Supplementary Figure legends

**Figure S1 - Bak is present in the cytosol of human tissue samples. Related to Figure 1.** Western blot analysis of Bak in the cytosol (C) and the heavy membrane (HM) fraction of human kidney and lung tissue. Equal sample portions of fractionated protein were loaded according to photodensitometric analysis. LDH and Tom20 serve as fractionation and loading controls.

**Figure S2 - Ectopic Bax expression. Related to Figure 2.**

A Expression levels of GFP-Bak in the presence or the absence of Bcl-xL overexpression in Bax/Bak DKO cells analyzed by Western blot using mouse α-Bcl-xL, rabbit α-Bak and rabbit α-Tom20 antibodies. Cells transfected with pcDNA 3.1- vector serve as control. \( n = 3 \).

B Western blot analysis of endogenous Bax levels in HCT116 wild-type and Bak -/- cells compared to the levels of stable (center) and transient (right) GFP-Bax expression in HCT116 Bax/Bak DKO cells. Unspecific protein detection is marked (*). Actin served as loading control. \( n = 4 \).

C Representative images showing GFP fluorescence in HCT116 Bax/Bak DKO cells transfected with GFP-Bak (left) and GFP-Bax (second from right) constructs. Phase contrast images are to the right of the respective fluorescence image.

D Transfection rates of HCT116 Bax/Bak DKO cells transiently expressing GFP-Bak or GFP-Bax constructs. \( n = 4 \).
Figure S3 - Bcl-xL overexpression increases cytosolic Bak levels. Related to Figure 3.

A Western blot analysis of Bak localization in HCT116 Bax/Bak DKO cells expressing untagged Bak with or without overexpressed Bcl-xL or Bcl-xL G138A. Cytosol (C) and heavy membrane fraction (HM) of HCT116 Bax/Bak DKO cells are displayed. GAPDH and Tom20 serve as fractionation controls. n = 3.

B FLIP of mitochondrial GFP-Bak in the presence (●, straight line) of overexpressed Bcl-xL G138A. Fluorescence of a neighboring cell is shown as control (○, dotted line). Data represent averages ± SEM from 24 ROI measurements.

C Retrotranslocation rates measured for GFP-Bak in the presence Bcl-xL and Mcl-1 in the presence or the absence of the BH3 mimic ABT-737, inhibiting interactions between BH3 domains and the hydrophobic grooves of Bcl-2 and Bcl-xL. Data represent averages ± SD.

D Levels of Flag-DD-Bcl-xL in HeLa cells in the presence or the absence of Shield 1 with or without MOMP initiated by STS in presence of the pancaspase inhibitor qVD were analyzed by Western blot using an anti-FLAG antibody. Depicted is a long and a short signal detection. Actin served as loading control.

E Cell death measured by propidium iodide (PI) staining of HeLa cells expressing DD-Bcl-xL in the presence or the absence of Shield 1. Cell death was initiated by ActD treatment. The pancaspase inhibitor qVD was used to block apoptotic cell death. n = 3.

F Analysis of endogenous Bak localization in cytosol (C) and heavy membrane fraction (HM) of HeLa cells exposed to 20 µg/ml CHX for 0, 1, 3 or 5h. Akt1 and Tom20 serve as fractionation controls. n = 3.
Figure S4 - Expression of Bax and Bak variants. Related to Figure 4.

A, B Expression levels of Bax (A), BaxTBak (A), Bak (B) or BakTBax (B) with or without Bcl-x<sub>L</sub> overexpression in HCT116 Bax/Bak DKO cells analyzed by SDS-PAGE and Western blot. Actin served as loading control.

C FLIP of BaxTBak in the presence (○, straight line) or the absence (●, broken line) of overexpressed Bcl-x<sub>L</sub>. Fluorescence of a neighboring cell is shown as control (▼, dotted line). Data represent averages ± SEM from 20 (-Bcl-x<sub>L</sub>) and 16 (+Bcl-x<sub>L</sub>) ROI measurements.

D FLIP of mitochondrial BakTBax without (●, broken line) or with (○, straight line) overexpressed Bcl-x<sub>L</sub>. Fluorescence bleaching control is shown (▼, dotted line). Data represent averages ± SEM from 20 (-Bcl-x<sub>L</sub>) and 16 (+Bcl-x<sub>L</sub>) ROI measurements.

