Supplemental Figure Legends

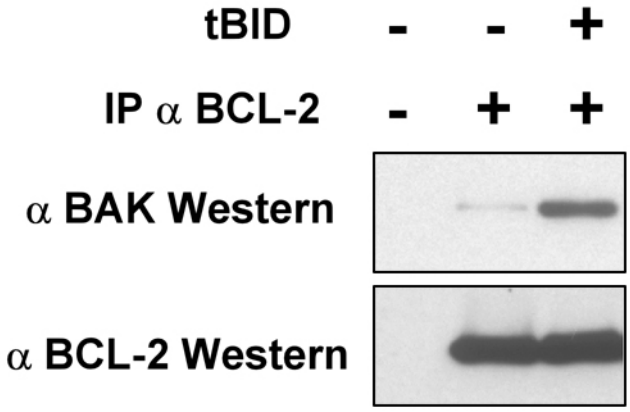
Fig. S1. BCL-2 does not substantially interact with BAK. Mitochondria (100 μg) isolated from FL5.12 cells expressing BCL-2 were untreated or treated with recombinant tBID protein at 30^0C for 30 min, and then lysed in 1% CHAPS buffer (1% CHAPS, 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 20 mM Tris, pH7.5). The lysates were immunoprecipitated with an anti-BCL-2 Ab. The immunoprecipitates were analyzed by immunoblot developed with anti-BAK and anti-BCL-2 Ab.

Fig. S2. *Bax* -/- or *Bak* -/- MEFs, 2 days following retroviral expression of VDAC1, VDAC2, or GFP (MIG vector control) were treated with 10 μg/ml etoposide. Cell death was quantitated by flow cytometric detection of Annexin-V staining at indicated time points. Values shown are mean ± 1 SD of three independent experiments.

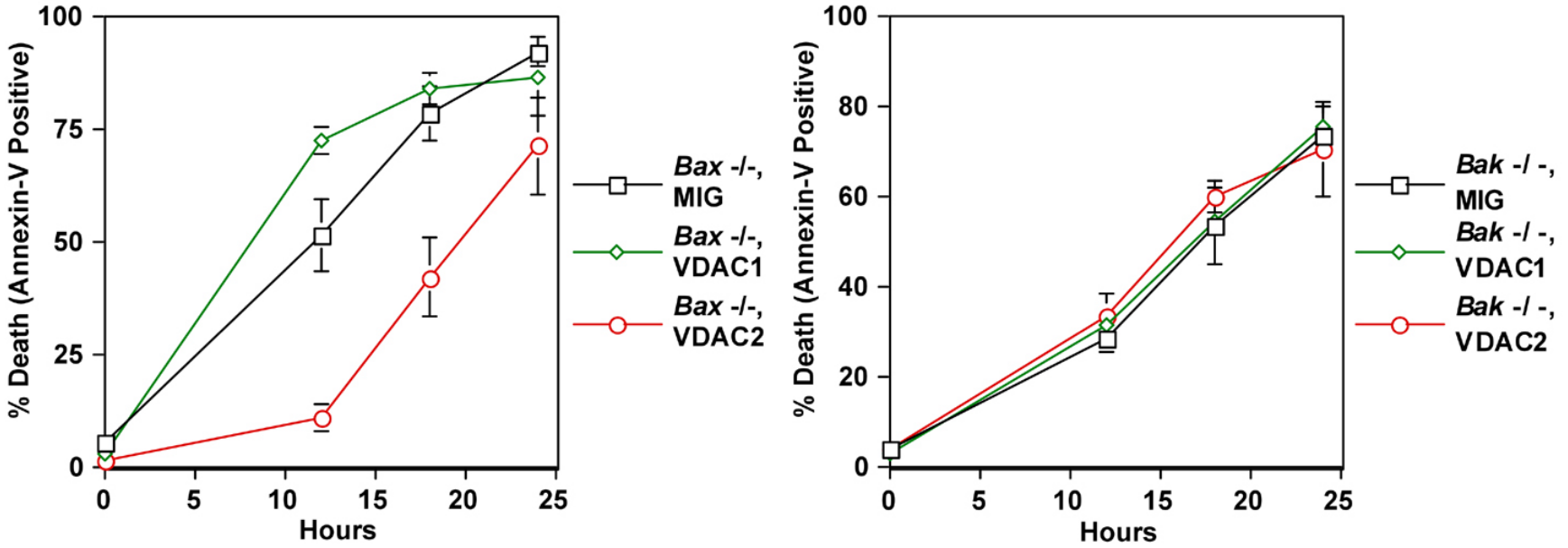
Fig. S3. (**A**) Primary wt or *vdac2* -/- MEFs were treated with 100 μM etoposide. Cell death was quantitated by flow cytometric detection of Annexin-V staining at indicated time points. Values shown are mean ± 1 SD of three independent experiments. (**B**) Primary wt or *vdac2* -/- MEFs were treated with tunicamycin (2 μg/ml). Cell death was quantitated by flow cytometric detection of Annexin-V staining. Values shown are mean ±1 SD of the three independent experiments at 12 hrs (wt 5.5 ± 2.3% vs. *vdac2*-null 18.0 ± 2.4%; p≤0.0015). (**C**) Primary wt and *vdac2* -/- MEFs were quantitatively assessed for spontaneous apoptosis by flow cytometric detection of Annexin-V staining after 24hrs. of culture. (**D**) Down-regulation of BAK in primary *vdac2-/-* MEFs. Primary *vdac2*-null cells are difficult to establish and expand, reflecting their increased

