SUPPLEMENTARY INFORMATION

Iyer et al,

Bak apoptotic pores involve a flexible C-terminal region and juxtaposition of the C-terminal transmembrane domains

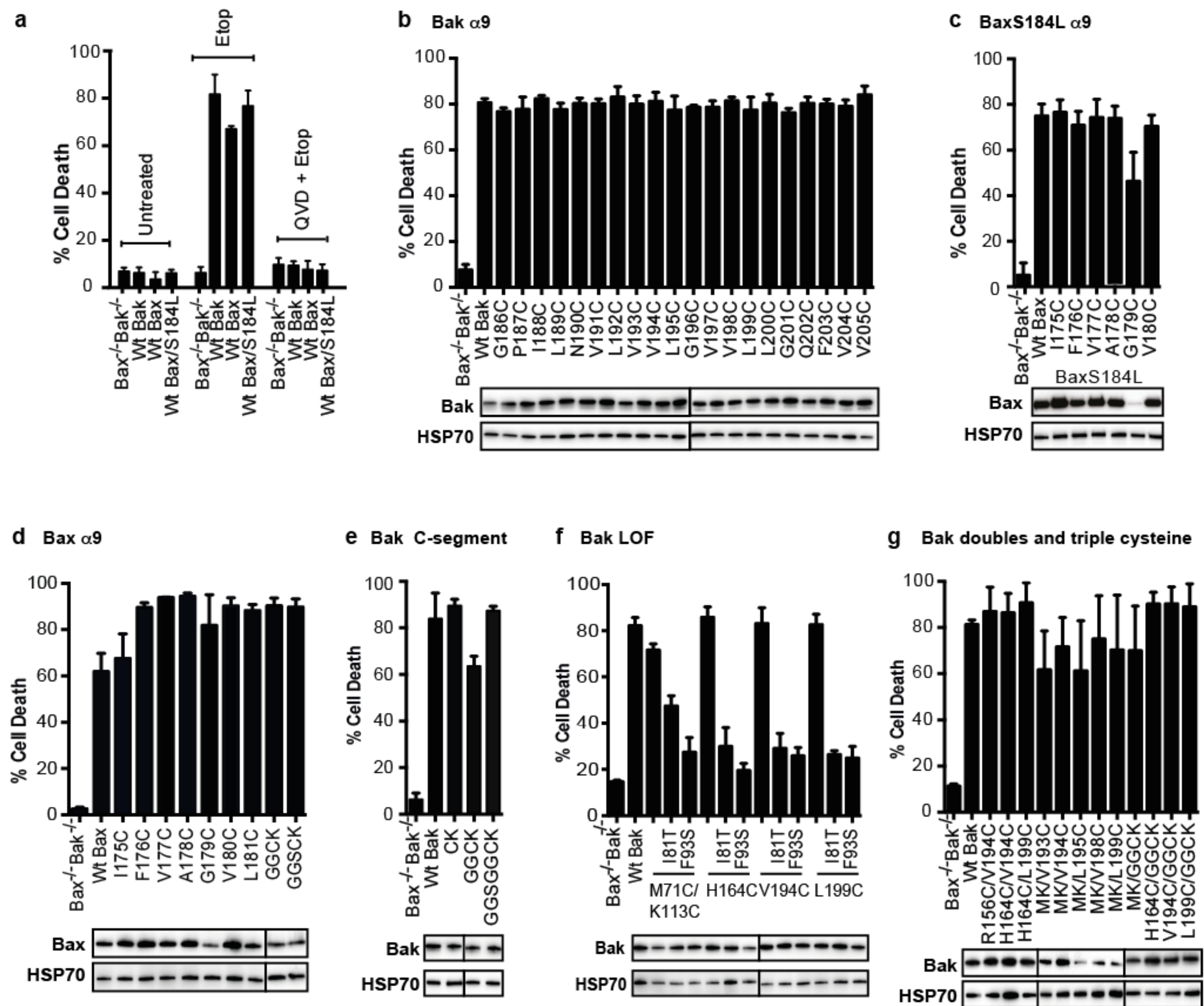

Figure S1. Most cysteine substitutions in Bak and Bax do not alter protein expression or pro-apoptotic function. (Relates to Figs 1-7)

(**a**) Cell death induced by etoposide is apoptotic. *Bak*$^{-/-}$*Bax*$^{-/-}$ MEFs stably expressing hBak, hBax or hBaxS184L were untreated or treated with etoposide (10 μM) for 24 h and cell death assessed by uptake of propidium iodide. Note that samples pre-incubated for 1 h with Q-VD-OpH (pan-caspase inhibitor; 50 μM) prevented propidium iodide uptake indicating etoposide-induced death is apoptotic.

