Supplementary materials and methods

Mitochondrial calcium measurements with Rhod2-AM

To evaluate mitochondrial calcium levels, cells were incubated for 45 min at 37ºC in serum-free DMEM containing Rhod2-AM (5 μM) predissolved in the detergent Pluronic (20%). After repeated washes, cultures were further incubated in complete medium for 5 h to allow for complete desterification of the dye and incorporation to mitochondria. At this point, most of the fluorescence showed a perinuclear, dotted distribution, consistent with mitochondrial staining (not shown). Cells were then detached, resuspended in HBSS (without sodium bicarbonate and phenol red) containing 10%FBS and measured using a Tecan Ultra Evolution fluorescence reader to record emission intensities at 581 nm after excitation at 552 nm. Unstained cells were always measured in parallel to assess background fluorescence.

ATP measurements

Cells were plated in 6-well plates and treated the next day with Bapta (15 μM) or 2-APB (75 μM) for 9 h, the same conditions as used in Fig. 5. As controls for ATP depletion, cells were incubated for 20 min in DMEM minus glucose and sodium pyruvate in the presence of sodium azide (10 mM) and 2-deoxy-glucose (6 mM). ATP levels were assessed using the ATP Bioluminescence Assay Kit HSII (Roche), following manufacturer's instructions.

Quantitation of cells showing mitochondrial depolarization

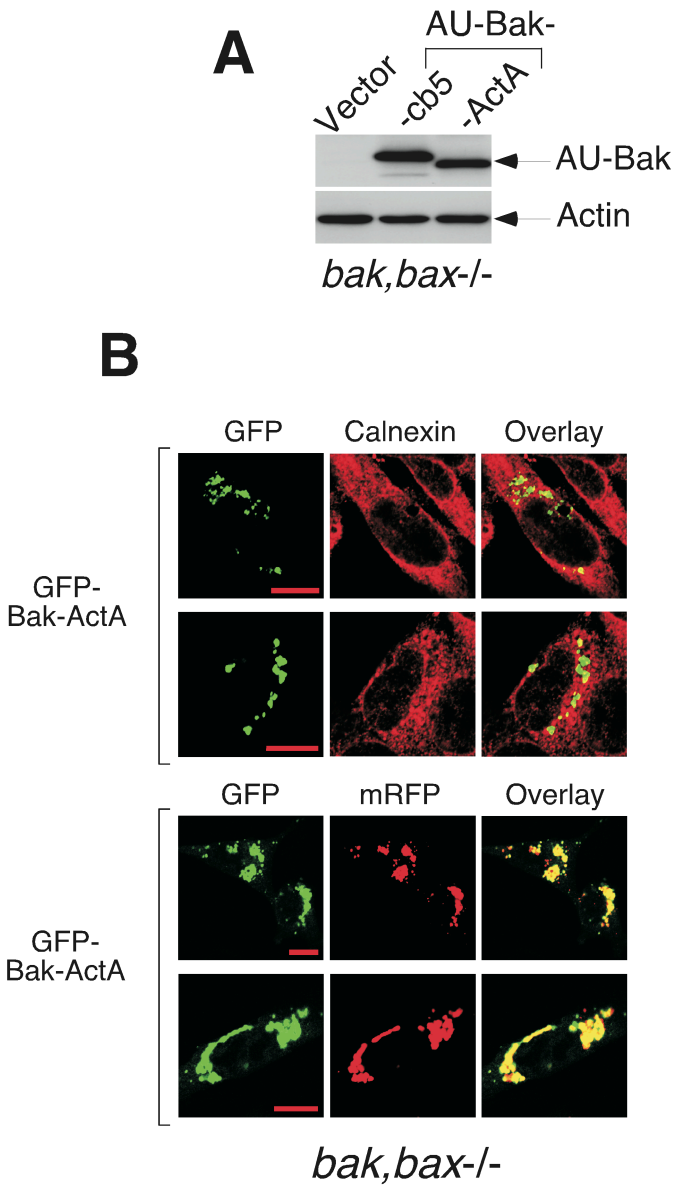
Cells previously plated onto poly-L-lysine-treated coverslips were transduced in the presence of 100 μM zVAD.fmk and, at the end of the experiment, stained with

MitoTracker Red (Molecular Probes) by incubating them for 30 min at 37ºC in medium containing 75 nM of the dye. Cells were fixed in 4% paraformaldehyde. The percentage of cells exhibiting mitochondrial depolarization was quantitated by blindly evaluating the percentage of cells per field showing a diffuse MitoTracker Red staining pattern accompanied by poor mitochondrial signal. Counts and statistics were done as for evaluation of cytochrome c release (see main Materials and methods).

