2 allKO cells (Fig. 6D,E). In contrast, both chimeric proteins predominantly exist as monomer or dimers when expressed in DKO cells (Supplemental Fig. S22). These results suggested that mitochondrial association is sufficient to trigger Bax/Bak homo-oligomerization in these cells (Fig. 6E). As expected, while the ΔTM mutants completely lost the apoptotic activities, the wild-type Bax/Bak and their chimeric proteins with Bcl-xL helix 9 efficiently caused apoptosis in Bcl-2 allKO cells even when their levels were barely detectable (Fig. 6F; Supplemental Fig. S23). In contrast, when wild-type Bax/Bak and their mutants were expressed in the DKO cells, no or a modest level of apoptosis was observed following strong expression (Fig. 6G). Together, these results

Figure 5. Generation of Bcl-2 allKO cells and the constitutive activities of Bax/Bak. (A) Diagram of strategy for the generation of Bcl-2 allKO cells. B Western blot analysis of the wild-type (WT), OctaKO clone (cln) B, and two Bcl-2 allKO clones for Bcl-2 family proteins. The asterisk indicates a nonspecific protein. C Genomic sequences for A1 at the targeted region (underlined). The dotted line indicates a deletion, and the asterisk indicates an insertion. D) The wild-type and mutant clones of HCT116 were treated with 25 ng/mL TRAIL for 5 h or a combination of 500 J/M² UV and 2.5 µM ABT-737 for 16 h. Following each treatment, cell lysates were Western-blotted against PARP. A representative of two independent experiments is shown. (E,F) Bcl-2 allKO clone B grown in 35-mm plates were transiently transfected with plasmids expressing GFP fusion proteins of wild-type Bax, Bak with or without z-VAD-fmk, and their respective BH3 mutants. Sixteen hours after transfection, whole-cell lysates were harvested for Western blotting against GFP or PARP. (G) DKO and Bcl-2 allKO cells carrying the tetracycline (Tet)-inducible expression system for GFP, G-Bax, or G-Bak were induced by the addition of 2 µg/mL doxycycline (Dox). Six hours after induction, cells were harvested for Western blot analysis. (H,I) Bcl-2 allKO cells carrying the Tet-inducible expression system for Bax (H) or Bak (I) were induced by 2 µg/mL Dox. At the indicated time points after induction, cells were harvested for Western blot analysis.
strongly suggest that mitochondrial association triggers Bax/Bak activation following neutralization of the anti-apoptotic Bcl-2 family proteins.

Discussion

All proapoptotic BH3-only proteins are known to bind and neutralize the anti-apoptotic Bcl-2 proteins in response to various apoptotic stimuli [Willis and Adams 2005; Chippuk and Green 2008; Youle and Strasser 2008]. In this study, we provide genetic evidence to suggest that, following inactivation of the anti-apoptotic Bcl-2 family proteins, the OMM—but not the BH3-only proteins, p53, or Rb—directly triggers Bax/Bak activation.

**BH3-only proteins, p53, and Rb are not necessary for a direct activation of Bax/Bak**

Mouse embryonic fibroblasts (MEFs) deficient for Bid, Bim, and Puma, the three major direct activator BH3-
only proteins, have been previously generated and tested for their response to various apoptotic stimuli or overexpression of BH3-only proteins [Willis et al. 2007; Ren et al. 2010]. However, due to the presence of additional putative direct activator BH3-only proteins [i.e., Noxa, Bmf, Bik, and Hrk] [Dai et al. 2011; Du et al. 2011], it has been difficult to assess the role of direct activation following inactivation of the anti-apoptotic Bcl-2 proteins in vivo. Using a cellular system in which all potential direct activator BH3-only proteins and the tumor suppressors p53/Rb were eliminated, the present study demonstrated that the BH3-only proteins are not necessary for Bax/Bak activation once any combination of them successfully neutralizes Bcl-xL and Mcl-1 (Fig. 2–4). Likewise, the tumor suppressor p53 has been proposed to directly bind and activate Bax/Bak after it is released from Bcl-xL’s sequestration by the BH3-only protein Puma [Chipuk et al. 2004, 2005]. However, the robust apoptosis in Octa/p53/Rb knockout cells following elimination/suppression of Bcl-xL/Mcl-1 [Fig. 3] suggests that such a mechanism is not essential for Bax/Bak activation and supports the notion that p53-mediated transcriptional up-regulation of Puma/Noxa is sufficient for p53-dependent apoptosis [Jeffers et al. 2003; Villunger et al. 2003].