(**b-g**) Etoposide-induced cell death. *Bak*$^{-/-}$*Bax*$^{-/-}$ MEFs stably expressing the indicated Bak (**b**), BaxS184L (**c**), Bax (**d**), Bak C-segment extensions (**e**), Bak loss-of-function (**f**) or Bak double and triple cysteine (**g**) variants were incubated with etoposide (10 μM) for 24 h and apoptosis assessed by uptake of propidium iodide.

Data are mean ± SD of three independent experiments. Protein expression levels were assessed by immunoblotting whole cell lysates for Bak or Bax, and for HSP70 as loading control.

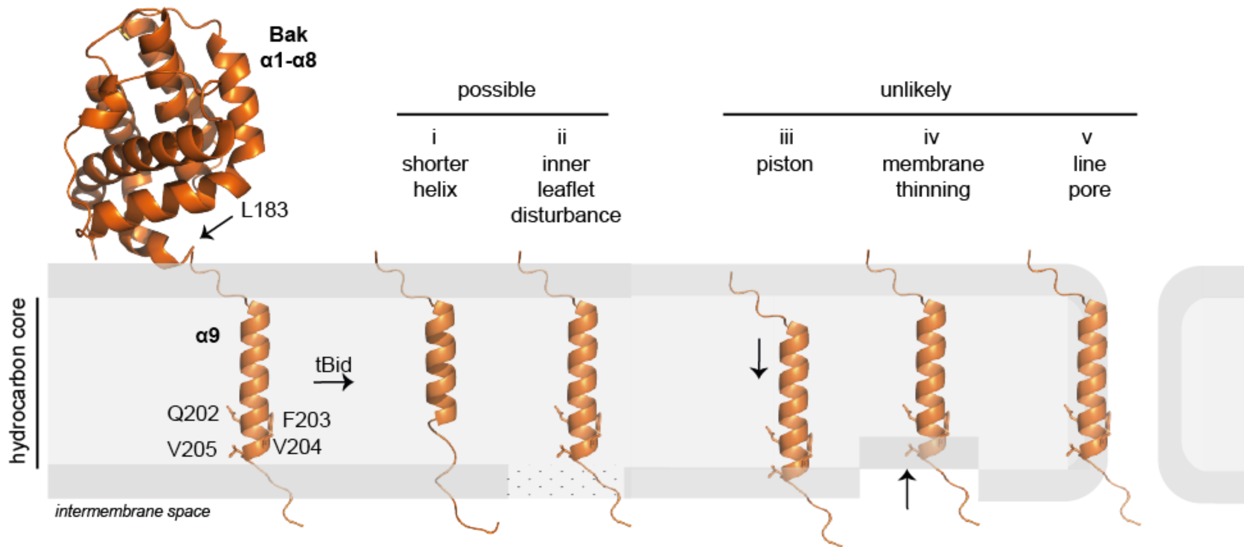

Figure S2. Possible membrane topology of the Bak C-terminus after pore formation. (Relates to Fig 2)

Diagram of changes in the Bak C-terminus after tBid-induced activation and pore formation, as discussed in the text. After Bak activation and pore formation, the most significant change in α9 labeling (apart from decreased labeling due to greater insertion) was increased labeling of four residues (Q202C-V205C) at the carboxy terminus. Increased labeling of these 4 residues might be explained by (i) the α9-helix becoming shorter, by (ii) IASD penetrating further into the inner leaflet of the MOM as a consequence of pore formation, or by α9 dissociating from neighbouring proteins (not shown). The labeling is not consistent with (iii) α9 positioning deeper in the membrane, as labeling of the amino-terminus (G186C-N190C) did not decrease after tBid. In addition, (iv) membrane thinning as observed in Bax-permeabilized vesicles [1] is also unlikely as G201C labeling did not increase. Finally, as the center of α9 did not label along one edge after tBid treatment, it is unlikely that (v) α9 lines the apoptotic pore formed by Bak. The C-terminus is modeled as in Figure 2C. Cartoon of Bak α1-α8 (aa21-183) is from the structure of nonactivated Bak (2IMT [2]).