Assessment of mitochondrial permeability transition using the calcein approach

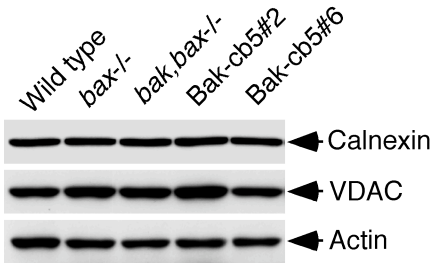
Calcein assays to measure MPT were conducted essentially as previously described (Poncet et al., 2003). Briefly, cells were washed with HBSS (without sodium bicarbonate and phenol red) supplemented with 5 mM Hepes 7,3 and subsequently incubated in the presence of 1mM $CoCl_2$ and 1 μM Calcein-AM (Molecular Probes) for 30 min at 37ºC. After staining, cells were washed, incubated in complete medium for retroviral transduction or treatment with stimuli (always in the presence of 100 μM zVAD-fmk) and, at the end of the experiment, detached resuspended in HBSS and analyzed by flow cytometry. At the time of analysis, the calcein signal showed an exclusively punctuated distribution consistent with mitochondrial staining (not shown).

Klee et al. Supplementary Figure 1



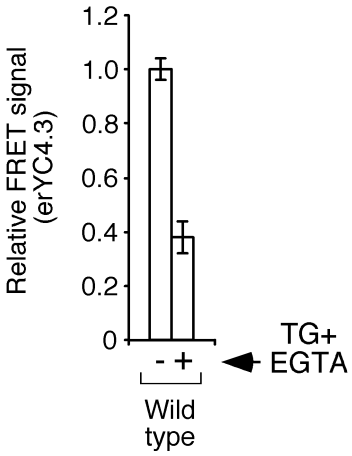
Supplementary Fig. 1. Expression of cb5 and ActA-tagged versions of Bak in *bak,bax*-/- cells. (A) Expression levels of Bak-cb5 and Bak-ActA in polyclonal cultures of retrovirally- transduced cells. Bak and Bax-double deficient cells were transduced with viruses encoding AU-tagged versions of Bak-cb5 or Bak-ActA and subsequently selected in the presence of 1 μg/ml of puromycin for about 10 days. Cells were then lysed for anti-AU Western-blot. **(B)** Bak-ActA localizes to mitochondria. Cells were transfected with GFP-Bak-ActA in the absence (top panels) or presence (bottom panels) of mRFP and, 24 h later, stained for calnexin (top) or mounted without further manipulation (bottom). The confocal pictures show no colocalization of GFP-Bak-ActA with calnexin, but substantial colocalization with mRFP. Two representative examples of each case are provided.

Klee et al. Supplementary Figure 2



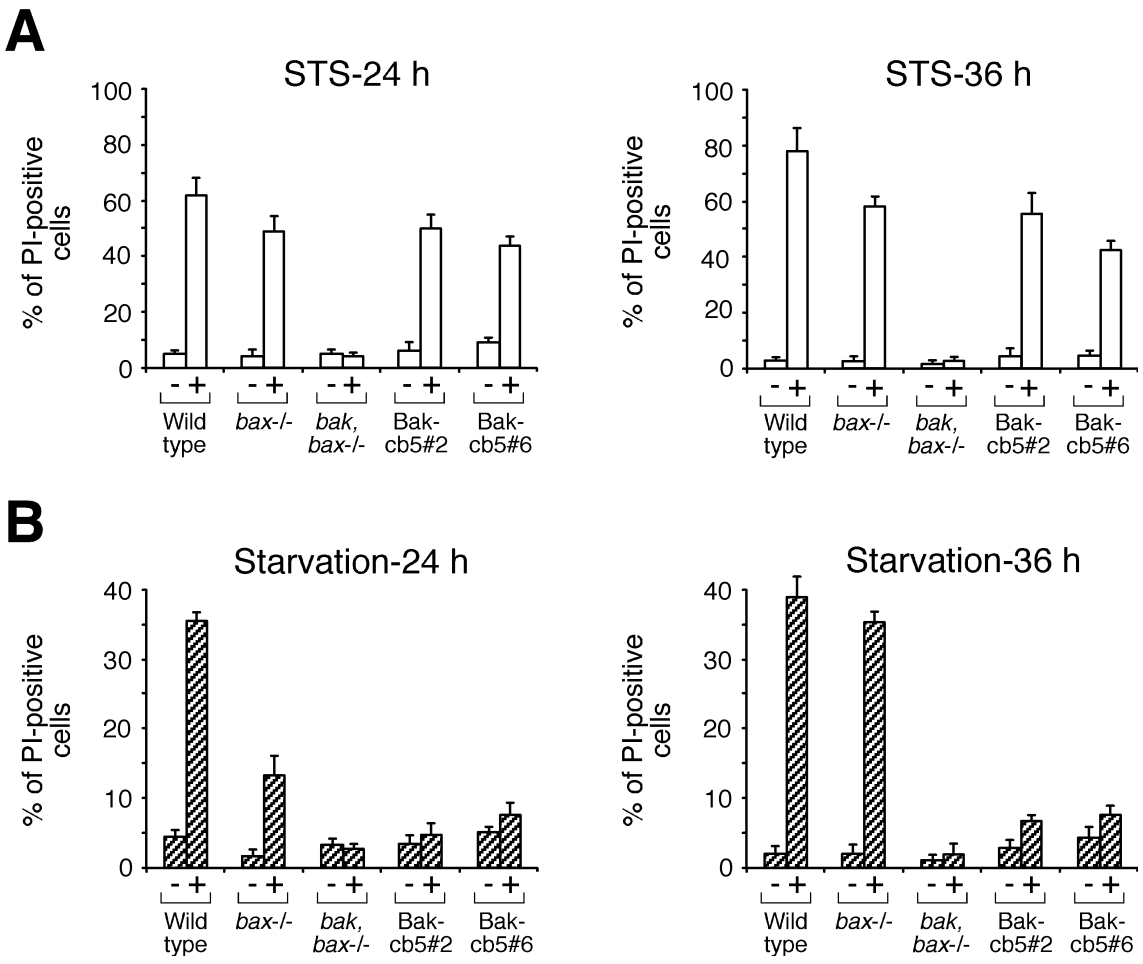
Supplementary Fig. 2. Expression levels of calnexin and VDAC in the different MEF strains. The indicated cells were lysed and subjected to anti-calnexin and anti-VDAC Western-blots. The figure shows no fundamental differences in the expression levels of both proteins. Clone #2 seems to have slightly higher levels of VDAC than clone #6, but the similar behavior of both strains all across the present study suggests that this difference is probably irrelevant.