The observed BH3-only-independent Bax/Bak activation following neutralization of Bcl-xL/Mcl-1 (Figs. 2–4) is mostly consistent with the “indirect activation” model, which suggests that BH3-only proteins indirectly activate Bax/Bak by inactivating the anti-apoptotic Bcl-2 proteins and releasing the active Bax/Bak molecules from their sequestration [Chen et al. 2005; Willis and Adams 2005; Willis et al. 2007]. However, this model has difficulty in explaining the activation of Bax. Since Bax normally resides in the cytosol as an inactive monomer [Wolter et al. 1997], what is the direct trigger for Bax activation?

**Bax and Bak are both OMM proteins in the absence of other Bcl-2 family proteins**

Generation of Bcl-2 allKO cells allows one for the first time to examine the in vivo behavior of Bax/Bak in the absence of all other Bcl-2 proteins. Surprisingly, in Bcl-2 allKO cells, both Bax and Bak appeared to be membrane proteins that target to the OMM through their C-terminal tail [α9] without any stress signals [Fig. 6]. This observation, combined with the membrane-dependent homo-oligomerization and the constitutive activities of Bax/Bak in these cells, eliminates the theoretical need for a “direct activator” outside the closed system of Bax/Bak–OMM.

The constitutive OMM localization of Bax in Bcl-2 allKO cells is consistent with results from Youle and co-workers [Edlich et al. 2011] that demonstrated that Bax has a tendency to constantly move to the OMM in healthy cells and that the prosurvival Bcl-2 proteins, such as Bcl-xL, possess a “retrotranslocation activity,” shuttling Bax from the OMM to the cytosol. Interestingly, Bak was also found to be retrotranslocated by Bcl-xL, albeit at a much slower rate [Todt et al. 2015]. Although the mechanism of retrotranslocation is not well understood, it is safe to suggest that the different abilities to be retrotranslocated by the anti-apoptotic Bcl-2 family proteins likely determine the differential subcellular localization of Bax and Bak before apoptotic stimulation.

It is conceivable that the cytosolic Bax molecules, presumably with their helix 9 constantly associating with and dissociating from the hydrophobic groove [Suzuki et al. 2000; Gahl et al. 2014], frequently collide with the OMM through free diffusion. While such a collision is likely not consequential in the presence of anti-apoptotic Bcl-2 proteins, their inactivation by the BH3-only proteins and the concomitant loss of the retrotranslocation activities should allow helix 9 to anchor Bax to the OMM and trigger the membrane-mediated Bax/Bak activation.

**OMM as the direct activator of Bax/Bak following neutralization of anti-apoptotic Bcl-2 proteins**

The OMM has been suggested to be an active participant of MOMP by allowing or enhancing various interactions among Bcl-2 family proteins [Leber et al. 2007]. How does OMM suddenly become an activator of Bax/Bak during apoptosis? Our data in OctaKO and Bcl-2 allKO cells suggest that, due to the BH3-only-mediated inactivation of anti-apoptotic Bcl-2 proteins but not the direct interaction with the BH3-only proteins, p53, or Rb, both Bax and Bak gain full access to the OMM through their helix 9 [Fig. 6]. After this initial anchoring, as can be speculated, other potential Bax/Bak–OMM interactions may help rearrange Bax/Bak helices and allow the formation of homo-oligomers through helices α2–α5. Consistent with this speculation, helices 4–6 of Bax have been found to associate with the OMM when they are individually expressed in the cells [George et al. 2010]. Interestingly, several detergents have been found to cause conformational changes and activation of Bax/Bak [Hsu and Youle 1998]. It is conceivable that these detergents to some extent mimic the actions of the OMM, and such stable lipid–protein interactions may help overcome a steep energy barrier to achieve Bax/Bak activation [Suzuki et al. 2000].

