Supporting Information

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SI Experimental Procedures

Development of *Bok*$^{-/-}$ Mice and MEFs. Three portions of the *Bok* locus were cloned into the pFlexible plasmid (kind gift from S. Orkin, Dana–Farber Cancer Institute, Boston) by genomic PCR: a 2.4-kb 5′ arm (1:93,683,887–6,249) upstream of the LoxP site, a 4.6-kb middle arm (1:93,686,249–90,915) between the LoxP site and the puromycin cassette, and a 5-kb 3′ arm (1:93,690,930–5,897). Construct DNA was linearized with ClaI and electroporated into RW4 embryonic stem cells. Clones were selected with 1 μg/mL puromycin and screened by Southern blotting and PCR. A correctly targeted clone was karyotyped and injected into C57BL/6 blastocysts to generate chimeric and ultimately germline-transmitted heterozygous *Bok*-targeted mice. Mating to ubiquitously expressing Cre-recombinase mice (Jackson Laboratory; 003724) generated *Bok*$^{+/-}$ mice. Mice were genotyped by PCR with 5′-gctgggatctgcgagattta-3′, 5′-gcgattcaaacaacacagga-3′, and 5′-gtaaagcctgctggtgcttc-3′ primers to generate 123-, 199-, and 414-bp products for the WT, floxed, and knockout alleles, respectively. Murine embryonic fibroblasts (MEFs) were generated from *Bok*$^{+/-}$ matings at 13.5 d after conception and either used directly as primary MEFs or immortalized with SV40 genomic DNA as described (1). Mice were backcrossed to C57BL/6 for eight generations. All mice were handled in accordance with the Dana–Farber Cancer Institute and Yale University institutional guidelines on animal care.

Animal Experiments and Tissue Sample Analysis. Adult WT and *Bok*$^{-/-}$ mice were intraperitoneally injected with 100 μL of 150 mM dextrose alone or with TG (1 mg/kg body weight) or etoposide (ETOP) (10 mg/kg body weight). After 24 h, the mice were euthanized by isoflurane inhalation. Liver, kidneys, and lungs were removed and fixed overnight in 4% (wt/vol) PBS-buffered formalin, followed by 10%, 20%, and 30% (wt/vol) sucrose solution for 3 d before embedding in OCT and 10-μm sections mounted on glass slides. In addition, 5 mg of each organ was washed with chilled 1× PBS, and Dounce homogenized on ice in lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 mM Tris·HCl, pH 7.4) and protease inhibitor mixture EDTA-free (Roche). After 30 min on ice, the samples were sonicated 2–5 min at 180 W (in 10-s on/off cycles). Finally, the samples were centrifuged at 10,000 × *g* for 20 min at 4 °C, and supernatant was collected. Samples were then used for immunoblot analysis.

Cell Culture. WT and *Bok*$^{-/-}$ MEF cell lines were grown in DMEM (high glucose; Invitrogen), containing 4.5 g/L glucose, 4 mM L-glutamine, 200 units/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 10% heat-inactivated FBS.

Immunoblot Analysis. WT and *Bok*$^{-/-}$ MEFs were grown to 90% confluence, washed twice with PBS, and disrupted in lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 mM Tris·HCl, pH 7.4) and protease inhibitor mixture EDTA-free (Roche). Total protein concentration was quantitated with the BCA protein assay kit (Thermo Scientific). Proteins were separated by SDS/PAGE and transferred onto a nitrocellulose membrane. Primary antibodies used included anti-cytochrome *c* (D18C7; 1:1,000; Cell Signaling), anti-GAPDH (14C10; 1:1,000; Cell Signaling), anti-COX IV (4D11-B3-E8; 1:500; Cell Signaling), anti-calnexin (1:1,000; Cell Signaling), anti-BOK (1:200; Abcam), anti-BAX (1:2,000; N20; Santa Cruz), anti-BAK (NT; 1:500; Calbiochem), anti–caspase-3 (1:1,000; Cell Signaling), anti–P-PERK (Thr-981; 1:200; Santa Cruz), anti-PERK (H-300; 1:200; Santa Cruz), anti–P-eIF2-α (1:500; Abcam), anti–eIF2-α (phospho S51; 1:50; Abcam), anti–P-IRE1-α (phospho S724; 1:1,000; Abcam), anti–ATF-4 (D4B8) mAb (1:1,000; Cell Signaling), anti-CHOP (L63F7) mAb (1:1,000; Cell Signaling), anti-FLAG (1:1,000; Sigma), anti-tubulin (DM1A; 1:10,000; Abcam), and anti-actin (1:5,000; Sigma). For secondary antibodies, anti-mouse IgG–peroxidase and peroxidase-conjugated goat anti-rabbit IgG antibodies (Sigma) were used. The nitrocellulose membrane was visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

XTT Cell Viability Assay. WT and *Bok*$^{-/-}$ MEFs (0.5–1 × 10^4 cells per well) were seeded in a flat-bottom 96-well microtiter plate. After treatment, 50 μL of XTT detection solution (Cell Signaling) was added to each well, and the plate was returned to the CO_2 incubator for 3 h, followed by measurement of absorbance at 450 nm with a Wallac 1420 VICTOR^2 multilabel counter.