Klee et al. Supplementary Figure 3



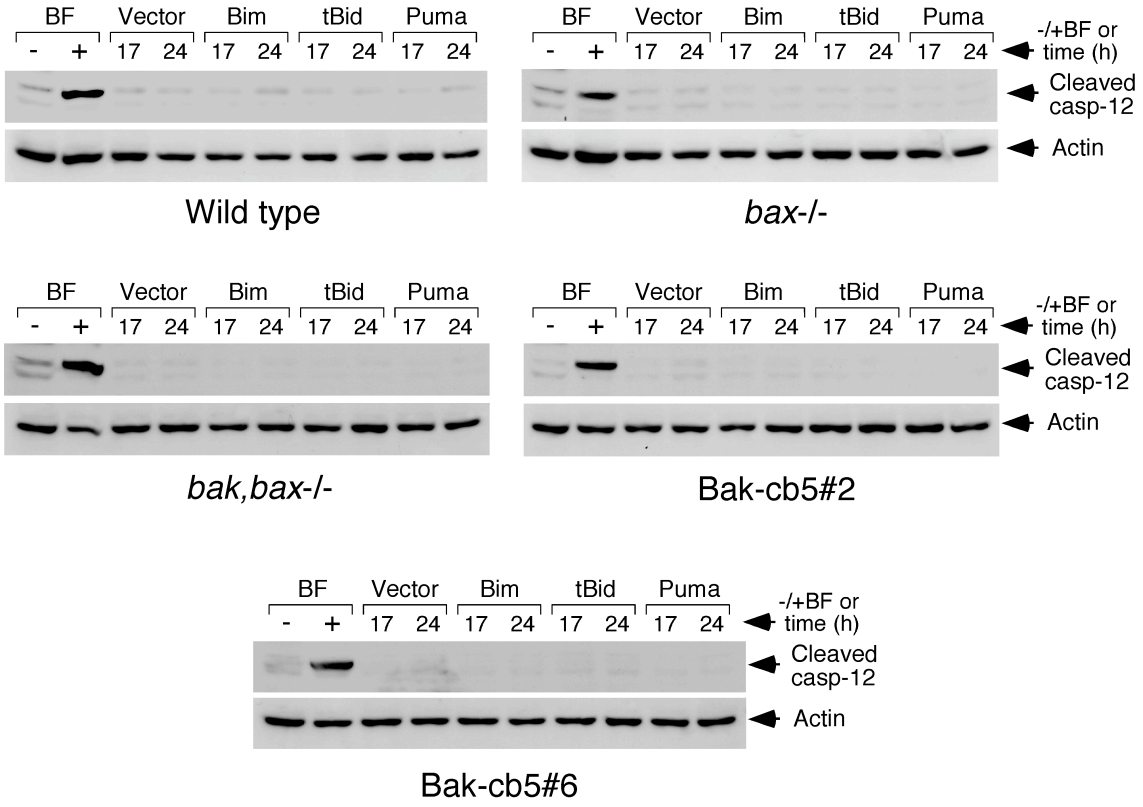
Supplementary Fig. 3. The reticular calcium reporter erYC4.3 responds to ER calcium depletion induced by TG in the presence of EGTA. Wild-type MEFs were transduced twice with retroviruses encoding erYC4.3 and, 36 h later, processed for in vivo capturing the FRET intensities before and after simultaneous treatment with TG (1 μM) and EGTA (2 mM) for 25 min. Data are represented as a fraction of the values obtained before treatment. Shown are average values and standard deviations (error bars) of the relative FRET levels shown by four to six cells present in the same microscopic fields of three independent assays.