Although the OMM has traditionally been viewed as the target of Bax/Bak’s destruction, our study provided evidence to suggest that it is also the primary, if not the only, direct instigator for such activities. Despite the fact that helices α2–α5 of Bax/Bak are the minimal structural unit sufficient for homo-oligomerization [George et al. 2007; Dai et al. 2011; Czabotar et al. 2013; Brouwer et al. 2014], the present study for the first time demonstrated the critical role of α9 in inducing Bax/Bak’s homo-oligomerization [Fig. 6]. Our results suggest that α9 is the trigger site of Bax/Bak and that a stable interaction between α9 and the OMM is sufficient to initiate the series of conformational changes and homo-oligomerization. Consistent with this conclusion, a recent study with reconstituted membranes suggested that, once in the membrane, Bax molecules form dimers, which further self-assemble into homo-oligomers [Subburaj et al. 2015]. Furthermore, an α9–α9 interface was recently detected in the homo-oligomers of Bax and Bak [Bleicken et al. 2014; Gahl et al. 2014; Iyer et al. 2015]. It can be speculated that, while the OMM lipids [e.g., cardiolipin, and sphingolipid products] are potential
Bok/Bak triple-knockout cells further demonstrated that et al. 2001). Recent data from Bok knockout and Bax/Bak DKO cells (Wei 2001). However, this hypothesis was not supported by the overwhelmingly strong apoptotic phenotype of Bax/Bak DKO cells (Wei et al. 2001). Recent data from Bok knockout and Bax/Bak/Bok triple-knockout cells further demonstrated that Bok’s apoptotic activity is Bax/Bak-dependent and that it does not play a role as an effector protein during MOMP [Echeverry et al. 2013; Ke et al. 2013; Carpio et al. 2015]. Interestingly, a new study provided evidence to suggest that Bok is a noncanonical effector of MOMP in Bax/Bak DKO cells when its degradation is blocked by proteasome inhibition (Llambi et al. 2016). As shown in Figure 2, the loss of Bax and Bak completely abolished the robust apoptosis in DKO and OctaKO cells upon elimination of Bcl-xL and Mcl-1, excluding Bok’s involvement as an effector under these conditions. Importantly, Bok’s apoptotic activity is insensitive to suppression by anti-apoptotic Bcl-2 proteins [Llambi et al. 2016], suggesting that Bok’s function and its regulation are independent of typical Bcl-2 family proteins. Thus, these findings strongly suggest that Bok does not function as an effector during BH3-only-mediated MOMP.

Based on Bok’s Bax/Bak-dependent apoptotic activity, it has also been suggested that Bok may function as a BH3-only protein [Echeverry et al. 2013; Carpio et al. 2015]. However, this proposition is in conflict with two important findings about Bok. First, Bok does not interact with the anti-apoptotic Bcl-2 proteins [Echeverry et al. 2013; Llambi et al. 2016]. Second, the proapoptotic activity of Bok is not dependent on the presence of the BH3 domain [Echeverry et al. 2013]. Collectively, these data appear to disqualify Bok as a proapoptotic BH3-only protein.

In summary, genetic knockout approaches combined with put-back experiments were used to demonstrate for the first time that, in the presence of OMM, both Bax and Bak are constitutively active without interference from the BH3-only proteins, the anti-apoptotic Bcl-2 family proteins, and the two tumor suppressors p53/Rb. We also demonstrated that the primary function of the BH3-only proteins in Bax/Bak activation is to inactivate or neutralize the anti-apoptotic Bcl-2 family proteins. Importantly, we found that the spontaneous association with the OMM provides the driving force for Bax/Bak activation following inactivation of anti-apoptotic Bcl-2 family proteins. Our data establish a “membrane-induced spontaneous Bax/Bak activation model” in which, upon BH3-mediated inactivation of anti-apoptotic Bcl-2 proteins, the free diffusion-based, C-terminal tail (α9)-mediated, spontaneous association of Bax/Bak with the OMM directly triggers their activation (Fig. 7). Although more work is needed to delineate the cascade of conformational changes in the membrane following inactivation of the anti-apoptotic Bcl-2 proteins and the initial membrane association during OMM-mediated Bax/Bak activation, our study supports the notion that the anti-apoptotic Bcl-2 family proteins and the OMM may be primary targets of therapeutic intervention aimed at potentiating or suppressing apoptosis.