Clonogenic Cell Survival Assay. WT and *Bok*$^{-/-}$ MEFs (2–5 × 10^5 cells/well) were seeded in six-well plates. The next day, the proapoptotic agents were added at the indicated concentrations and incubated for 24 h. After treatment, cells were trypsinized, counted, and 100-cell suspensions were replated in a six-well plate. After at least 1 wk, the media was removed and the cells were rinsed with 10 mL of PBS before being fixed in a 3-mL mixture of 6.0% (wt/vol) glutaraldehyde and 0.5% crystal violet for 30 min. The plates were then carefully rinsed with water and air dried before taking pictures with a DS5000 Nikon camera. Colonies were counted using ImageJ 1.46 software.

Proliferation Assay. WT and *Bok*$^{-/-}$ MEFs (1–3 × 10^3 cells/well) were seeded in 100 μL in a flat-bottom 96-well microtiter plate and incubated for 24 h, followed by addition of the indicated proapoptotic agents and 10 μL of PrestoBlue Cell Viability reagent (Molecular Probes/Life Technologies). Fluorescence was measured at multiple time points using a Wallac 1420 VICTOR^2 multilabel counter (wavelength 535-nm excitation and 615-nm emission filters).

Caspase-3/7 Activation Assay. WT and *Bok*$^{-/-}$ MEFs (0.5–1 × 10^4 cells per well) were seeded in 100 μL of media in a flat-bottom 96-well microtiter plate. The next day, the indicated drugs and 5 μL of CellEvent Caspase-3/7 Green Detection Reagent (Molecular Probes/Life Technologies) were added to each well. Fluorescence was measured at multiple time points using a Wallac 1420 VICTOR^2 multilabel counter (wavelength 502-nm excitation and 530-nm emission filters).

Immunofluorescence. For cytochrome *c*, WT and *Bok*$^{-/-}$ MEFs were grown on glass coverslips to 60% confluence and incubated for 24 h, followed by addition of the indicated drugs. Cells were washed twice with PBS, fixed with 3% (wt/vol) formaldehyde and 4% (wt/vol) sucrose in PBS for 30 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and then treated with 5% (wt/vol) BSA for 1 h before addition of antibodies. Cells were incubated overnight at 4 °C with cytochrome *c* (6H2.B4) mouse monoclonal primary antibody (1:300; Cell Signaling) and for 1 h at room temperature with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (1:1,000; Molecular Probes). All antibodies were diluted in PBS containing 1% BSA. TO-PRO-3 far red-fluorescent counterstain was used to stain cellular nuclei.

For mouse organs, 10-μm sections were washed twice with 1× PBS and then treated with 1% BSA, 0.3% Triton X-100 in 1× PBS for 1 h before addition of antibodies. The sections were incubated with anti-CHOP mouse monoclonal antibody (1:3,200) or anti-cleaved caspase-3 rabbit polyclonal antibody (1:400) overnight at 4 °C and incubated for 1 h with Alexa Fluor 546-conjugated goat anti-mouse IgG (1:1,000) or Alexa Fluor 546-conjugated donkey anti-rabbit IgG (1:1,000) secondary antibodies, respectively. Both antibodies were diluted in 1× PBS containing 1% BSA and 0.3% Triton X-100. TO-PRO-3 far red-fluorescent counterstain was used to stain cellular nuclei and Alexa Fluor 488-phalloidin for cytoskeleton staining.

TUNEL Assay. Immunostaining of DNA fragmentation of apoptotic cells in tissues was performed using the APO-BrdU TUNEL Assay Kit (Molecular Probes; Invitrogen) according to the manufacturer's protocol. Briefly, 10-μm sections were washed twice with 1× PBS and then treated with ice-cold 70% (vol/vol) ethanol for 30 min at –20 °C. The sections were incubated in the DNA-labeling solution (reaction buffer, TdT enzyme, and BrdUTP) for 60 min at 37 °C, washed twice with 1× PBS, and incubated with Alexa Fluor 488 dye-labeled anti-BrdU antibody and propidium iodide/RNase A for 30 min at room temperature.

Image Acquisition. Confocal images were captured with an inverted Zeiss LSM 510 Meta laser confocal microscope (Carl Zeiss), using a 63/1.4 Plan-Apochromat objective. A Zeiss LSM 510 Meta laser confocal microscope equipped with a 25-mW argon excitation laser emitting at 488 nm was used. Emissions were collected using a 505- to 530-nm bandpass filter for Alexa 488.

Annexin V Binding. WT and *Bok*$^{-/-}$ MEFs (4×10^5 cells per well) were seeded in six-well plates, and the indicated drugs were added the next day. After 12 h, cells were harvested with 2 mM EDTA, washed in PBS, pelleted, and resuspended in incubation buffer containing 1% annexin V and 1% 7AAD (Annexin V-PE Apoptosis Detection Kit I; BD Pharmingen). Samples were kept in the dark for 15 min prior and then analyzed on a FACSCanto II flow cytometer (BD Biosciences) using BD FACSDiva software and FlowJo, version 10.