Klee et al. Supplementary Figure 4



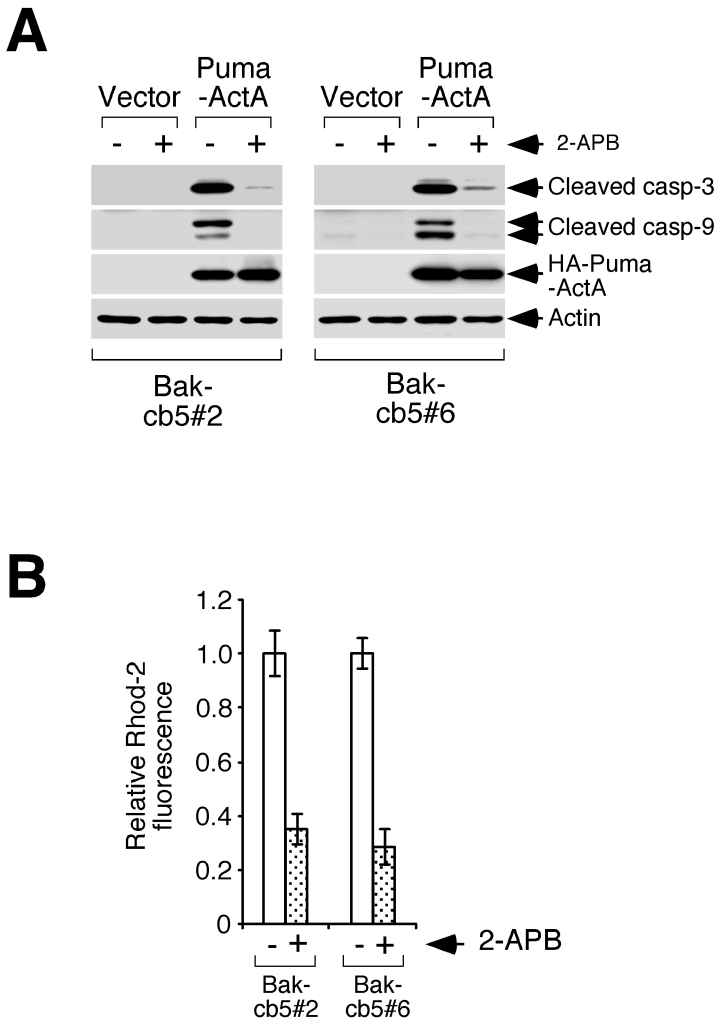
Supplementary Fig. 4. Clones expressing Bak-cb5 are susceptible to cell death induced by STS but not serum starvation. (A) Susceptibility of Bak-cb5-expressing clones to STS-induced death. Cells were subjected to STS (1μM) for the indicated times and the extent of cell death was evaluated by PI-staining. **(B)** Resistance of Bak-cb5-expressing clones to serum starvation-induced death. Cells were subjected to serum starvation for the indicated times and the extent of cell death measured as in (A).

Klee et al. Supplementary Figure 5



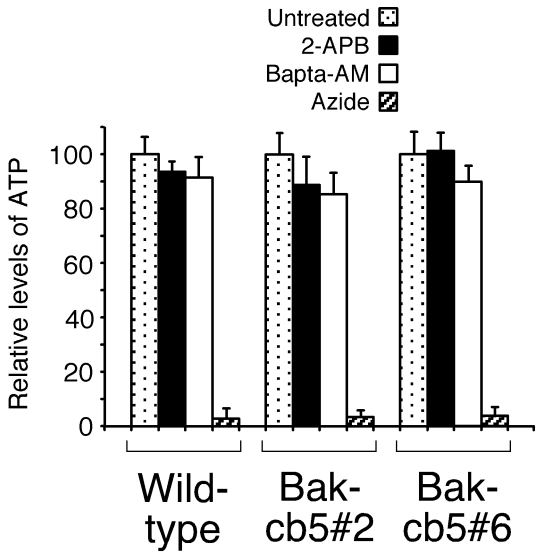
Supplementary Fig. 5. Absence of caspase-12 cleavage induced by Bim and Puma in the Bak-cb5-expressing clones. Cells were transduced with the indicated constructs and lysed for anti-caspase-12 Western-blot 17 or 24 h later, as shown. Brefeldin A (BF) treatment (10 μg/ml for 24 h) was used as a positive control for caspase-12 processing.