susceptibility to apoptosis. In the absence of inhibitory VDAC2, they have apparently compensated by having lower amounts of BAK. (**E**) Primary wt, *vdac1-/-*, or *vdac3-/-* MEFs were treated with 1.2 μM staurosporine. Cell death as quantitated by flow cytometric detection of Annexin-V was similar in all cells.

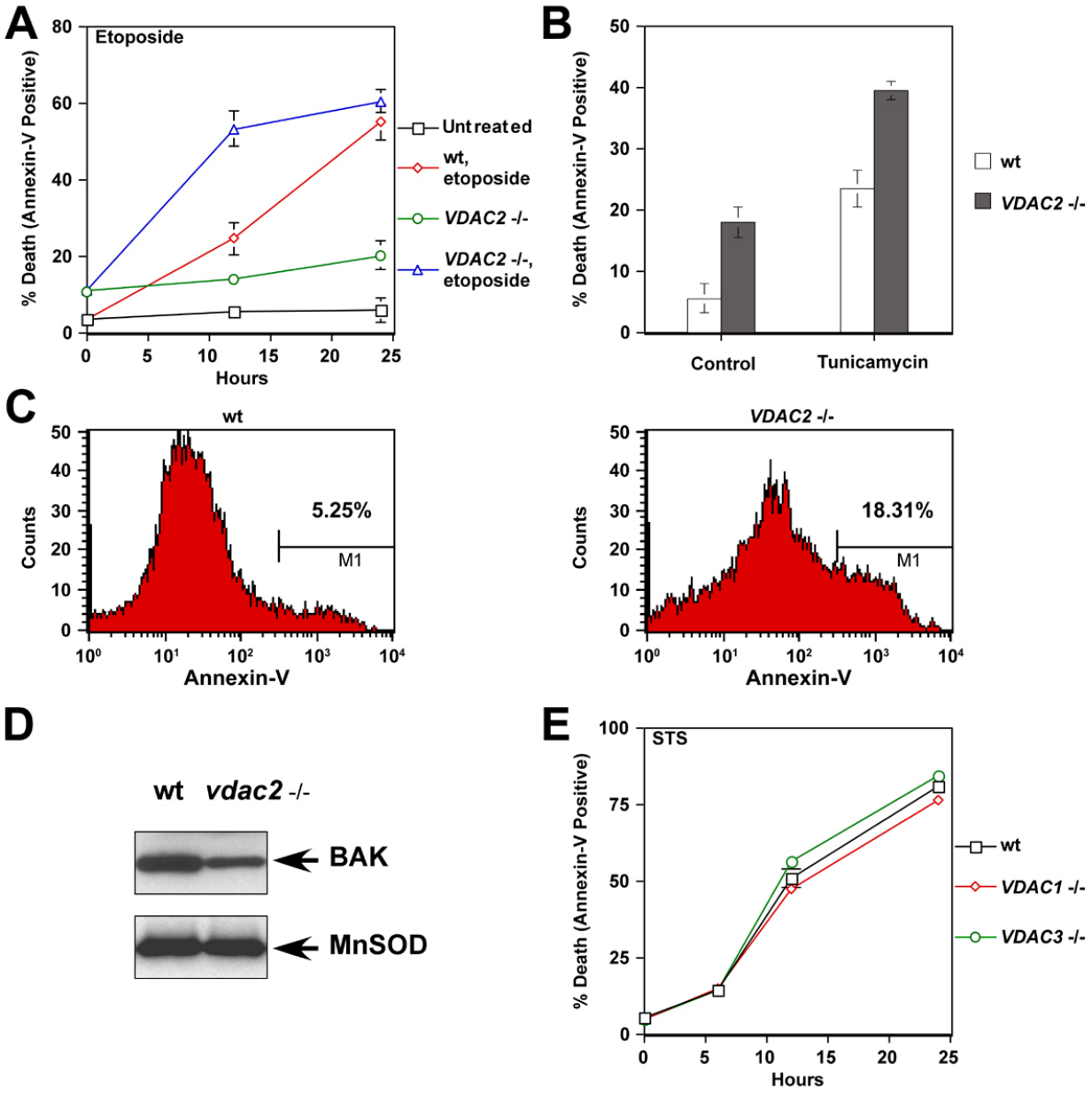
Fig. S4. Time course of staurosporine-induced caspase activation. Lysates from wt or *vdac2* -/- MEF lines treated with 1 μM staurosporine at indicated time points were assessed for DEVD-AFC cleavage activity. Data presented are the fold activation of DEVD-AFC cleavage activity compared to the basal activity at 0 hour.