Cellular Fractionation. WT and *Bok*$^{-/-}$ MEFs (2.5×10^5 cells per well) were seeded in six-well plates. The next day, the indicated proapoptotic agents were added and incubated for 24 h. After treatment, cells were trypsinized, collected by centrifugation (211 × *g*; 3 min) and washed twice with 1× PBS. The cells were incubated with 0.005% digitonin in 1× PBS for 5 min on ice and centrifuged (21,130 × *g*; 3 min) to collect the cytosolic fraction (supernatant) and membrane fraction (pellet). The pellet was washed twice with 1× PBS and disrupted in lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 mM Tris·HCl, pH 7.4) and protease inhibitor mixture EDTA-free (Roche). Samples were then used for immunoblot analysis.

Quantitative RT-PCR. WT and *Bok*$^{-/-}$ MEFs were seeded in 10-cm^2 dishes. The next day, the proapoptotic agents were added at the indicated concentrations and incubated for 24 h. After treatment, total mRNA was isolated with the RNeasy Mini Kit (Qiagen) and reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. The cDNA was amplified using iQ SYBR Green Supermix (Bio-Rad), and expression levels of ATF4, CHOP, BIM, and β-actin mRNA were determined using these primers: ATF4 forward, 5′-TCGATGCTCTGTTTCGAATG-3′; reverse, 5′-GGCAACCTGGTCGACTTTTA-3′; CHOP forward, 5′-CATACACCACCACACCTGAAAG-3′; reverse, 5′-CGTTCCTTCTTGATCCTTTGCC-3′; BIM forward, 5′-CGACAGTCTCAGGAGGAACC-3′; reverse, 5′-CATTTGCAAACACCCTCCTT-3′; actin forward, 5′-GACCCAGATCATGTTTGAGACC-3′; reverse, 5′-ATCTCCTTCTGCATCCTGTCG-3′. Cycle conditions were as follows: 48 °C for 30 min, 95 °C for 3 min, followed by 40 cycles of 15 s at 95 °C, 20 s at 60 °C, and 30 s at 72 °C on an iCycler (Bio-Rad). PCRs for each sample were done in triplicate for all of the target genes and actin.

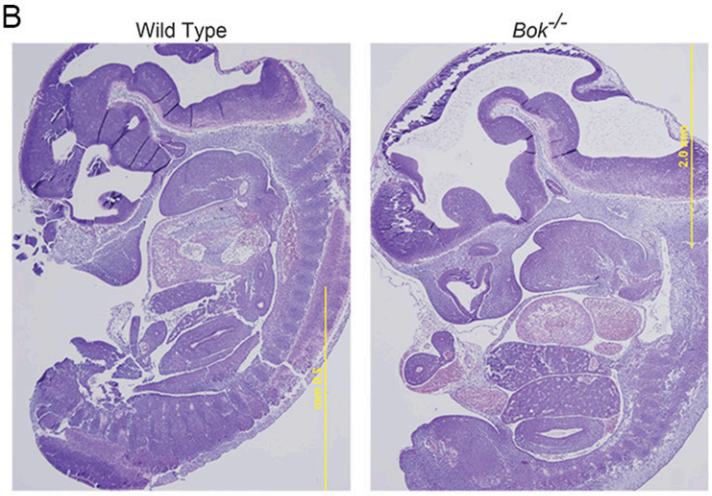
Immunoprecipitation. Cells were transfected using Fugene (Roche) according to the manufacturer's protocol with 2.5 μg of plasmid encoding an HA/FLAG-tagged BOK under the MSCV promoter and followed by an IRES-GFP cassette. These cells were treated with vehicle, 10 μM TG, or 1 μM STS for 16 h before lysed in RIPA buffer (25 mM Tris·HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors. For each condition, 4 μL of anti-FLAG polyclonal Ab were incubated for 1 h at 4 °C with 25 μL of protein G-Sepharose (Amersham Biosciences) in 1 mL of RIPA buffer. The suspension was briefly centrifuged, and the pellet was washed twice with RIPA buffer. Then, the whole-cell lysate (300–500 μg of protein) was added to the suspension, and the mixture was rotated overnight at 4 °C. The beads were pelleted, washed, and eluted in SDS loading dye. Samples were run on a gel, transferred, and immunoblotted.

1. Wei MC, et al. (2001) Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. *Science* 292(5517):727–730.

A

	Male (Actual)	Male (Expected)
Bok $^{-/-}$	6	10.25
Bok $^{+/-}$	26	20.5
Bok $^{+/+}$	9	10.25
	Female (Actual)	**Female (Expected)**
Bok $^{-/-}$	12	12.75
Bok $^{+/-}$	25	25.5
Bok $^{+/+}$	14	12.75

B



C

	WT (n=11)	KO (n=11)	p value
WBC	8.02 ± 1.02	7.59 ± 1.55	0.45
RBC	11.00 ± 0.39	10.73 ± 0.36	0.11
Hgb	15.37 ± 0.42	15.62 ± 0.42	0.19
Hct	56.64 ± 1.11	56.50 ± 1.98	0.84
MCV	51.51 ± 1.46	52.67 ± 1.75	0.11
MCH	13.97 ± 0.60	14.57 ± 0.51	0.02
MCHC	27.14 ± 0.74	27.68 ± 0.61	0.08
CHCM	25.47 ± 0.59	25.93 ± 0.68	0.11
CH	13.09 ± 0.50	13.63 ± 0.52	0.02
RDW	12.79 ± 0.50	12.39 ± 0.54	0.09
HDW	1.55 ± 0.11	1.57 ± 0.05	0.46
PLT	1283.82 ± 122.26	1288.73 ± 112.92	0.92
MPV	7.19 ± 0.14	7.27 ± 0.06	0.10
Neut	6.75 ± 2.26	10.54 ± 4.83	0.03
Lymph	88.09 ± 2.55	84.36 ± 5.15	0.04
Mono	1.79 ± 0.48	2.01 ± 0.71	0.41
Eo	2.12 ± 0.89	2.05 ± 0.59	0.82
Baso	0.40 ± 0.09	0.38 ± 0.14	0.72
LUC	0.86 ± 0.28	0.68 ± 0.11	0.06

