BCL-2 family member BOK promotes apoptosis in response to endoplasmic reticulum stress

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B-cell lymphoma 2 (BCL-2) ovarian killer (BOK) is a BCL-2 family protein with high homology to the multidomain proapoptotic proteins BAX and BAK, yet $Bok^{-/-}$ and even $Bax^{-/-}Bok^{-/-}$ and $Bak^{-/-}Bok^{-/-}$ mice were reported to have no overt phenotype or apoptotic defects in response to a host of classical stress stimuli. These surprising findings were interpreted to reflect functional compensation among the BAX, BAK, and BOK proteins. However, BOK cannot compensate for the severe apoptotic defects of $Bax^{-/-}Bak^{-/-}$ mice despite its widespread expression. Here, we independently developed $Bok^{-/-}$ mice and found that $Bok^{-/-}$ cells are selectively defective in their response to endoplasmic reticulum (ER) stress stimuli, consistent with the predominant subcellular localization of BOK at the ER. Whereas $Bok^{-/-}$ mouse embryonic fibroblasts exposed to thapsigargin, A23187, brefeldin A, DTT, geldanamycin, or bortezomib manifested reduced activation of the mitochondrial apoptotic pathway, the death response to other stimuli such as etoposide, staurosporine, or UV remained fully intact. Multiple organs in $Bok^{-/-}$ mice exhibited resistance to thapsigargin-induced apoptosis in vivo. Although the ER stress agents activated the unfolded protein response, both ATF4 and CHOP activation were diminished in $Bok^{-/-}$ cells and mice. Importantly, BAX and BAK were unable to compensate for the defective apoptotic response to ER stress observed in SV40-transformed and primary $Bok^{-/-}$ cells, and in vivo. These findings support a selective and distinguishing role for BOK in regulating the apoptotic response to ER stress, revealing—to our knowledge—the first bona fide apoptotic defect linked to Bok deletion.

BOK | apoptosis | BCL-2 family | endoplasmic reticulum stress | unfolded protein response

BAX and BAK are essential B-cell lymphoma-2 (BCL-2) family proteins that porate the mitochondrial outer membrane in response to a broad range of apoptotic stimuli (1). BCL-2 ovarian killer (BOK) exhibits ∼70–80% sequence homology to BAX and BAK, sharing BH1-3 domains and a carboxy-terminal transmembrane domain (2). Like BAX and BAK, BOK is widely expressed and induces cell death upon transient overexpression (2–9). In the context of BOK overexpression, BOK-induced cell death manifests classical apoptotic features, including the release of cytochrome *c*, activation of caspase-3, and nuclear and DNA fragmentation (6, 9–11). In addition, overexpression of antiapoptotic MCL-1, BFL-1/A1, and BHRF-1 inhibit BOK-mediated cell death (2, 7, 12, 13). Based on these similarities, it has been suggested that BOK acts in a redundant fashion to BAX and BAK and may be responsible for the apparently normal development of numerous organs in $Bax^{-/-}Bak^{-/-}$ mice (14).

Despite these initial observations, the physiologic role of BOK in responding to cell stress has remained enigmatic. Indeed, the development and characterization of Bok-deficient mice revealed little to no phenotype (3). Moreover, leukocyte subsets from $Bok^{-/-}$ mice showed no alteration in their death response to classical proapoptotic agents, such as etoposide, dexamethasone, Fas ligand, ABT-737, or cytokine deprivation (3). Even $Bok^{-/-}Bax^{-/-}$ and $Bok^{-/-}Bak^{-/-}$ doubly deficient mice are mostly normal, and cells

derived from these mice are equally susceptible to death stimuli (15), in stark contrast to the severe apoptotic blockade of $Bax^{-/-}Bak^{-/-}$ cells. Also intriguing, a functional role for Bok as a tumor suppressor was suggested by its genetic location in 1 of the 20 most deleted regions in all human cancers (16).

To investigate a role for BOK in the apoptotic pathway, we generated $Bok^{-/-}$ mice and cells. Based on the recent localization of BOK at the endoplasmic reticulum (ER) (4), we focused our inquiry on ER stress pathways, including the unfolded protein response (UPR). We find that $Bok^{-/-}$ cells manifest reduced activation of the classical mitochondrial apoptotic pathway in response to a battery of ER stress stimuli. Moreover, the apoptotic deficiency is selective, with no observed differences in response to other inducers of apoptosis. From a mechanistic standpoint, we find that BOK requires downstream BAX/BAK activation to induce mitochondrial apoptosis, as BOK expression can rescue the defective response of $Bok^{-/-}$ cells, but not $Bax^{-/-}Bak^{-/-}$ cells. These findings support a selective role for BOK in regulating the apoptotic response to ER stress, a functional activity that corresponds to its recent subcellular localization at the ER.