Klee et al. Supplementary Figure 6



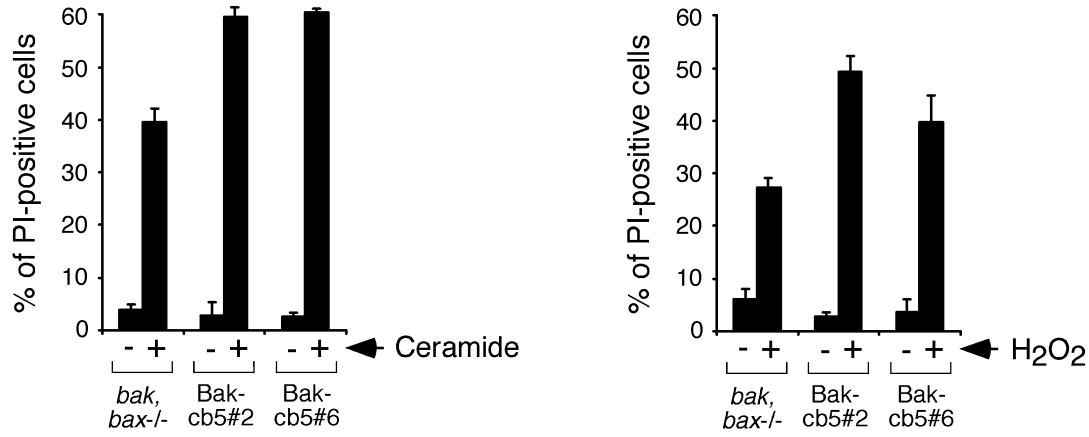
Supplementary Fig. 6. The apoptotic effect of Puma-ActA in Bak-cb5-expressing clones #2 and #6 is calcium-dependent. (A) 2-APB inhibits processing of caspases 3 and 9 induced by Puma-ActA. Cells were transduced, treated with 2-APB (75 μM) 8 h later, and lysed for Western-blotting 17 h after transduction. **(B)** 2-APB reduces the basal level of mitochondrial calcium. Cells were treated with 2-APB (75 μM) for 4 h, stained with the indicator Rhod2-AM, and further incubated for an additional 5 h in the presence of 2-APB (the total time of 2-APB treatment was 9 h). Shown are averages and standard deviations (error bars) of the values obtained in one representative experiment performed in triplicate. The experiment was repeated three times with similar results. Data are represented as a fraction of the values obtained in the absence of 2-APB.

Klee et al. Supplementary Figure 7



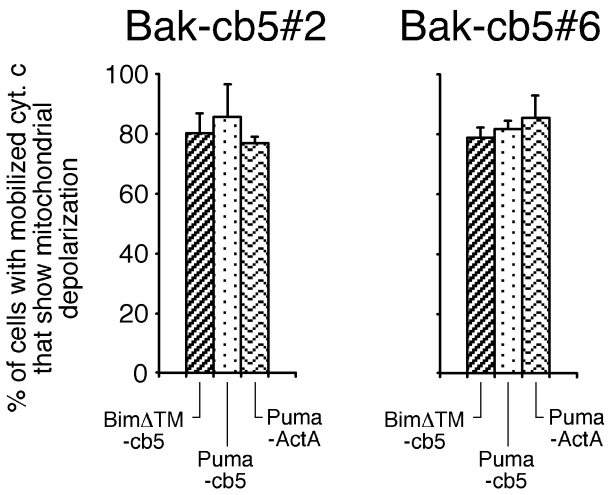
Supplementary Fig. 7. Levels of ATP in cells treated with 2-APB or Bapta-AM. The indicated cells were treated for 9 h with 2-APB (75 μM) or Bapta-AM (15 μM). Cells were also incubated for 20 min in the presence of sodium azide (10 mM) and 2-deoxy-D-glucose (6 mM) to provide a positive control for ATP depletion. Data are represented as fractions of the values obtained in untreated cells. Shown are average values and standard deviations (error bars) of one representative experiment performed in triplicate.