D

	WT (n=11)	KO (n=15)	p value
Sodium	152.18 ± 2.60	152.13 ± 2.36	0.96
Potassium	4.73 ± 0.59	4.66 ± 0.52	0.78
Chloride	108.55 ± 2.25	106.53 ± 3.14	0.08
CO2	16.11 ± 1.27	16.75 ± 2.09	0.43
anion gap	28.11 ± 4.28	29.08 ± 5.76	0.68
Glucose	139.50 ± 41.41	129.08 ± 40.34	0.55
BUN	32.56 ± 5.77	27.92 ± 3.00	0.03
Cre	0.12 ± 0.04	0.09 ± 0.07	0.17
Ca	9.94 ± 0.25	10.09 ± 0.60	0.50
Phos	7.25 ± 0.99	6.88 ± 1.65	0.54
Mag	2.6 ± 0.21	2.74 ± 0.26	0.22
AST	69.6 ± 24.48	73.15 ± 16.68	0.68
ALT	35.55 ± 22.38	44.53 ± 42.33	0.53
LDH	659.00 ± 127.92	697.22 ± 88.55	0.48
Alk Phos	101.55 ± 24.68	102.67 ± 48.95	0.95
Alb	3.36 ± 0.16	3.33 ± 0.32	0.78
Total Protein	5.43 ± 0.31	5.58 ± 0.65	0.55
Bilirubin, total	0.06 ± 0.05	0.09 ± 0.03	0.05
Bilirubin Direct	0.02 ± 0.04	0.04 ± 0.05	0.25

Fig. S1. Loss of *Bok* does not affect mouse viability. *Bok*$^{-/-}$ mice (*A*) survival, (*B*) hematoxylin and eosin histology at embryonic day 12.5, (*C*) blood indices, and (*D*) serum chemistries, as compared to WT. Values are mean ± SD.