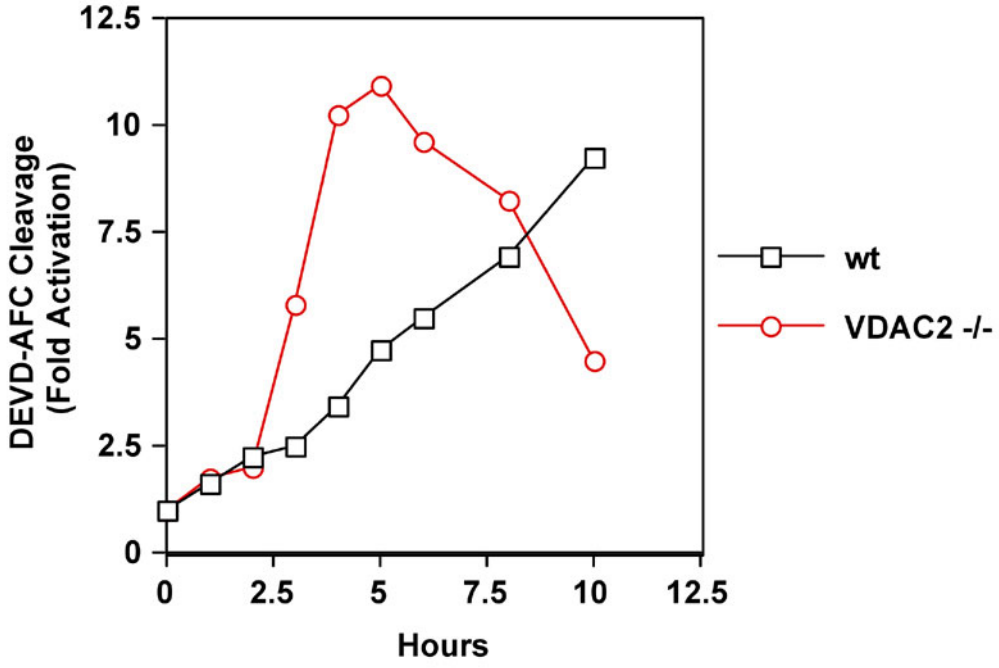


Cheng, et al. Supp. Fig. 1



Cheng, et al. Supp. Fig. 2


A
Etoposide
% Death (Annexin-V Positive)
Hours
Untreated
wt, etoposide
VDAC2 -/-
VDAC2 -/-, etoposide
B
% Death (Annexin-V Positive)
Control
Tunicamycin
wt
VDAC2 -/-
C
wt
VDAC2 -/-
Counts
5.25%
18.31%
M1
Annexin-V
D
wt vdac2 -/-
BAK
MnSOD
E
STS
% Death (Annexin-V Positive)
Hours
wt
VDAC1 -/-
VDAC3 -/-



Cheng, et al. Supp. Fig. 4

Materials and Methods

Plasmid Construction and Retrovirus Production

Murine *vdac1*, *vdac2*, N-terminal HA-tagged *vdac2*, N-terminal-HA tagged *Bax* or *Bak*, as well as wild type and mutant *Bak* (mBH1, W122A, G123E, R124A; mBH3, L75E) were cloned into the retroviral expression vector MSCV-IRES-GFP (pMIG). PcDNA3-tBID (wt and mutants) was described previously (*7*). Generation of amphotropic retroviruses and retroviral infection were described previously (*4*). Retroviral expression of each indicated protein was confirmed by immunoblot.

Purification of BAK-VDAC2 Cross-linked Complex

All purification steps were carried out at 4^0C. The chromatographic step of Hydroxyapatite (Calbiochem) was carried out using conventional stepwise chromatography. The chromatographic steps of Q-sepharose and Mono-Q were performed on an automatic fast protein liquid chromatography (FPLC) station (Pharmacia). FL5.12 cells expressing the N-terminal Flag-tagged BAK and BCL-2, treated with 5 mM DSS at room temperature for 30 min, were subjected to subcellular fractionation as described previously. Lysates (50 mg protein in buffer A-1% CHAPS, 100 mM KCl, 20 mM Tris, pH 7.8, supplemented with protease inhibitors) of heavy membrane fraction were applied onto a HiTrap Q Sepharose column (Pharmacia). The bound materials were step eluted with buffer A containing 0.2 M, 0.6 M, 0.8 M, and 1 M KCl. The Flag-BAK-X complex was followed by an immunoblot for BAK in various fractions. Flag-BAK-X was present predominantly in the 0.6 M KCl eluent which was subjected to dialysis against buffer A containing 10 mM potassium phosphate and then