Figure S5 - BaxTBak activation. Related to Figure 5.

A Western blot analysis of PARP cleavage in Bax/Bak DKO cells overexpressing Bax, BaxTBak, Bak or BakTBax with or without Bcl-x<sub>L</sub> overexpression in the absence of STS. Comparable sample loading is controlled by actin. n = 3.

B PARP cleavage after STS treatment in Bax/Bak DKO cells overexpressing Bax, BaxTBak, Bak or BakTBax with or without Bcl-x<sub>L</sub> overexpression analyzed by Western blot. Actin is used as loading control. n = 3.

C Co-IP of wild-type Bcl-x<sub>L</sub> and wild-type Bax, BaxD68R, Bax 1-2/L-6 or BaxTBak precipitating myc-Bcl-x<sub>L</sub> from cell extracts of HCT116 Bax/Bak DKO cells in the presence of 0.2% Triton X100. The Bax D68R substitution prevents interactions between Bax BH3 and the hydrophobic groove of Bcl-x<sub>L</sub> (Fletcher et al, 2008). Bax 1-2/L-6 contains two intramolecular disulfide bonds even in
the reducing environment of the cytosol that prevent conformational changes required for Bax/Bcl-xL heterodimerization (Edlich et al, 2011). Thus, both Bax variants serve as controls. The input is shown in the upper two panels. Bcl-xL was detected by anti-myc antibodies and Bax by anti-Bax antibodies. n = 3.

**Figure S6 - MA hydrophobicity and thus Bax shuttling rate determine Bax activation. Related to Figure 6.**

**A** The influence of MA hydrophobicity on the activation of BaxTBak was tested by introducing valine to serine substitutions into the Bak MA sequence. The variant was designed to match the properties of Bax S184V. The hydrophobicity (H), hydrophobic moment (µH) and non-polar residue content of the different MA sequences according to HeliQuest are displayed. For clarity, the substitutions are denoted according to the residue position in the wild-type protein.

**B** FLIP of BakTBax S184V in the presence (●, straight line) or the absence (○, broken line) of overexpressed Bcl-xL. Fluorescence of a neighboring cell is shown as control (▼, dotted line). Data represent averages ± SEM from 12 (-Bcl-xL) and 12 (+Bcl-xL) ROI measurements.

**Figure S7 - Retrotranslocation regulates Bax and Bak. Related to Figure 7.**

**A** Western blot analysis of Bcl-xL localization in Bax/Bak DKO cells in the presence or the absence of ectopic Bak or Bax expression. Fractionation of cytosol (C) and heavy membrane fraction (HM) of HCT116 Bax/Bak DKO is controlled by GAPDH and Tom20. n = 5.

**B** Co-IP of Bax and wild-type GFP-Bcl-xL, GFP-Bcl-xLTBax and myc-Bcl-xL G138A, precipitating Bax from HCT116 cell extracts in the presence of 0.2% Triton X100 or CHAPS. The Bcl-xL G138A
substitution blocks interactions between Bax BH3 and the hydrophobic groove of Bcl-xL. Wild-type GFP-Bcl-xL and GFP-Bcl-xLTBax interact with Bax in the presence of Triton X100. The input is shown on the left. Actin serves as loading control. Unspecific bands detected by the anti-Bax and the anti-Bcl-xL antibodies are indicated (*). n = 3.

C Carbonate extraction of the wild-type Bax pool in the HM fraction of HCT116 Bax/Bak DKO cells separates OMM-associated Bax (in the supernatant, S) and OMM-integral Bax (in the extraction pellet, P) in the presence or the absence of ectopic Bcl-xL expression. In presence of ectopically expressed Bcl-xL, Bax is almost completely shifted into the cytosol. Equal amounts of P and S are loaded. Smac and VDAC serve as fractionation controls. n = 3.

D Carbonate extraction of OMM-associated (S) and the OMM-integral Bax S184V (P) from the HM fraction of HCT116 Bax/Bak DKO cells in the presence or the absence of ectopic Bcl-xL expression. Equal amounts of P and S are loaded. Smac and VDAC serve as fractionation controls. n = 3.

E IP of endogenous Bax from cell extracts of HCT116 precipitated by active Bax-specific anti-Bax (6A7, Sigma Aldrich) in the presence of 0.2% Triton X100 (TX) or CHAPS (CH). The input is indicated on the left. Actin serves as loading control. n = 3.