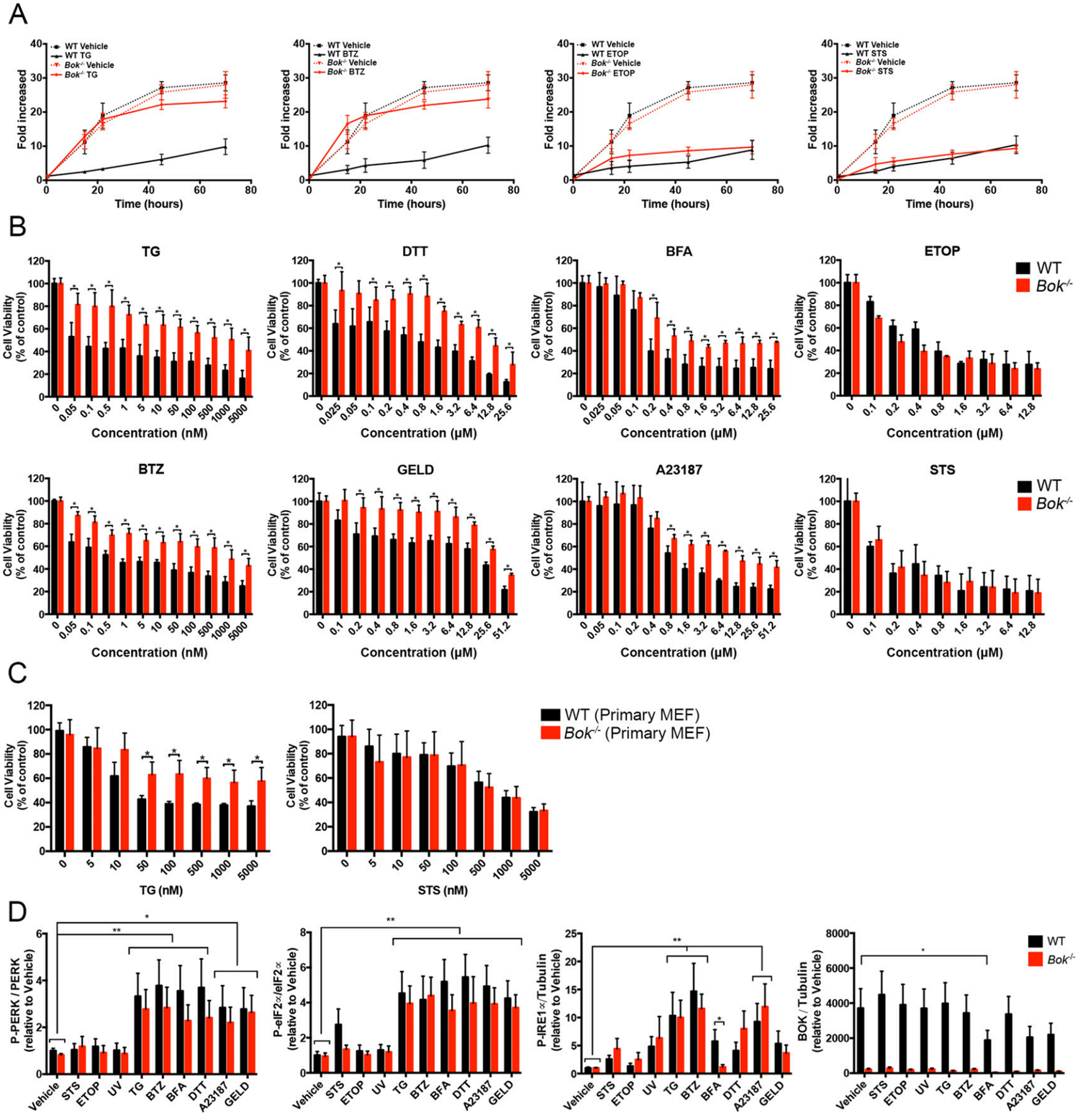


Fig. S2. *Bok*$^{-/-}$ MEFs exhibit resistance to ER stress. (*A*) Cell proliferation was measured at 18, 24, 48, and 72 h using Presto blue cell viability reagent for SV40-immortalized WT and *Bok*$^{-/-}$ cells incubated in the presence of vehicle, 10 μM thapsigargin (TG), 10 μM bortezomib (BTZ), 10 μM etoposide (ETOP), or 1 μM staurosporine (STS). Lines represent the fold increase of fluorescence intensity measured. (*B*) Cell viability of SV40-immortalized WT and *Bok*$^{-/-}$ cells was measured at 24 h by XTT over a broad dose range for each drug: TG, DTT, brefeldin A (BFA), ETOP, BTZ, geldamycin (GELD), A23187, and STS. (*C*) Cell viability was measured at 24 h by XTT of primary WT and *Bok*$^{-/-}$ MEFs ($n = 5$) with a dose curve for TG and STS. (*D*) Quantification of Western blot analysis for phosphorylated PERK relative to total PERK, phosphorylated eIF2-α relative to total eIF2-α, phosphorylated IRE1-α relative to tubulin, and BOK relative to tubulin in WT and *Bok*$^{-/-}$ MEFs ($n = 3$) 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. Error bars represent mean ± SD (*A*–*C*) or SEM (*D*). Significance was calculated using an ANOVA test (*$P < 0.05$, **$P < 0.005$).