Of note, a recent study presented data to suggest that, while Bax and Bak are directly activated by tBid, Bim, Puma, or Noxa, the activated Bax/Bak are suppressed by anti-apoptotic Bcl-2 proteins through complex formation but are liberated by the simultaneous suppression of Mcl-1 and Bcl-xL (Chen et al. 2015). In contrast, our data suggest that, following a BH3-only-mediated neutralization of the anti-apoptotic Bcl-2 proteins, Bax/Bak undergo spontaneous activation due to their affinity for the OMM, thus eliminating the need for a direct recruitment and
activation of Bax/Bak by any BH3-only proteins as well as the need for preformed stable complexes between Bcl-xL/ Mcl-1 and Bax/Bak.

Materials and methods

Cell culture

HCT116 cells were purchased from American Type Culture Collection and maintained in McCoy’s 5A medium supplemented with penicillin/streptomycin and 10% fetal bovine serum (FBS) (Atlanta Biologicals, no. S11150). 293GP cells were cultured in DMEM without penicillin/streptomycin and 10% fetal bovine serum (FBS) (Atlanta Biologicals, no. S11150). 293GP cells were cultured in DMEM medium supplemented with penicillin/streptomycin and 10% FBS. All cells used in this study were maintained at 37°C with 5% CO₂.

Reagents

ABT-737 was purchased from Cayman Chemical. Human recombinant TRAIL was generated as previously described (Zhang et al. 2011). Thapsigargin was purchased from Adipogen. z-VAD(OMe)FMK was purchased from Fischer Scientific. Dox hydrochloride was purchased from Fisher Scientific. Propidium iodide solution was purchased from Biolegend. Crystal Violet was purchased from Sigma-Aldrich. FMK was purchased from MP Biomedicals. Dox hydrochloride was purchased from Fischer Scientific. Promipid iodine solution was purchased from Biolegend. Crystal Violet was purchased from Fisher Scientific. The antibodies used included anti-Tom-20 (Santa Cruz Biotechnology, no. sc-11415), anti-rabbit Alex fluor 594 (Molecular Probes, no. 11012), anti-Bcl-xL (Santa Cruz Biotechnology, no. sc-8392), anti-Bcl-2 (Santa Cruz Biotechnology, no. sc-6082-1), anti-Mcl-1 (Santa Cruz Biotechnology, no. sc-819), anti-Bad (Santa Cruz Biotechnology, no. sc-8044), anti-Bak (Santa Cruz Biotechnology, no. sc-11425; Calbiochem, no. 202000), anti-Bid (Luo et al. 1998), anti-Bax (Santa Cruz Biotechnology, no. sc-493), anti-p53 (Santa Cruz Biotechnology, no. sc-393), anti-RB (Santa Cruz Biotechnology, no. sc-50), anti-GFP (Santa Cruz Biotechnology, no. sc-459), anti-Bak (Santa Cruz Biotechnology, no. sc-832), Cell Signaling Technologies, no. 3814), anti-Flag (Santa Cruz Biotechnology, no. sc-65625), anti-Noxa (Santa Cruz Biotechnology, no. sc-56169; Imagenex Technologies Corp., no. IMG-349A), anti-Puma (Santa Cruz Biotechnology, no. sc-28226; Pro-Sci, 3041), anti-Bcl-w (Cell Signaling Technologies, no. 2724), anti-Bax (Cell Signaling Technologies, no. 5324), anti-p-actin (Sigma-Aldrich, no. A5441), and anti-TetR (Clonitech, no. 631131).