Results

Targeted Disruption of the Murine *Bok* Gene Results in Normal Development. To disrupt the *Bok* gene, we electroporated RW4 ES cells with a targeting vector that introduces *LoxP* sites in the introns between exons 1 and 2, and exons 3 and 4 (Fig. 1*A*). Properly targeted ES cells with both the 5′ LoxP site and the puromycin cassette (Fig. 1*B*) were injected into host blastocysts to generate chimeric mice. Targeted mice were bred to mice ubiquitously expressing Cre recombinase to generate an allele that deletes exons 2 and 3, which includes the ATG start site as well as the BH4, BH3, and one-half of the BH1 BCL-2 homology (BH) regions. Heterozygous $Bok^{+/-}$ mice were interbred to generate viable $Bok^{-/-}$ mice. Analysis of $Bok^{-/-}$ mice revealed minimal differences in embryonic histology, complete blood count, and serum chemistries (Fig. S1). Although a shortened transcript was

Significance

The role of B-cell lymphoma-2 (BCL-2) ovarian killer (BOK) in apoptosis regulation has been a long-standing enigma. Despite the homology to BAX and BAK, BOK has yet to be linked to a definitive physiologic function in the classical apoptotic pathway. Here, we report a selective role for BOK in promoting mitochondrial apoptosis in response to endoplasmic reticulum stress.
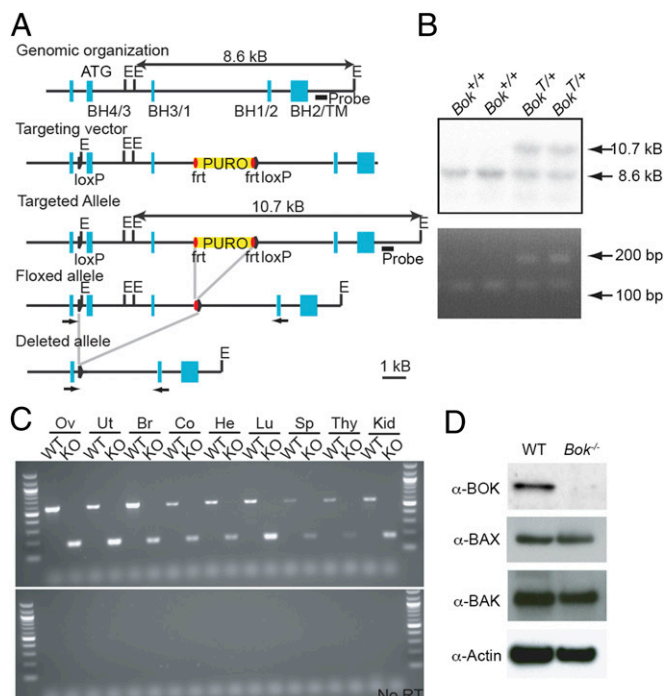
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Fig. 1. Targeted disruption of the mouse *Bok* gene. (*A*) Partial map of the murine *Bok* locus (*Top*), *Bok* targeting vector (*Top Middle*), targeted homologous recombination (*Middle*), removal of the puromycin resistance (puro) cassette (*Bottom Middle*), and complete deletion of the locus (*Bottom*). *Bok* exons are shown as blue boxes with positions of BCL-2 homology (BH) and transmembrane (TM) domains indicated below. Solid triangles represent LoxP sites, and red ovals indicate FRT sites. The probe used in Southern blotting is shown as a black line. Arrows represent primers used for PCR genotyping in *B* and RT-PCR in *C*. E, EcoRI. (*B*) Southern blot analysis of ES cell DNA showing the presence of WT (+/+) and heterozygous (+/Targeted) mutant genotypes (*Top*). PCR analysis of ES cell DNA showing the presence of the 5′ LoxP site in targeted clones (*Bottom*). (*C*) RT-PCR of *Bok* in multiple tissues from WT and *Bok*⁻/⁻ mice: ovary (Ov), uterus (Ut), brain (Br), colon (Co), heart (He), lung (Lu), spleen (Sp), thymus (Thy), and kidney (Kid). (*D*) Western blot for BOK, BAX, BAK, and Actin from WT and *Bok*⁻/⁻ MEFs.

detected in *Bok*⁻/⁻ mice by RT-PCR (Fig. 1*C*), Western blot revealed the absence of BOK protein (Fig. 1*D*). RT-PCR analysis confirmed that BOK exhibits a wide distribution of expression.

Loss of BOK Partially Protects Cells from ER Stress-Induced Cell Death.
A series of viability assays were performed to determine whether BOK has a proapoptotic function. Wild-type (WT) and *Bok*⁻/⁻ murine embryonic fibroblasts (MEFs) were generated by SV40 immortalization of cells isolated from embryonic day 13.5 liver. *Bok*⁻/⁻ MEFs grew similarly to WT MEFs as measured by PrestoBlue (Fig. S2*A*). Whereas the growth of WT MEFs was impaired by treatment with the ER stressors thapsigargin (TG) (a SERCA pump inhibitor) (10 μM) and bortezomib (BTZ) (a proteasome inhibitor) (10 μM), *Bok*⁻/⁻ MEFs continued to grow normally (Fig. S2*A*). In contrast, both WT and *Bok*⁻/⁻ MEFs exhibited decreased growth upon exposure to the DNA-damaging agent etoposide (ETOP) and the promiscuous protein kinase inhibitor staurosporine (STS) (Fig. S2*A*). In agreement with these data, loss of BOK partially protected the cells from treatment with TG or BTZ, but not STS or ETOP, in a dose- and time-dependent fashion, compared with WT cells in an XTT assay (Fig. 2 *A* and *B*). Clonogenic rescue analysis further documented that *Bok*⁻/⁻ cells treated for 48 h with 10 μM TG or 10 μM BTZ remained ~10-fold more viable than WT cells (Fig. 2*C*). To further validate these findings, we tested additional ER stress agents including