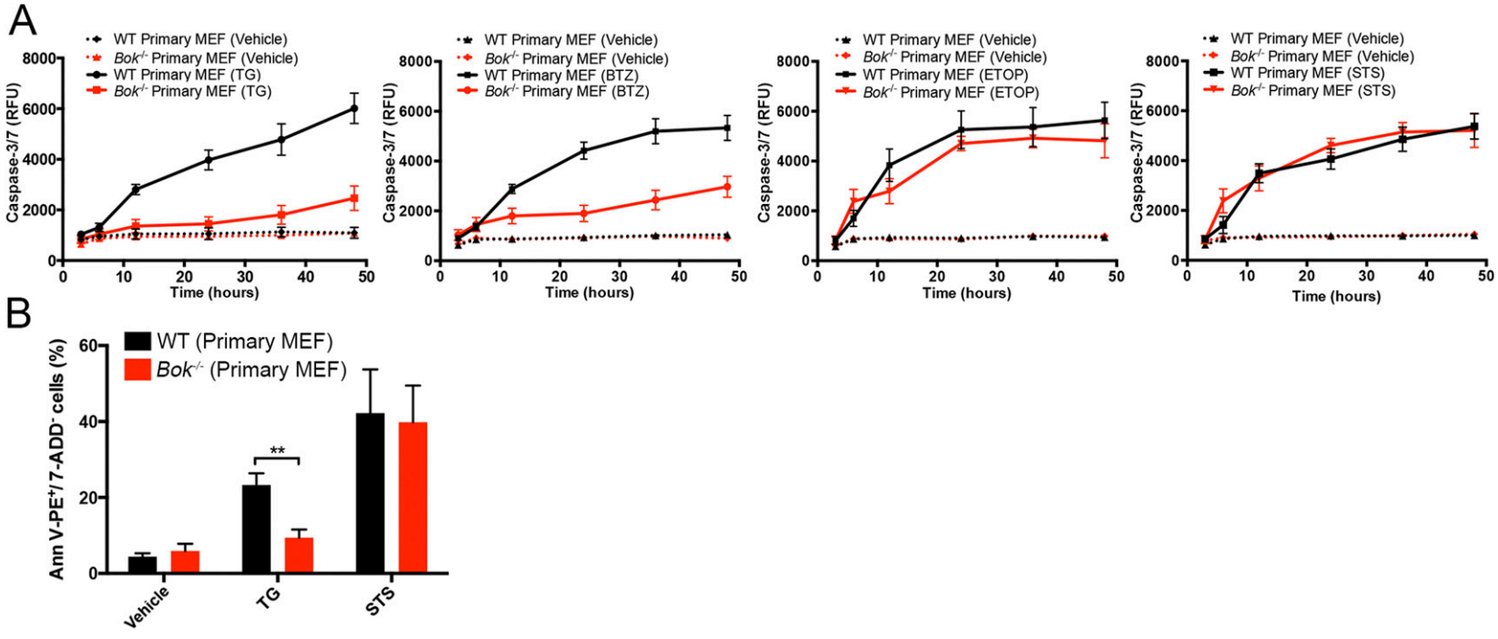


Fig. S3. Loss of BOK protects cells from ER stress-induced caspase-3/7 activation and annexin V exposure in primary MEFs. (*A*) Caspase-3/7 activation in primary WT and $Bok^{-/-}$ MEFs ($n = 5$) at multiple time points with 10 μM TG, 10 μM BTZ, 10 μM ETOP, or 1 μM STS. (*B*) Percent annexin V-positive/7AAD-negative primary WT and $Bok^{-/-}$ MEFs ($n = 5$) treated for 12 h with vehicle, 10 μM TG, or 1 μM STS. TG, thapsigargin; BTZ, bortezomib; ETOP, etoposide; STS, staurosporine. Error bars represent mean ± SD (*A*) or SEM (*B*). Significance was calculated using an ANOVA test (*$P < 0.05$, **$P < 0.005$).

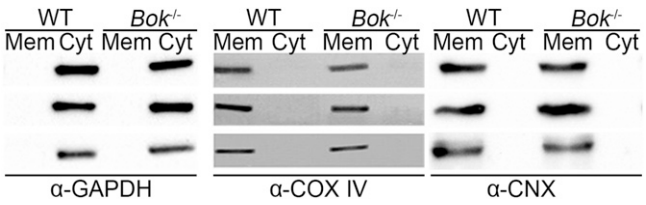


Fig. S4. Cytosolic and membrane preparations for Fig. 4*C*. Western analyses documented the presence of GAPDH in the cytosolic fraction (Cyt), and COX IV (mitochondria), and calnexin (ER) in the membrane (Mem) fraction. The subcellular markers also served as protein loading controls.

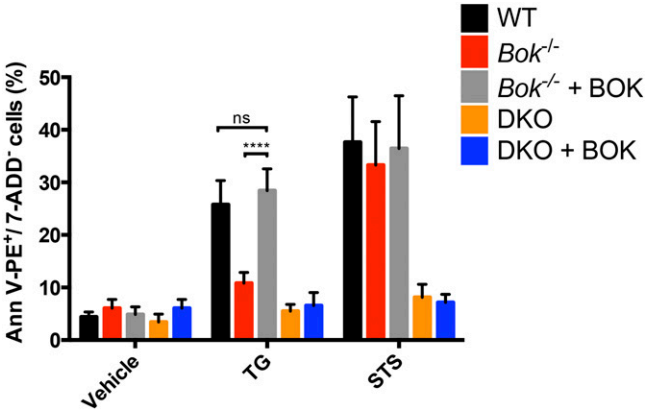


Fig. S5. BOK rescues ER-stress induced annexin V positivity in $Bok^{-/-}$ but not $Bax^{-/-}Bak^{-/-}$ cells. Percent annexin V-positive/7AAD-negative WT, $Bok^{-/-}$, and $Bax^{-/-}Bak^{-/-}$ (DKO) MEFs, which were either not transfected or transfected with a BOK expression vector, and after 24 h treated for 12 h with 10 μM TG or 1 μM STS. Three independent experiments were performed, and the means ± SEM were plotted. Significance was calculated using an ANOVA test (****$P < 0.0001$; ns, not significant).

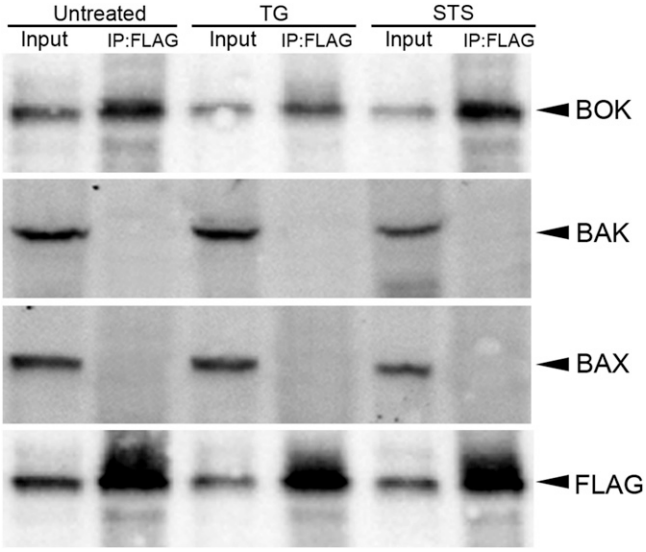


Fig. S6. BOK does not coimmunoprecipitate with BAX or BAK. $Bok^{-/-}$ cells transiently transfected with FLAG-BOK and treated with vehicle, 10 μM TG, or 1 μM STS do not show association of BOK with BAK or BAX, as assessed by FLAG immunoprecipitation and anti-BAK and -BAX Western analysis.