Immunoblotting

Harvested cells were lysed in EBC buffer (50 mM Tris, 120 mM NaCl, 1 mM EDTA, 0.5% NP40 at pH 7.5) supplemented with 0.1% PMSF and protease inhibitors (5 mg/mL peptatin A, 10 mg/mL leupeptin) with a constant rotation for 1-2 h at 4°C. Following centrifugation at 22,000g for 10 min at 4°C, supernatant was collected as the whole-cell lysate. The protein concentrations of the whole-cell lysates were measured using Comassie protein assay solution [Thermo Scientific, no. 1856209]. Approximately 50 µg of total protein from each whole-cell lysate was resolved by SDS-PAGE and transferred onto a nitrocellulose membrane before incubation with primary and secondary antibodies for Western blot. Proteins of interest were detected by chemiluminescence.

Plasmids

The TALEN expression vector for Bim was designed and constructed by Seoul National University, TALEN Library Resource [order no. H39213]. A pair of DNA oligos for the sgRNA targeting site designed for gene and target regions of interest [Ran et al. 2013b] were annealed and ligated into Bbs1-cut px330 or px335 (AddGene, nos. 42230 and 42335). Oligo sequences cloned into px330 and px335 are in Supplemental Table S2.

Pairs of oligos containing the TALEN, CRISPR, or nickase recognition sites were ligated into EcoR1- and BamH1-cut reporter plasmid mRFP-TS-2A-HYG-EGFP (PNA Bio, Inc.). Sequences ligated into the mRFP-TS-2A-HYG-EGFP reporter plasmid included Mcl-1 ([tcggAGACGATTTGAACTCGAGACGAGCACGAGGTCA], Bcl-xL ([ggcgACGAGTTTGAACCTCGAGACGAGGCGGCGG]), Bcl-2 ([GATTGAGACATTTGAACTCGAGACGAGGCGGCGG]), Nix ([tcggAGACGATTTGAACTCGAGACGAGGCGGCGG]), Puma ([tcggAGACGATTTGAACTCGAGACGAGGCGGCGG]), Bim ([tcggAGACGATTTGAACTCGAGACGAGGCGGCGG]), and Bcl-xL ([tcggAGACGATTTGAACTCGAGACGAGGCGGCGG]).

The retroviral expression plasmids pMIG-GFP, pMIG-Bcl-xL, and pMIG-FMk were generated by ligating DNA fragments containing GFP, Bcl-xL, or FMk into the Xho1 and EcoR1 sites. The retroviral expression plasmids pMIG-GFP, pMIG-Bcl-xL, and pMIG-FMk were generated by ligating DNA fragments containing GFP, Bcl-xL, or FMk into the Xho1 and EcoR1 sites.

Plasmid transfection

FugeneHD reagent (Promega, no. E2311) was used for transfection in HCT116 cells according to the manufacturer’s instructions.
For CRISPR and nickase transfections, $3 \times 10^5$ to $4 \times 10^5$ cells were seeded in a 35-mm plate 24 h prior to transfection. Three-hundred nanograms to 750 ng of each CRISPR or nickase construct was cotransfected with 400 ng of the corresponding mRFP-TS-2A-HYG-EGFP reporter plasmid. For the Bim TALEN transfection, 1.5 µg of TALEN construct was used along with 400 ng of mRFP-TS-2A-HYG-EGFP reporter. pcDNA3.1 was added to reach a total DNA concentration of 2 µg per transfection. The transfected cells were split 1:2 24 h later followed by 1 mg/ml hygromycin B (Calbiochem, no. 400050) selection or flow cytometry sorting for RFP/GFP-positive cells. The FACS-sorted or hygromycin-selected cells were subsequently plated for single clones on 15-mm plates. FugeneHD was also used for transfection of wild-type or mutant HCT116 cells with Bad-expressing plasmids. Cells ($3 \times 10^5$ to $4 \times 10^5$) were seeded in a 35-mm plate 24 h prior to transfection. Five-hundred nanograms of each construct was used, with pcDNA3.1 added to maintain 1.5 µg of total DNA per plate. Cells were analyzed 20–24 h after transfection.