DTT (reducing agent), geldanamycin (GELD) (Hsp90 inhibitor), brefeldin A (BFA) (Golgi/ER transport inhibitor), and A23187 (calcium ionophore), and again found that loss of BOK partially protected cells from these ER stress stimuli but not 5 min of 100 J/m² UV irradiation (Fig. 2*D* and Fig. S2*B*). We confirmed that the observed selectivity in apoptotic defect was also present in primary *Bok*⁻/⁻ MEFs (Fig. 2*E* and Fig. S2*C*). To interrogate whether the blunted ER stress response derived from a defect in afferent stress sensing, we performed Western blot analysis of PERK, phospho-PERK, eIF2-α, phospho–eIF2-α, and phospho–IRE1-α. For each of the six ER stress stimuli tested, both WT and *Bok*⁻/⁻ cells manifested intact, upstream signaling pathway responses as assessed by appropriate phosphorylation of PERK and eIF2-α (Fig. 2*F* and Fig. S2*D*). Phosphorylation of IRE1-α was detected for both WT and *Bok*⁻/⁻ cells treated with TG, BTZ, and A23187, but there was decreased phosphorylation in *Bok*⁻/⁻ cells treated with BFA (Fig. 2*F* and Fig. S2*D*). Of note, based on densitometry from three independent experiments, there were no statistically significant changes in BOK expression in WT cells as a result of drug treatment except for mildly decreased levels upon BFA treatment (Fig. 2*F* and Fig. S2*D*). Taken together, these results demonstrate that *Bok*⁻/⁻ cells are selectively deficient in the apoptotic response to ER stress but not STS (kinase inhibition), ETOP (DNA damage), or UV irradiation. Strikingly, the presence of BAX and BAK does not compensate for the loss of BOK in this context.

BOK Mediates ER Stress-Induced Caspase-3/7 Activation and Annexin V Exposure. To determine whether the observed apoptotic deficiency in *Bok*⁻/⁻ cells places BOK upstream of caspase activation, we monitored caspase-3/7 activation in response to ER stress in *Bok*⁻/⁻ vs. WT MEFs. Loss of BOK prevented both the time- and dose-dependent increase in caspase-3/7 activation observed in WT cells exposed to TG or BTZ (Fig. 3 *A* and *B*). As a measure of specificity, *Bok*⁻/⁻ cells treated with STS or ETOP maintained normal levels of caspase-3/7 activation (Fig. 3*A*). Similar results were obtained in primary MEFs (Fig. S3*A*) and for each of the ER stress agents, as evaluated by Western blot for cleaved caspase-3 (Fig. 3*C*). Correspondingly, *Bok*⁻/⁻ MEFs treated with TG or BTZ manifested reduced annexin V positivity compared with WT MEFs, as measured by FACS analysis; however, no differences were observed in response to treatment with ETOP or STS (Fig. 3*D*). Primary *Bok*⁻/⁻ MEFs treated with TG, but not STS, also showed reduced annexin V positivity compared with WT MEFs (Fig. S3*B*). Thus, upon exposure to ER stress stimuli, the presence of BOK correlates with canonical activation of the apoptotic cascade, as reflected by caspase-3/7 and annexin V positivity.