loaded onto a hydroxyapatite column (bed volume 5ml) equilibrated with the same buffer. The bound materials were step eluted with buffer A containing 0.15 M or 0.5 M potassium phosphate. Most of the Flag-BAK-X complex was present in the 0.15 M potassium phosphate eluent, which was subjected to dialysis against buffer A, then loaded onto Mono Q (HR 5/5, Pharmacia). The Flag-BAK-X complex was present predominantly in the 0.4 M KCl eluent, which was subjected to dialysis against buffer A, then loaded onto an anti-Flag (M2, Sigma) column. The bound proteins were eluted with Flag peptide (1 mg/ml in buffer A). Aliquots of eluent were analyzed by silver stain (BIO-RAD) and by an immunoblot developed with Anti-BAK Ab. The ~60 kD band corresponding to Flag-BAK-X was excised from a silver stained 4-12 % NuPAGE gel, subjected to combined liquid chromatography and tandem mass spectrometry analysis (Harvard Medical School Taplin Biological Mass Spectrometry Facility).

Determination of BAK Conformation by Limited Proteolysis

Isolated mitochondria (50 μg) were incubated with 30 μg/ml trypsin in MRM buffer (250 mM sucrose, 10 mM Hepes, 1 mM ATP, 5 mM succinate, 0.08 mM ADP, 2 mM K2HPO4, 40 mM KCl, 1 mM MgCl2, pH 7.5) on ice for 20 minutes. Soybean trypsin inhibitor (100 μg/ml) was added, and the mitochondria were pelleted and solubilized in SDS-PAGE buffer. Samples were analyzed by immunoblots using an anti-BAK NT Ab (Upstate Biotechnology) recognizing the N-terminal 34 aa residues, or the anti-BAK G23 Ab (Santa Cruz) reacts with a peptide of 20 aa residues spanning the BH3 domain.