siRNA transfection

siRNA transfections were performed on $3 \times 10^5$ to $4 \times 10^5$ cells in 35-mm culture dishes with antibiotic-free McCoy’s 5A medium supplemented with 10% FBS and DharmaFECT2 transfection reagent (Dharmacon, Inc., no. T-2002-01). On-Target Plus siMcl-1, siBcl-xl, or siMcl-1 (Dharmacon, Inc., nos. L-004501-00, L-003458-00, and D-001810-10-05) was suspended in 1× siRNA buffer (Dharmacon, Inc., no. B-002000-UB-100) and added to a plate at a final concentration of 22 nM. Cells transfected with either siBcl-xl or siMcl-1 individually were harvested 48–72 h after transfection for Western blot analysis. Double siRNA knockdown (Bcl-xl and Mcl-1) was performed sequentially. Cells were first transfected with siBcl-xl or siControl. Forty-eight hours later, cells were split 1:2, with one half transfected with Mcl-1 siRNA and the other with siControl. Twenty-four hours later, cells from the double-knockdown plates and the siControl plates were harvested for Western blot analysis with PARP antibody or stained by Hoechst dye for quantification of the condensed nuclei.

Flow cytometry

Transfected cells were sorted according to fluorescence on a BD FACSAria or a BD FACSAria II cell sorter with BD FACSDiva 6.1.2 software (Flow Cytometry Research Facility, University of Nebraska Medical Center). Cells with RFP and/or GFP signals were collected for plating.

Retrovirus production

pMIG, PIG, pRetroX-Tet-on Advanced, and pRetroX-Tight-Pur retroviruses were produced in 293GP packaging cells as previously described (Lopez et al. 2010). Virus infection was performed in HCT116 cells using 10 µg/mL polybrene (American Bioanalytical, no. AB01643).

Inducible retroviral gene expression

Advanced cell lines were made in DKO and Bcl-2 allKO cell lines by infection with pRetroX-Tet-on Advanced virus. Cells ($1 \times 10^5$) were infected in a 35-mm plate using a 1:1 ratio of virus and medium and 10 µg/mL polybrene. DKO-infected cells were selected with 2 mg/mL G418 for 14 d. Bcl-2 allKO-infected cells were plated for single clones, and the single clones were screened for Tet-R expression via Western analysis. Advanced cell lines were subsequently infected with pRetroX-Tight-Pur viruses containing the desired gene. Infected Bcl-2 allKO Adv cells were plated for single clones, and single clones were screened via 2–3 µg/mL Dox induction and GFP detection using a fluorescence microscope and Western analysis. Due to high infection efficiency and protein expression after Dox induction, DKO Adv pRetroX-Tight-Pur-infected pools were used for experiments.

Genotyping

Genomic DNA was isolated from HCT cells using DNeasy blood and tissue kit (Qiagen, no. 69504) and subjected to PCR to amplify genomic target regions of interest using Phusion or Taq polymerase (New England Biolabs, nos. E0553S and M0273L). PCR was performed according to the manufacturer’s instructions for each polymerase, and thermocycler settings were adjusted according to product size and optimal primer annealing temperatures. Sequences for primers used for genomic PCR are in Supplemental Table S3. PCR products were cloned into the pGEM T-easy vector (Promega, no. A1360) using T4 ligase. Following transformation, blue/white selection (Fisher Scientific, no. BP4200-10) was used to enrich for colonies containing PCR inserts. Miniprep DNAs (Wizard Plus SV MiniPreps DNA purification system, Promega, no. A1460) from the selected colonies were digested by EcoR1 to determine the sizes of inserts. Plasmids with inserts were then sent for Sanger sequencing using T7 or SP6 primers (High-Throughput DNA Sequencing and Genotyping Core Facility, University of Nebraska Medical Center). In addition, if the genomic PCR reaction yielded a single PCR product on the agarose gel, primers from the original PCR or inner primers were used to directly sequence the PCR product to confirm the results from the corresponding pGEM clones.