BOK Engages the Intrinsic Mitochondrial Pathway of Apoptosis. Previous reports indicate that, although BOK is a multidomain proapoptotic BCL-2 family member like BAX and BAK, it is predominantly localized to the ER (4). This raised the question of whether BOK-mediated cell death involves the mitochondria. Indeed, we found that ER stress-induced cytochrome *c* release was blunted in *Bok*⁻/⁻ cells compared with WT cells, as monitored by anti-cytochrome *c* immunofluorescence (Fig. 4 *A* and *B*). Diminished cytochrome *c* release was further demonstrated by Western analysis of membrane and cytosolic fractions from *Bok*⁻/⁻ cells treated with 10 μM TG (Fig. 4*C* and Fig. S4). Whereas WT cells demonstrated BAX translocation in response to ER stress, the *Bok*⁻/⁻ cells failed to show an increase in BAX translocation (Fig. 4*C* and Fig. S4). Moreover, BOK did not show any change in localization from the membrane fraction, which contained ER and mitochondria (Fig. S4), in response to TG or STS (Fig. 4*C*). No differences in BAX translocation or cytochrome *c* release were observed in WT and *Bok*⁻/⁻ cells treated with STS (Fig. 4*C* and Fig. S4), confirming that the canonical proapoptotic machinery in *Bok*⁻/⁻ cells is otherwise intact. Importantly, overexpression of
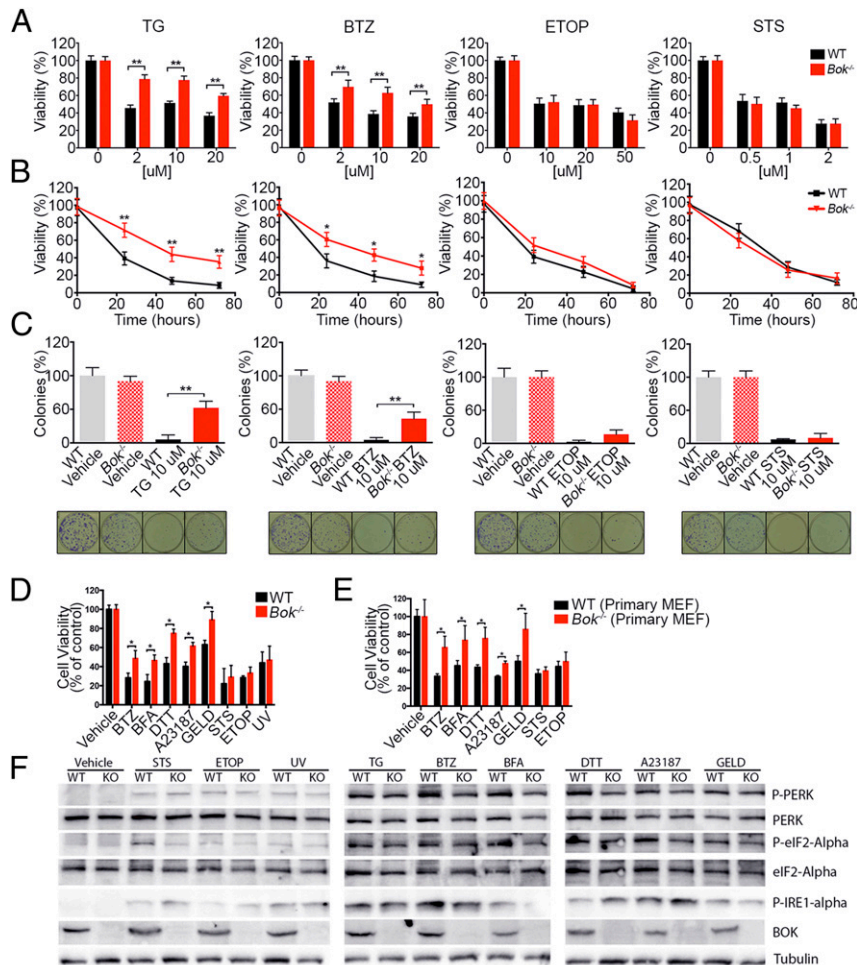
Fig. 2. Loss of *Bok* protects cells from ER stress-induced death. (*A* and *B*) Cell viability of SV40-immortalized WT and *Bok*$^{-/-}$ MEFs as measured by the XTT assay at 24 h using the indicated concentrations of thapsigargin (TG), bortezomib (BTZ), etoposide (ETOP), or staurosporine (STS) (*A*) and at multiple time points with 10 μM TG, 10 μM BTZ, 10 μM ETOP, or 1 μM STS (*B*). Bars and lines represent percent cell viability with respect to untreated cells. (*C*) Number of colonies formed when SV40-immortalized WT and *Bok*$^{-/-}$ cells were plated at clonal densities after 48 h of treatment with 10 μM TG, 10 μM BTZ, 10 μM ETOP, or 1 μM STS, as compared to untreated cells. Representative wells are shown below. (*D* and *E*) Cell viability was measured at 24 h by XTT of SV40-immortalized MEFs (*D*) and primary WT and *Bok*$^{-/-}$ MEFs (n = 5) (*E*) at 1 μM BTZ, 2 μM brefeldin A (BFA), 1 μM DTT, 2 μM A23187, 1 μM geldanamycin (GELD), 1 μM STS, 1 μM ETOP, and after 5 min of 100 J/m^2 UV (UV light; SV40-immortalized MEFs only). All experiments were performed at least in triplicate. Error bars represent SD. Significance was calculated using an ANOVA test (*$P < 0.05$, **$P < 0.005$). (*F*) Representative Western blot analysis for ER stress response pathway in WT and *Bok*$^{-/-}$ (KO) MEFs 6 h after treatment with vehicle, 1 μM STS, 1 μM ETOP, 5 min of 100 J/m^2 UV, 2 μM TG, 1 μM BTZ, 2 μM BFA, 1 μM DTT, 2 μM A23187, and 1 μM GELD.

BOK restored the capacity of *Bok*$^{-/-}$ cells to fully respond to ER stress stimuli, as reflected by annexin V positivity and loss of viability responses (Fig. 5*A* and Fig. S5). However, added BOK was unable to alter the apoptotic resistance of *Bax*$^{-/-}$*Bak*$^{-/-}$ cells, highlighting the downstream requirement of BAX/BAK for BOK's proapoptotic activity (Fig. 5*B* and Fig. S5). Although we do not detect a specific interaction between overexpressed FLAG-BOK with native BAX or BAK by coimmunoprecipitation from untreated cells or cells exposed to TG or STS (Fig. S6), we cannot rule out the role of a transient proapoptotic interaction or intramembrane engagement that is disrupted by cell lysis. Nevertheless, we find that BOK selectively regulates the mitochondrial apoptotic response to ER stress upstream of BAX/BAK.

BOK Is Required for Activation of ATF4 and CHOP. To characterize the signaling defect between ER stress and mitochondrial apoptosis in *Bok*$^{-/-}$ cells, we first interrogated the UPR. By 6 h after initiation of ER stress with TG or BTZ, the UPR is activated in both WT and *Bok*$^{-/-}$ cells as reflected by the phosphorylation of PERK and eIF2-α (Figs. 2*F* and 6*A* and Fig. S7). At this early

time point, eIF2-α has not yet stimulated the transcription of ATF4, which subsequently activates CHOP (Fig. 6*A* and Fig. S7). However, by 16 and 24 h after induction, WT cells exhibited decreased PERK and eIF2-α phosphorylation and increased expression of ATF4 and CHOP, whereas *Bok*$^{-/-}$ cells have markedly increased PERK and eIF2-α phosphorylation and blunted expression of ATF4 and CHOP (Fig. 6*A* and Fig. S7). WT and *Bok*$^{-/-}$ cells treated with STS showed no differences in the expression of UPR components. Decreased expression of ATF4 and CHOP mRNA in *Bok*$^{-/-}$ cells compared to WT cells treated with TG or BTZ, further suggests that BOK's regulatory role occurs between eIF2-α phosphorylation and ATF4 transcription (Fig. 6*B*). In agreement with the reported activation of BIM by CHOP (17), the *Bok*$^{-/-}$ cells, which have diminished CHOP expression, display concomitant loss of BIM expression (Fig. 6*B*). Thus, we find that loss of BOK results in decreased UPR-induced expression of ATF4, CHOP, and BIM.