Gel Filtration Analyses

The chromatographic step of Superdex 200 (HR 10/30, Amersham-Pharmacia) was performed on an automatic fast protein liquid chromatography (FPLC) station (Amersham-Pharmacia) at 4^0C. The column was equilibrated with buffer containing 2% CHAPS, 300 mM NaCl, 0.2 mM DTT, and 25 mM Hepes, pH 7.5 (*16*) and calibrated with thyroglobulin (669 kD, Amersham-Pharmacia), ferritin (440 kD, Amersham-Pharmacia), catalase (232 kD, Amersham-Pharmacia), aldolase (158 kD, Amersham-Pharmacia), bovine serum albumin (66 kD, Calbiochem), and cytochrome *c* (14 kD, Sigma). 200 μl of mitochondrial lysates (1 mg/ml protein in the same buffer as above) was loaded onto the column, eluted at a flow rate of 0.3 ml/minute. Fractions of 0.6 ml were collected, precipitated by trichloroacetic acid, and analyzed by an immunoblot (fractions 16-31) developed with an anti-BAK Ab.

Antibodies

Antibodies used for immunoblot included anti-BAK NT (Upstate Biotechnology), anti-BAK G23 (Santa Cruz), anti-cytochrome c (Pharmingen), anti-BCL-2 (/100, Pharmingen), anti-HA-Biotin (12CA5, Roche), anti-BAX (651), and anti-VDAC2. Antibodies used for immunoprecipitation included anti-HA (12CA5, Roche) and anti-BCL-2 (6C8).

Generation of *vdac2* -/- Mouse Embryonic Fibroblasts

vdac1 -/- and *vdac2* -/- embryonic stem (ES) cells were described previously (*14*). 129/SvEv derived ES cell clones with homozygous deletion of *vdac2* alleles were

microinjected into blastocysts from C57BL/6 female mice. Mouse embryonic fibroblasts derived from E13.5 day chimeric embryos were treated with 0.3 mg/ml G418 (GIBCO-BRL) at second passage for 5 days to select for *vdac2* -/- cells. Normally, 100% of MEFs died at 3 days in the presence of 0.3 mg/ml G418. Deficiency of VDAC2 in cells following G418 selection was confirmed by an immunoblot developed with an anti-VDAC2 Ab.

Fluorogenic Caspase Activity Assay

Cellular extracts from 10^5 cells were incubated with DEVD-AFC at 37^0C for 1 hour. The release of fluorescent AFC was measured at excitation of 400 nm and emission of 505 nm using a luminescence spectrometer (LS50B, Perkin Elmer).

Flow Cytometric Analysis of Mitochondrial Membrane Potential

MEF cell lines with or without staurosporine treatment were harvested and incubated with PBS containing 150 nM tetramethylrhodamine ethyl ester (TMRE, Molecular Probes) at 37^0C for 30 minutes. Cells were pelleted and resuspended in PBS containing 15 nM TMRE. Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson). The uncoupler carbonyl cyanide chlorophenylhydrazone (CCCP) was added subsequently to validate the specificity of TMRE staining in reflecting mitochondrial membrane potential.

Cytochrome *c* Release Assay

MEF cell lines treated with 1 μM staurosporine were harvested at various time points and lysed for 5 min in isotonic buffer containing 10 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 20 mM Hepes, pH 7.2, 0.025% digitonin, and complete protease inhibitors (Roche). Organelles that contain mitochondria were removed from the soluble cytosolic fraction by pelleting at 15000 g. Supernatant and mitochondrial pellets were solubilized in SDS-PAGE loading buffer and analyzed by an immunoblot, developed with an anti-cytochrome *c* Ab. Protein cross-linking, immunoblot, immunoprecipitation, isolation of mitochondria, cell viability assays, and anti-cytochrome c immunohistochemistry were described previously (*4*).