cDNA amplification and analysis

RNA from HCT116 wild-type cells, each of the three OctaKO clones, and both Bcl-2 allKO clones was isolated with TRIzol reagent (Invitrogen, no. 1304078) following the manufacturer’s instructions. Total cDNA was made from each RNA sample using the GoScript reverse transcriptase system (Promega, no. A5000) with the oligo(dT) primer according to the manufacturer instructions. The total cDNAs from the wild-type and OctaKO cell lines were used as templates to amplify cDNAs of Bmf and Hrk with gene-specific primers. The forward and reverse primers for Bmf and Hrk cDNA were designed in different exons to ensure that amplification of CRISPR target regions was from cDNA but not from contaminating genomic DNA. The primers used for Bmf cDNA were outer (F-GATGGACCGCATTCGAGTGGTG and R-GTTCCTGTTGCTCCATGCTG) and nested [F-GAGACTGG AGATGATGTGT and R-TTCAAGAGCAGGTAGTGCAG]. The primers used for Hrk cDNA were outer [F-CACAAGGAG AAACCTTTGTTG and R-GCAAGAGTTGACAAAAGGAAG] and nested [F-AACTTGGTGTCCAGGGGAG and R-CGATCG CTCCAGGCGCTG]. Bmf and Hrk PCR products from the cDNA amplification were cloned into pGEM T-easy and subjected to PCR and nested colonies were digested by EcoR1 to determine the sizes of inserts. Plasmids with inserts were then sent for Sanger sequencing using T7 or SP6 primers (High-Throughput DNA Sequencing and Genotyping Core Facility, University of Nebraska Medical Center). In addition, if the genomic PCR reaction yielded a single PCR product on the agarose gel, primers from the original PCR or inner primers were used to directly sequence the PCR product to confirm the results from the corresponding pGEM clones.

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F-CAGTGAATTCTAGTCAGACCCAGGGAGCTG and R-CAGTCTCGAGTATTTAGCTGAAGTGA.}

**Apoptosis assays**

Cells (4 × 10^5 to 5 × 10^5) were seeded in 35-mm plates 16–20 h prior to apoptosis treatments. Cells were treated with 3 μM thapsigargin for 24 h, human recombinant 25 ng/mL TRAIL for 5 h, or 2.5 μM ABT-737 for 16 h before being harvested for analysis. Cells were also treated with 500 J/M^2 UV or 500 J/M^2 UV-ABT-737 with 2.5 μM ABT-737 added immediately following UV treatment and were harvested for analysis 16 h later. For serum starvation, the culture medium from cells seeded the previous day was completely removed and replaced by McCoy’s 5A medium without FBS. These cells were cultured for another 48 h before being collected for analysis by either FACS or Western blot against PARP. For Annexin V staining, treated cells were collected from medium in plates and trypsinization of attached cells in plates. Cells were stained with FITC Annexin V or APC Annexin V (Biolgend, nos. 640906 and 640920) according to manufacturer’s instructions. Ten-thousand cells from each sample were analyzed for FITC signal by flow cytometry. Cell counting and analysis were performed on a BD FACSCalibur flow cytometer with BD FACSDiva 8.0 software (Flow Cytometry Research Facility, University of Nebraska Medical Center). For detection of PARP cleavage in treated samples, whole-cell lysates were prepared as described above and subjected to Western blot with PARP antibody.

**Apoptosis quantification**

Quantification of apoptotic cells according to nuclear morphology was performed as previously described (George et al. 2007). Cells were stained with 1 μg/mL Hoechst 33342 (Molecular Probes, no. H-3570) for 10 min at 37°C. Pictures of three random viewing areas were taken for each plate. The percentage of cells undergoing nuclear condensation was determined for each viewing area. At least two independent experiments were performed for each transfection.

**Caspase 3/7 activity assay**

Cells were seeded in a 96-well plate at a concentration of 8 × 10^4 to 10 × 10^5 cells per well 40 h prior to treatment. After treatment, 50 μL of Apo-ONE Caspase 3/7 reagent (Promega, no. G7790) was added to 70 μL of medium in each well. Plates were rocked in the dark for 20 min and then frozen at −80°C before being thawed to achieve complete cell lysis. Plates were analyzed on a POLARStar Optima microplate reader (BMG Labtech) at 485-nm excitation and 520 emission.

**Mitochondrial staining**

MitoTracker Red CMXRos (Life Technologies, no. M7512) was added to cells cultured on coverslips at a final concentration of 50 nM. Following incubation for 5 min at 37°C, coverslips were mounted on slides, sealed, and photographed under a fluorescence microscope [Nikon Eclipse 50i]. Immunostaining was performed using Tom-20 primary antibody and anti-rabbit Alexa fluor 594 secondary antibody. Cells were plated on chamber slides and fixed with 3% paraformaldehyde, permeabilized with 0.15% Triton X-100 in PBS, and blocked with 2% BSA in PBS prior to antibody staining. After staining, coverslips were mounted and sealed on slides, and cells were then photographed under a fluorescence microscope [Nikon Eclipse 50i].