***Bok*$^{-/-}$ Mice Display Decreased Activation of CHOP and Apoptosis in Response to ER Stress.** To determine whether the apoptotic defect observed in *Bok*$^{-/-}$ MEFs is functionally relevant in an in
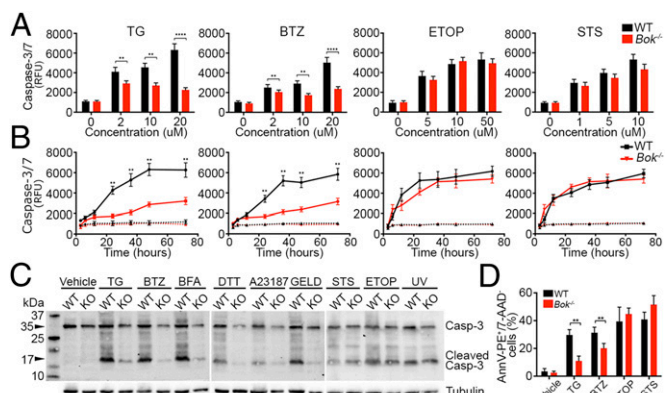
Fig. 3. Loss of *Bok* protects cells from ER stress-induced caspase-3/7 activation and annexin V exposure. (*A* and *B*) Caspase-3/7 activation in SV40-immortalized WT and *Bok*$^{-/-}$ cells at 24 h in response to the indicated concentrations of thapsigargin (TG), bortezomib (BTZ), etoposide (ETOP), or staurosporine (STS) (*A*), and at multiple time points with 10 μM TG, 10 μM BTZ, 10 μM ETOP, or 1 μM STS (*B*). Bars and lines represent relative fluorescence units (RFU). Dashed lines, vehicle; solid lines, treated. (*C*) Representative Western blot analysis for activated caspase-3 in SV40-immortalized WT and *Bok*$^{-/-}$ (KO) MEFs 16 h after treatment with vehicle, 2 μM TG, 1 μM BTZ, 2 μM BFA, 1 μM DTT, 2 μM A23187, 1 μM GELD, 1 μM STS, 1 μM ETOP, and 5 min of 100 J/m² UV. (*D*) Percent annexin V-positive/7AAD-negative SV40-immortalized WT and *Bok*$^{-/-}$ cells treated for 12 h with vehicle, 10 μM TG, 10 μM BTZ, 10 μM ETOP, or 1 μM STS. All experiments were performed at least in triplicate. Error bars represent (*A* and *B*) SD or (*D*) SEM. Significance was calculated using an ANOVA test (**$P < 0.005$, ****$P < 0.0001$).

vivo context, WT and *Bok*$^{-/-}$ mice were i.p. injected with vehicle (150 mM dextrose), TG (1 mg/kg), or ETOP (10 mg/kg). Mice were euthanized at 24 h, and full necropsies were performed. Interestingly, a subset of WT mice treated with TG, but none of the *Bok*$^{-/-}$ mice, died before the 24 h end time point. Western blot of liver extracts revealed activation of the UPR as evidenced by phosphorylation of PERK in both WT and *Bok*$^{-/-}$ mice treated with TG, but not vehicle or ETOP (Fig. 7*A* and Fig. S8). However, *Bok*$^{-/-}$ mice treated with TG showed diminished expression of CHOP and activated caspase-3 in the liver by Western blot (Fig. 7*A* and Fig. S8). Equivalent activated caspase-3 levels were detected in ETOP-treated mice. Immunofluorescence of the liver, kidney, and lung further confirmed diminished expression of CHOP and activated caspase-3, and decreased TUNEL positivity (Fig. 7*B* and Fig. S9). Taken together, these findings support a physiologic role for BOK in mediating ER stress-induced UPR activation and apoptosis in vivo.

Discussion

BOK was identified as a BAX/BAK homolog nearly two decades ago, but its physiologic role has remained enigmatic. Although BOK, BAX, and BAK are widely expressed, BOK does not compensate for the severe apoptotic defects that contribute to the embryonic lethality of *Bax*$^{-/-}$*Bak*$^{-/-}$ mice (18). Likewise, cells deficient in both BAX and BAK are highly resistant to a wide variety of apoptotic agonists despite the presence of BOK (19–21). In striking contrast, the intimate functional relationship between BAX and BAK is underscored by the fact that cells singly deficient for BAX or BAK exhibit little to no apoptotic resistance to death stimuli (20, 21). Given the normal growth and development of visceral tissues, such as liver, heart, and lung, in *Bax*$^{-/-}$*Bak*$^{-/-}$ mice, we and others hypothesized that perhaps BOK plays a major, independent apoptotic role in these tissues. Nevertheless, *Bok*$^{-/-}$ mice show no overt defects in these tissues either (3, 14, 15). Thus, we explored whether BOK could subserve a specific

apoptotic function independent from BAX and BAK at the ER, its site of predominant intracellular localization (4).