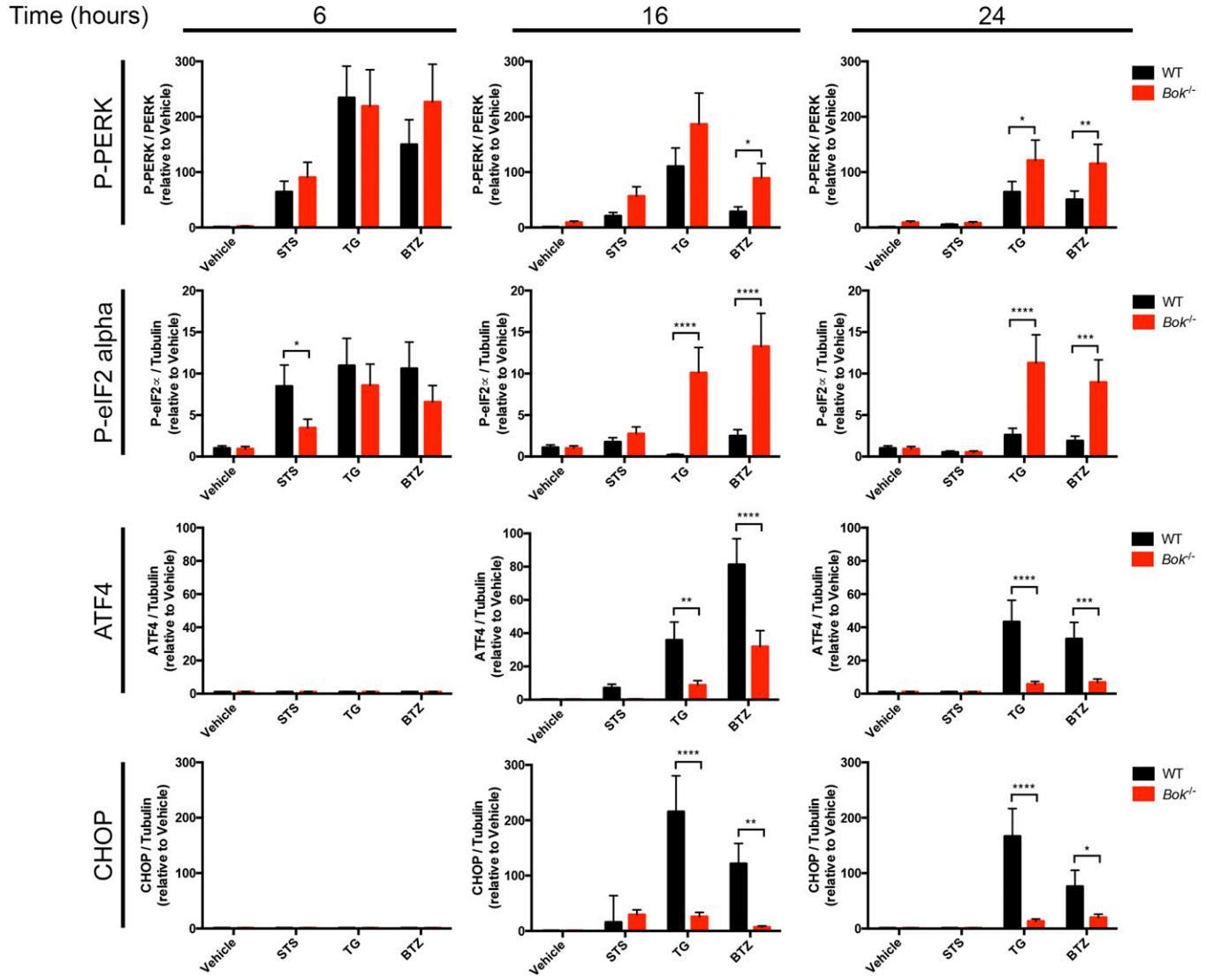


Fig. S7. ATF4 and CHOP expression is diminished in *Bok*$^{-/-}$ cells. Quantification of Western blot analysis for phosphorylated PERK relative to total PERK, phosphorylated eIF2-α relative to tubulin, ATF4 relative to tubulin, and CHOP relative to tubulin in WT and *Bok*$^{-/-}$ MEFs 6, 16, and 24 h after treatment with vehicle, 1 μM STS, 2 μM TG, and 1 μM BTZ. Three independent experiments were performed, and the means ± SEM were plotted. Significance was calculated using an ANOVA test (**P* < 0.05, ***P* < 0.005, ****P* < 0.003, *****P* < 0.0001).