Klee et al. Supplementary Figure 8



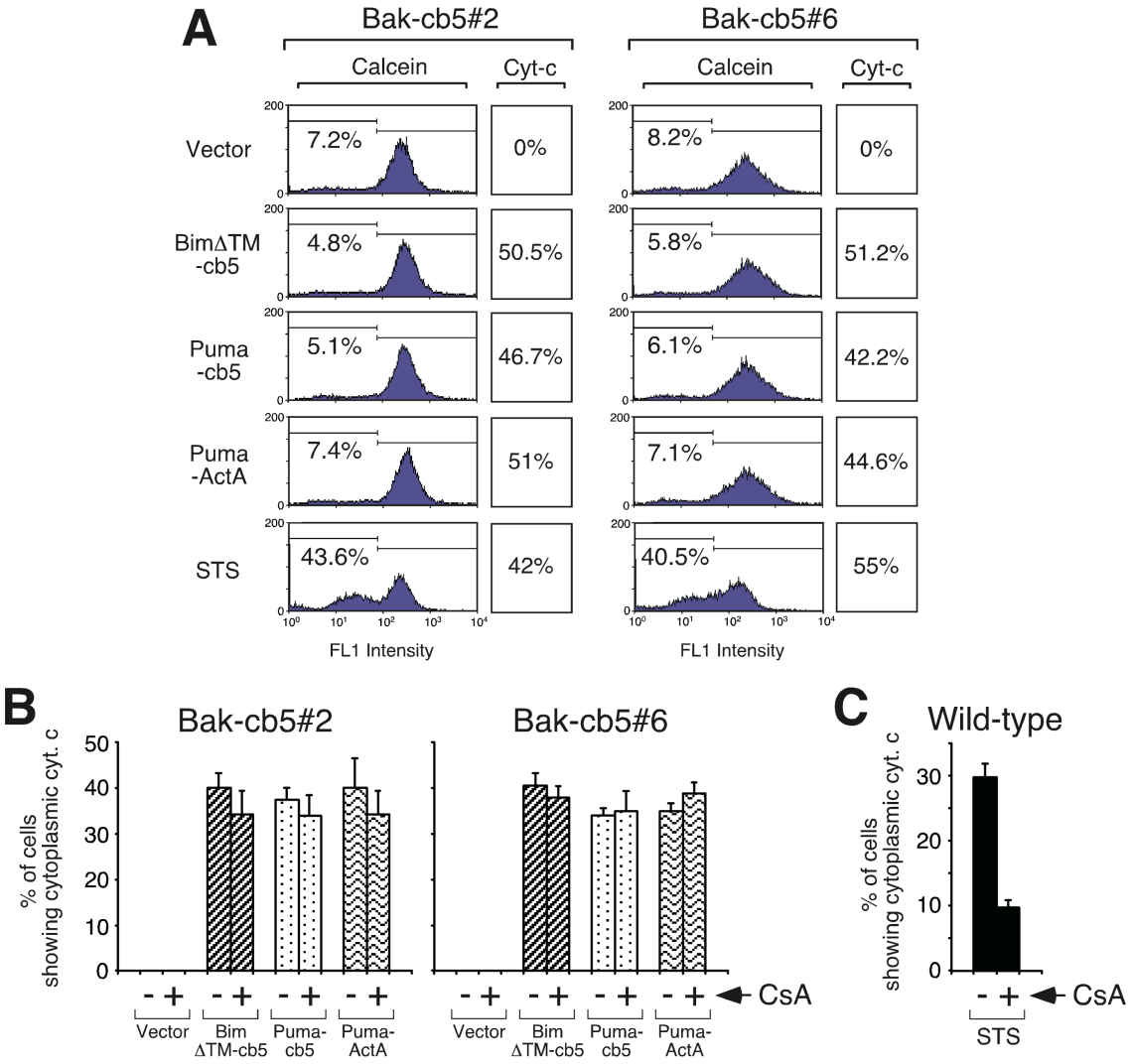
Supplementary Fig. 8. Increased cell death induced by ceramide and H_2O_2 in Bak-cb5-expressing cells compared to *bak,bax*-/- MEFs. The indicated cells were treated with C_2-ceramide (100 μM, 11 h treatment) or H_2O_2 (1 mM, 4 h treatment) and cell death was evaluated by PI staining.

Klee et al. Supplementary Figure 9



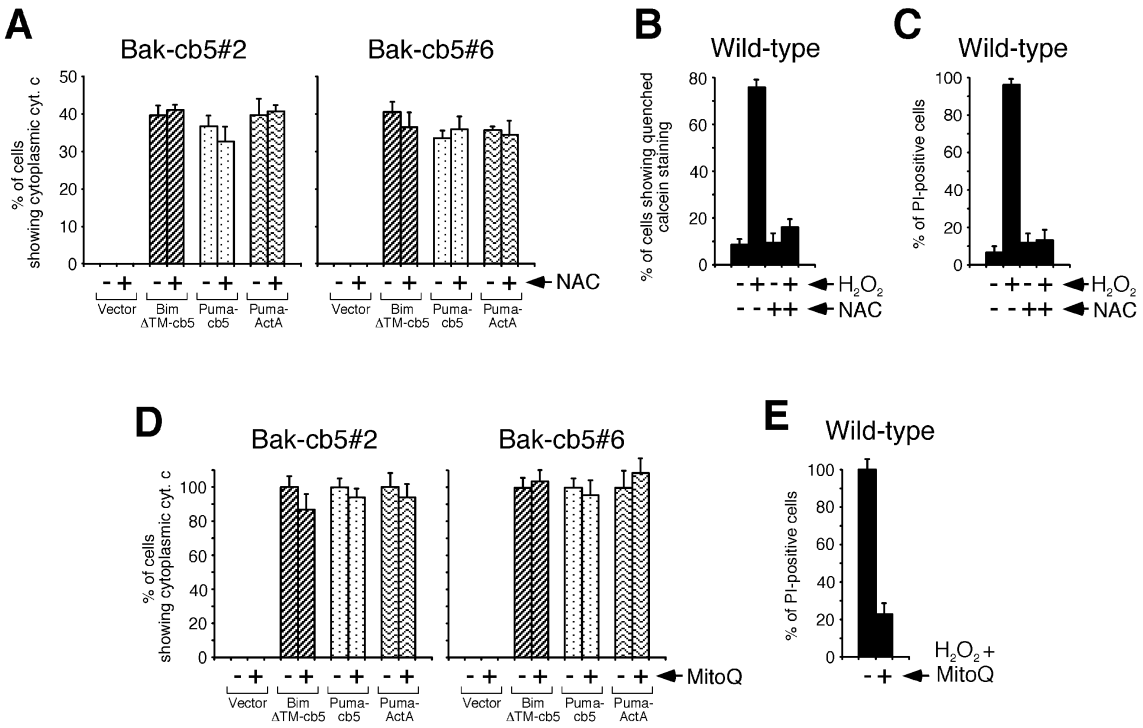
Supplementary Fig. 9. Mitochondrial depolarization in Bak-cb5-expressing cells that show mobilized cytochrome c. The indicated cells were transduced with the shown BH3-only derivatives in the presence of 100 μM zVAD.fmk and, 17 h later, stained with MitoTracker Red right before processing them for cytochrome c immunofluorescence. Shown are the percentages of cells that, showing cytochrome c mobilization, also exhibit mitochondrial depolarization assessed as the absence of MitoTracker staining in mitochondria.