A previous study reported no difference in annexin V positivity between WT and independently derived *Bok*$^{-/-}$ MEFs treated with TG, and even an increased sensitivity of *Bok*$^{-/-}$ cells to BFA exposure (4). In contrast, here we observe apoptotic resistance in SV40-transformed *Bok*$^{-/-}$ MEFs exposed to six different ER stress agents, including TG and BFA, at various time points and over a broad dose range, as analyzed by XTT, caspase-3/7, clonogenic, and annexin V assays. Importantly, we observe a similar profile of selective apoptotic resistance to ER stress stimuli in primary *Bok*$^{-/-}$ MEFs. Although Ke et al. (3) deleted one-half of exon 1 and all of exon 2, and we deleted exons 2 and 3, both studies document loss of BOK by Western blot and RT-PCR, so the source of the discrepancy is unclear. However, we demonstrate that reexpression of BOK fully rescues the capacity of *Bok*$^{-/-}$ cells to respond to the ER stress agents, confirming the dependence of the apoptotic defect on BOK. Furthermore, we observe the selective apoptotic defect to ER stress in multiple tissues of our *Bok*$^{-/-}$ mice. Indeed, protection from ER stress-induced apoptosis upon loss of BOK is consistent with its proposed proapoptotic functionality, as observed upon overexpression (2, 5–8, 10, 12).

It is especially striking that BOK confers at least partial apoptotic resistance independent of BAX and BAK, whereas only
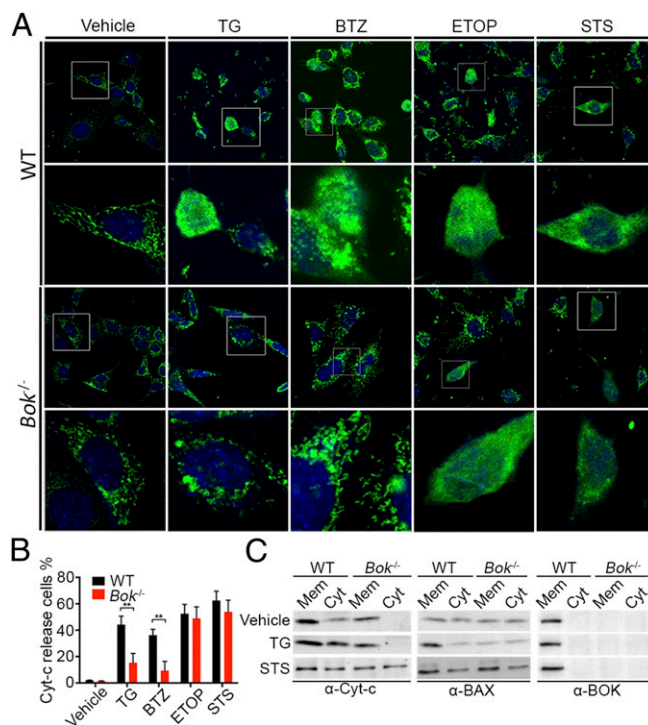

Fig. 4. BOK promotes cytochrome *c* release and BAX translocation in response to ER stress. (*A*) Immunofluorescence detection of cytochrome *c* release by SV40-immortalized WT and *Bok*$^{-/-}$ cells subjected to vehicle or 24-h exposure to 10 μM TG, 10 μM BTZ, 10 μM ETOP, or 1 μM STS. Cytochrome *c* release and cell nuclei were examined by confocal immunofluorescence microscopy using anti-cytochrome *c* antibody (green) and TO-PRO3 (blue) staining. (*B*) Quantitative analysis of cytochrome *c* release from *A*. For each condition, 100 cells were analyzed in each of five randomly chosen fields. Three independent experiments were performed, and the means ± SEM were plotted. Significance was calculated using an ANOVA test (**$P < 0.005$). (*C*) Representative Western analyses of cytosolic (Cyt) and membrane (Mem) fractions of WT and *Bok*$^{-/-}$ cells subjected to vehicle or 24-h treatment with 10 μM TG or 1 μM STS to monitor cytochrome *c* release (*Left*), and BAX (*Middle*) and BOK (*Right*) translocation. The presence of mitochondria and ER in the membrane preparation is further documented by Western analyses for COX IV and calnexin, respectively (Fig. S4).
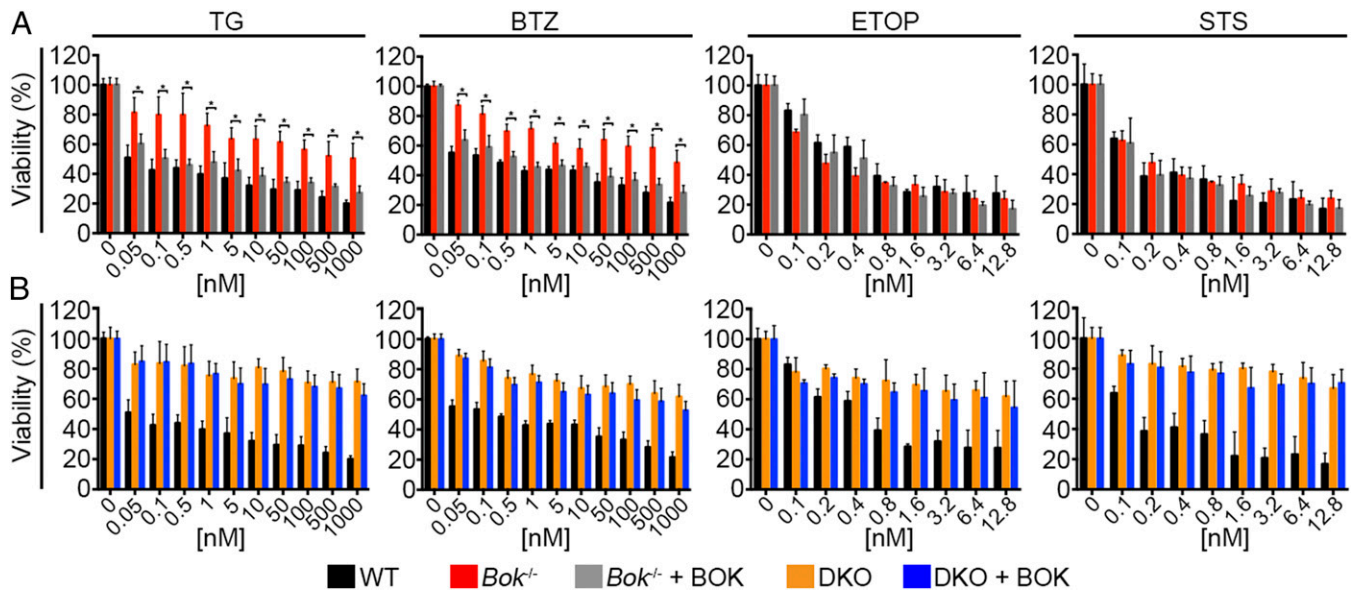
Carpio et al.