**Bax translocation assay**

Octa/Mel-I/Bax/Bak knockout/GFP-Bax cells were cotransfected with pDsRED and either pcFlag, pcFlag-Bad, or pcFlag-Bad^Δ114E, z-VAD-FMK (20 μM) was added to plates immediately prior to transfection. Twenty-four hours after transfection, pictures capturing six views from each plate were taken. Cells in each view were then counted for the presence of RFP signal (marking the transfected cells) and the punctate GFP staining (marking the cells with GFP-Bax mitochondrial translocation). Cell counts from each plate were combined to calculate the percent of transfected cells with Bax translocation. Counts from two independent experiments were used.

**Gel filtration analysis**

pRetro Adv cell lines were induced for 16 h using 2 μg/mL Dox. Bel-2 allKO cells carrying pRetro Bax, Bak, Bak-xL, and Bak-xL were induced in the presence of 20 μM z-VAD. Whole-cell lysates were obtained and lysed in lysis buffer [buffer A consisted of 20 mM HEPES-KOH, 10 mM KCL, 1.5 mM MgCl_2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol [DTT], 0.1 PMSF, 5 mg/mL pepstatin A, 10 mg/mL leupeptin]. Lysates were rotated for 30–60 min at 4°C and then centrifuged at 25,000g for 15 min at 4°C. The supernatant containing 1–2 mg/mL protein was then loaded on a FPLC Superdex 200 10/30 column equilibrated with 100 mM NaCl in buffer A with 2% CHAPS. Fractions were eluted at 0.5 mL per minute and collected as 1-mL fractions. Thirty microliters of each fraction was loaded on SDS-PAGE and transferred to nitrocellulose membrane after electrophoresis. Western blot analysis was then performed to detect GFP-tagged proteins.

**FACS cell cycle analysis**

HCT116 cells (2 × 10^6) in 60-mm plates were washed with PBS and fixed with 70% ethanol. After fixing, cells were incubated in Telford reagent [0.1 mM EDTA, 0.03 mg/mL RNase A, 0.05 mg/mL propidium iodide, 0.1% Triton X-100 in PBS] for 30–60 min on ice. Cells were then stained and analyzed for cell cycle phase using BD FACS Calibur flow cytometer and ModFit LT analysis software (Flow Cytometry Research Facility, University of Nebraska Medical Center).

**Cell growth assay**

Cells were plated at a concentration of 50,000 cells per plate. Every 24 h for six consecutive days, cells were trypsinized, centrifuged, resuspended, and counted on a hemocytometer to determine the number of cells per plate.

**Clonogenic survival assays**

Cells were plated at 4 × 10^5 per 35-mm plate 1 d prior to treatment. After treatment, cells were trypsinized, centrifuged, and serially diluted, and dilutions were placed separately in 10-cm plates. Eight days after plating, medium was removed, and cells were stained for 30 min at room temperature with Crystal violet fixing/staining solution [0.05% Crystal Violet, 1% formaldehyde, 1% methanol in 1× PBS solution]. Fixing/staining solution was then removed, and plates were dipped in water to remove excess Crystal violet.
Acknowledgments

We are grateful for the University of Nebraska Medical Center Mouse Genome Engineering Core facility, especially Rolen Quadros and Dr. Channabasaviah Gurumurthy, for their technical support, helpful discussions, and advice. We are also grateful for the University of Nebraska Medical Center DNA Sequencing and Flow Cytometry facilities, especially Victoria Smith, Samantha Wall, and Dr. Philip Hexley, for their excellent technical support. We thank Dr. David Kelly for his help with fluorescence reading of the caspase substrate assay. This work was supported by a pilot grant from the Nebraska Center for Cellular Signaling [P20-GM103489] and Nebraska Research Initiative Funds to X.L.

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