Klee et al. Supplementary Figure 10



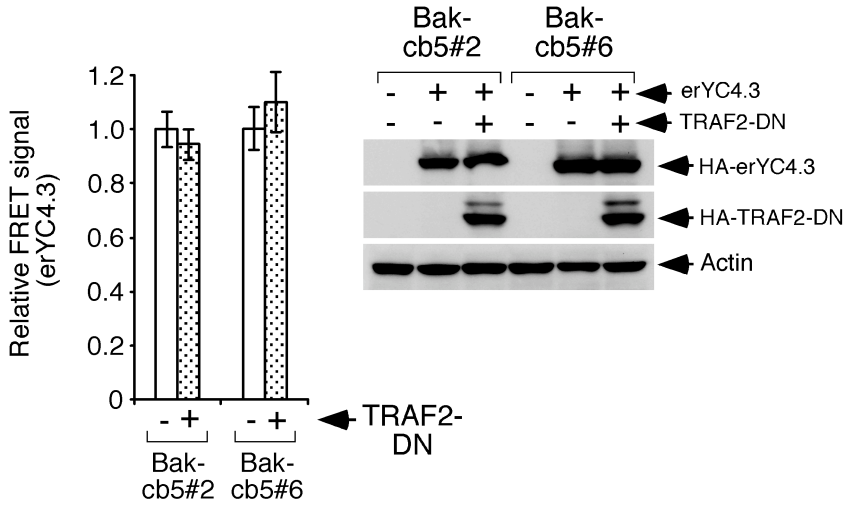
Supplementary Fig. 10. MPT is not involved in cytochrome c mobilization induced by reticular Bim and Puma (or Puma-ActA) in Bak-cb5-expressing cells. (A) Reticular Bim and Puma (or Puma-ActA) do not provoke cobalt-induced quenching of mitochondrial calcein in clones #2 and #6. Cells were pre-loaded with calcein/cobalt and transduced with retroviruses expressing the indicated molecules in the presence of zVAD.fmk (100 μM). Calcein fluorescence was measured 17 h later. Shown are the fluorescence profiles indicating the percentage of cells exhibiting quenched signal (left column). The same retroviral supernatants were used in identical conditions to assess the percentage of cells showing cytochrome c mobilization (right column). The figure shows total absence of MPT occurring in the same conditions that provoke substantial cytochrome c release. **(B)** Cyclosporin A does not inhibit cytochrome c liberation induced by reticular Bim and Puma (or Puma-ActA) in Bak-cb5-expressing cells. Cells were transduced in the presence of zVAD-fmk (100 μM) and CsA (2 μM) was added 1 h later. The cultures were processed for cytochrome c staining 17 h after transduction. CsA did not inhibit retroviral transduction in these conditions, as assessed in control experiments using a virus encoding GFP (not shown). **(C)** Cyclosporin A inhibits cytochrome c mobilization induced by STS. Wild-type MEFs were pre-incubated for 1 h with 2 μM CsA and subsequently treated with STS (1 μM) for 6 h before anti-cytochrome c immunofluorescence. This experiment shows that CsA can actually inhibit cytochrome c mobilization in some conditions.