Fig. 5. BOK rescues the ER stress-induced death response of $Bok^{-/-}$, but not $Bax^{-/-}Bak^{-/-}$, cells. (A and B) Cell viability was measured at 24 h by XTT for WT (A and B), $Bok^{-/-}$ (A), and $Bax^{-/-}Bak^{-/-}$ [double knockout (DKO)]. (B) MEFs were either not transfected or transfected with a BOK expression vector, and after 24 h were treated with a broad dose range of TG, BTZ, ETOP, and STS for an additional 24 h. All experiments were performed at least in triplicate. Error bars represent SD. Significance was calculated using an ANOVA test (*$P < 0.05$).

the combination of BAX and BAK deficiency can protect against ER stress (20). This suggests that at least one of BOK's functions involves a distinct step in the ER stress-induced apoptotic pathway, and this function cannot be compensated for by BAX or BAK. The absence of cytochrome c release and mitochondrial translocation of BAX in the stimulated $Bok^{-/-}$ cells, and the inability of overexpressed BOK to kill stimulated $Bax^{-/-}Bak^{-/-}$ cells, suggests that BOK functions upstream of BAX and BAK.

Although it is plausible that BOK could directly and/or indirectly activate BAX/BAK in an analogous fashion to a BH3-only protein via its conserved BH3 domain, we did not detect a BAX/BAK interaction by coimmunoprecipitation.

To determine how BOK connects ER stress signaling to the core apoptotic machinery, we further dissected the UPR. Whereas upstream UPR sensing was intact in $Bok^{-/-}$ cells, as measured by PERK and eIF2-α phosphorylation at 6 h, downstream effector
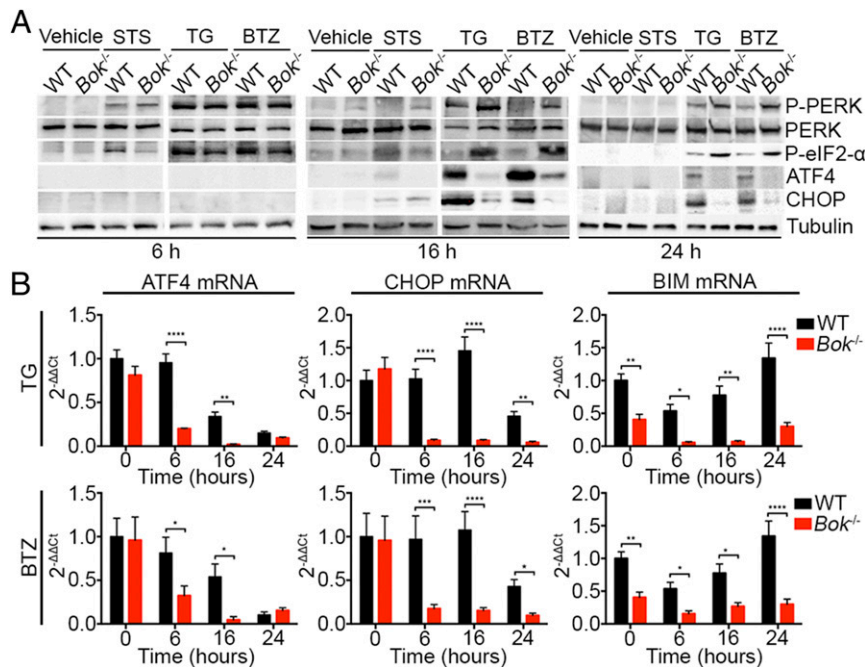