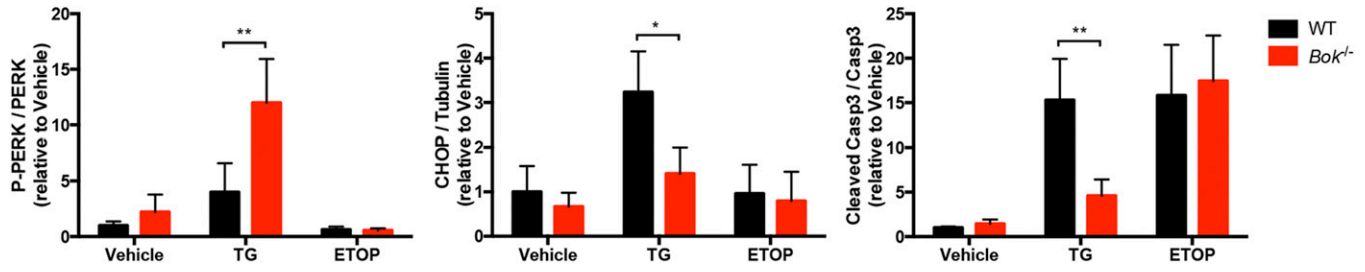


Fig. S8. Quantification of Western blot analysis for phosphorylated PERK relative to total PERK, phosphorylated CHOP relative to tubulin, and cleaved caspase-3 (casp3) relative to total caspase-3 in WT and *Bok*$^{-/-}$ livers ($n = 3$), from mice treated with vehicle, 1 mg/kg TG, or 10 mg/kg ETOP. Means ± SEM were plotted. Significance was calculated using an ANOVA test (**P* < 0.05, ***P* < 0.005).

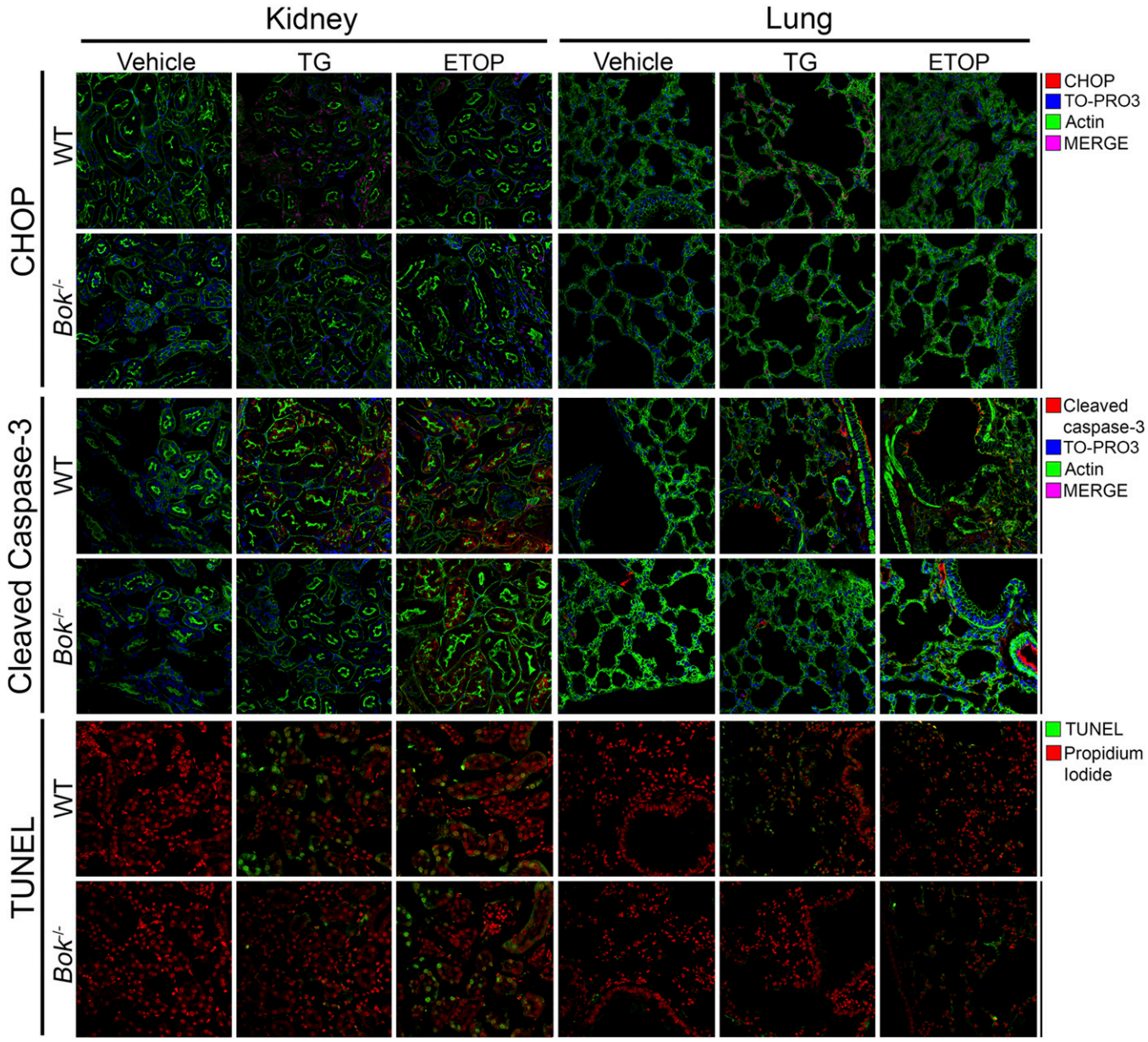


Fig. S9. Immunofluorescence detection of CHOP, activated caspase-3, and TUNEL in WT and *Bok*$^{-/-}$ kidneys and lungs from a representative mouse treated with vehicle, 1 mg/kg TG, or 10 mg/kg ETOP. Merge is between red (CHOP in rows 1 and 2; cleaved caspase-3 in rows 3 and 4) and blue (TO-PRO3).