Klee et al. Supplementary Figure 11



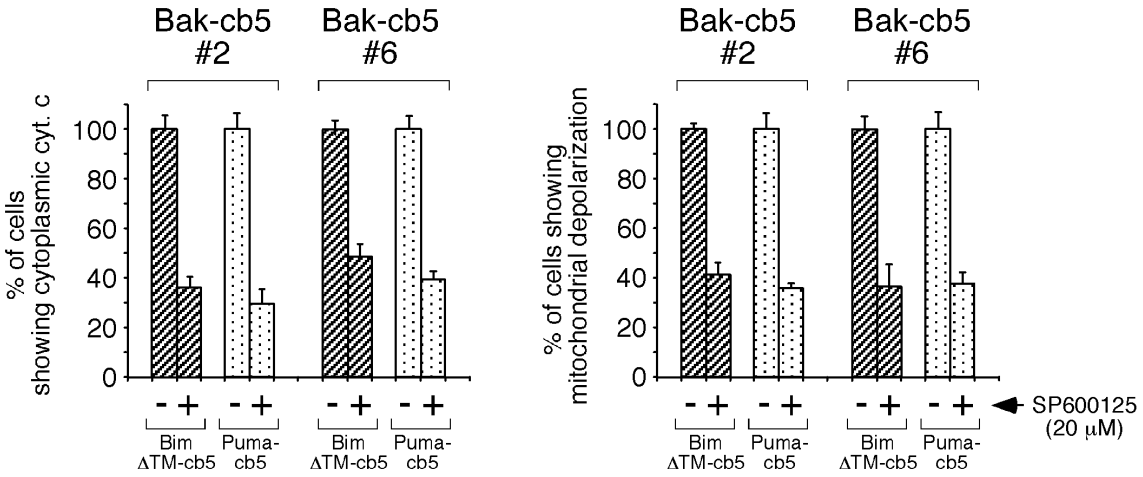
Supplementary Fig. 11. ROS inhibition by NAC or MitoQ does not prevent cytochrome c mobilization induced by reticular Bim and Puma (or Puma-ActA) in clones #2 and #6. (A) NAC does not inhibit cytochrome c mobilization. Cells were transduced in the presence of zVAD-fmk (100 μM) and NAC (7.5 mM) was added 1 h later. Cytochrome c immunostaining was done 17 h post-transduction. NAC did not affect retroviral transduction as evaluated in control experiments with a virus encoding GFP (not shown). **(B)** NAC prevents cobalt-induced calcein quenching provoked by exogenous oxidative stress. MEFs were loaded with calcein/cobalt, pre-incubated for 1 h with 7.5 mM NAC and further cultured for 4 h with H_2O_2 (1 mM) before measuring their calcein fluorescence intensities. Shown are percentages and standard deviations (error bars) of cells showing quenched fluorescence obtained from three independent experiments. **(C)** NAC blocks death induced by H_2O_2. MEFs were pre-incubated for 1 h with NAC (7.5 mM) and then treated for 9 h with H_2O_2 (1 mM) before assessing the percentage of death by PI staining. Experiments shown in (B) and (C) demonstrate that NAC can actually quench ROS in our conditions. **(D)** MitoQ does not inhibit cytochrome c release. MitoQ (0.2 μM) was added 1 h post-transduction and cells were stained for cytochrome c 17 h after infection. MitoQ used in these conditions did not affect retroviral transduction, whereas higher concentrations (1 μM) had a massive inhibitory effect (not shown). Data are shown as percentages of the values obtained in the absence of inhibitor. **(E)** MitoQ inhibits cell death induced by H_2O_2. Cells were pre-incubated for 1 h wit MitoQ (0.2 μM) and then treated with H_2O_2 (0.5 mM) for 24 h before assessing cell death by PI staining. Data are represented as in (D).

Klee et al. Supplementary Figure 12



Supplementary Fig. 12. Expression of TRAF2-DN does not alter ER calcium levels in Bak-cb5-expressing clones #2 and #6. Cells were co-transduced with retroviruses encoding an HA-tagged version of the ER calcium reporter erYC4.3 and, either an irrelevant insert (-) or TRAF2-DN (+), and 36 h later lysed for Western-blot (inset) or processed for in vivo capturing of confocal FRET intensities. Shown are averages and standard deviations (error bars) of the FRET measurements gathered from at least fifty cells in one representative experiment. Data are expressed as fractions of the values obtained in the absence of TRAF2-DN.

Klee et al. Supplementary Figure 13



Supplementary Fig. 13. The JNK inhibitor SP600125 reduces cytochrome c release and mitochondrial depolarization induced by reticular BimΔTM and Puma. Cells were transduced, treated 8 h later with the JNK inhibitor (20 μM) and stained for cytochrome c (left) or MitoTracker Red (right) 17 h post-transduction. Shown are the percentages of cells exhibiting cytochrome c mobilization (left), or showing mitochondrial depolarization assessed as the absence of MitoTracker staining in mitochondria (right). Data are represented as a fraction of the values obtained in the absence of SP600125.