Fig. 6. ATF4 and CHOP expression is diminished in $Bok^{-/-}$ cells. (A) Representative Western blot analysis for the ER stress response pathway and (B) quantitative RT-PCR analysis for ATF4, CHOP, and BIM in WT and $Bok^{-/-}$ MEFs 6, 16, and 24 h after treatment with vehicle, 1 μM STS, 2 μM TG, and 1 μM BTZ. RNA expression levels are fold change of the target gene normalized to β-actin and expressed relative to WT cells at time 0. Error bars represent SD. Significance was calculated using an ANOVA test (*$P < 0.05$, **$P < 0.005$, ****$P < 0.0001$).
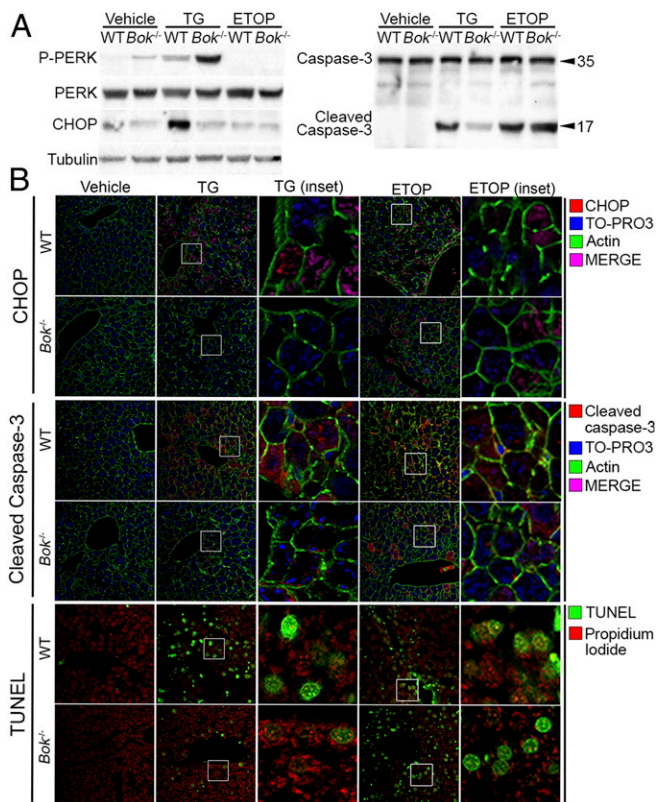
Fig. 7. Loss of *Bok* protects mice from ER stress-induced apoptosis. (*A*) Representative Western blot analysis (*n* = 3) for the ER stress response pathway and activated caspase-3. (*B*) Immunofluorescence detection of CHOP, activated caspase-3, and TUNEL in WT and *Bok*⁻/⁻ livers (*n* = 3) from a representative mouse treated with vehicle, 1 mg/kg TG, or 10 mg/kg ETOP. Merge is between red (CHOP in rows 1 and 2; cleaved caspase-3 in rows 3 and 4) and blue (TO-PRO3).

BIM levels are diminished in the ER-stressed *Bok*⁻/⁻ cells, the observed reduction in classical apoptotic hallmarks, such as cytochrome *c* release, caspase-3/7 activation, and annexin V positivity, may actually be due to BOK's regulation of CHOP. Thus, the requirement of BAX/BAK may result from BOK induction of BIM through CHOP and subsequent indirect and direct activation of BAX and BAK by BIM.

Additional afferent roles of BOK may operate at the level of the ER, the mitochondria, or both subcellular locations. Because BOK and BAX/BAK independently resist TG-induced death despite the other protein(s) being present, there are likely multiple BCL-2 family-regulated control points for the ER stress pathway. Indeed, in addition to their roles at the mitochondria, BAX and BAK have proximal functions at the ER in both regulating calcium homeostasis and the UPR (22, 23). As BOK binds inositol 1,4,5-trisphosphate receptors (24), it too might function in discrete homeostatic processes of the ER, and thereby contribute to the complex interplay of BCL-2 family functions at the intersection of ER and mitochondrial physiology. Our in vitro and in vivo loss-of-function studies demonstrate that BOK has a bona fide proapoptotic function in regulating ER stress-induced apoptosis, linking apoptotic sensing at the ER to apoptotic execution at the mitochondria.

Experimental Procedures

Cell Survival and Apoptosis Assays. XTT (Cell Signaling), PrestoBlue (Life Technologies), caspase-3/7 (Life Technologies), and annexin V (BD Pharmingen) assays were performed according to manufacturers' instructions and measured on a Wallac 1420 VICTOR² multilabel counter or a FACSCanto II (annexin V). Full details of these procedures and the clonogenic cell survival assay may be found in *SI Experimental Procedures*.

Molecular Biology. Immunoblot analysis, cellular fractionation, and quantitative RT-PCR were performed using standard techniques. A full description of buffers, antibodies, and primers may be found in *SI Experimental Procedures*.

Animal Work. Generation and handling of C57BL/6 backcrossed *Bok*⁻/⁻ mice, including generation of MEFs and treatment with proapoptotic agents, were approved by the institutional animal care and use committees of the Dana–Farber Cancer Institute and Yale University. These details as well as tissue fixative protocols, TUNEL (Invitrogen), antibodies, and a description of the imaging analysis may be found in *SI Experimental Procedures*.

signaling of the UPR was defective. In particular, by 16 h, ATF4 and CHOP transcriptional induction is greatly diminished in the *Bok*⁻/⁻ cells and eIF2-α phosphorylation is markedly increased. Importantly, the defects in downstream UPR signaling identified in MEFs were also found in the liver, kidneys, and lungs of *Bok*⁻/⁻ mice treated with TG. Because CHOP regulates BIM (17), and

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