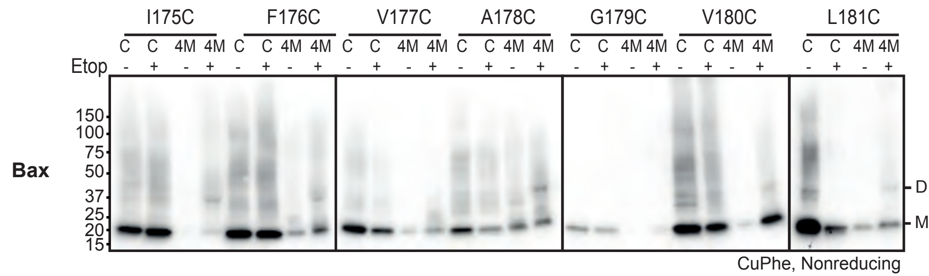

Figure S3. Intermolecular α9:α9 linkage can be captured in activated Bax at membranes. (Relates to Fig 3)
Bak$^{-/-}$*Bax*$^{-/-}$ MEFs expressing the indicated Bax cysteine variants were cultured with etoposide (10 μM), and were able to mediate cell death (Fig S1c). The cytosolic (C) and membrane (M) fractions were treated with CuPhe to induce disulfide bonding and then analyzed by nonreducing SDS-PAGE and western blot for Bax to reveal monomers (M) and linked dimers (D). Note that due to the low level of Bax that translocates to mitochondria during apoptosis, 4-fold membrane fraction (4M) was loaded.

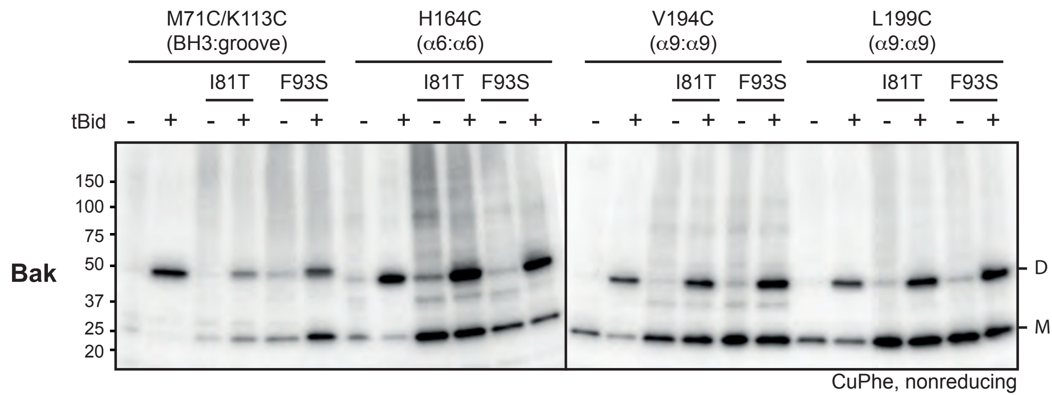

Figure S4. The Bak α9:α9 interface is partially inhibited when the BH3:groove interface is inhibited by mutagenesis. (Relates to Fig 5)

Membrane fractions from *Bak*$^{-/-}$*Bax*$^{-/-}$MEFs expressing the indicated cysteine variants were incubated without or with tBid. Some variants also contained a loss-of-function mutation in the BH3 domain (I81T) or groove (F93S) to inhibit BH3:groove dimerization [3]. Aliquots were assessed for cysteine linkage by CuPhe as in Figure S3.

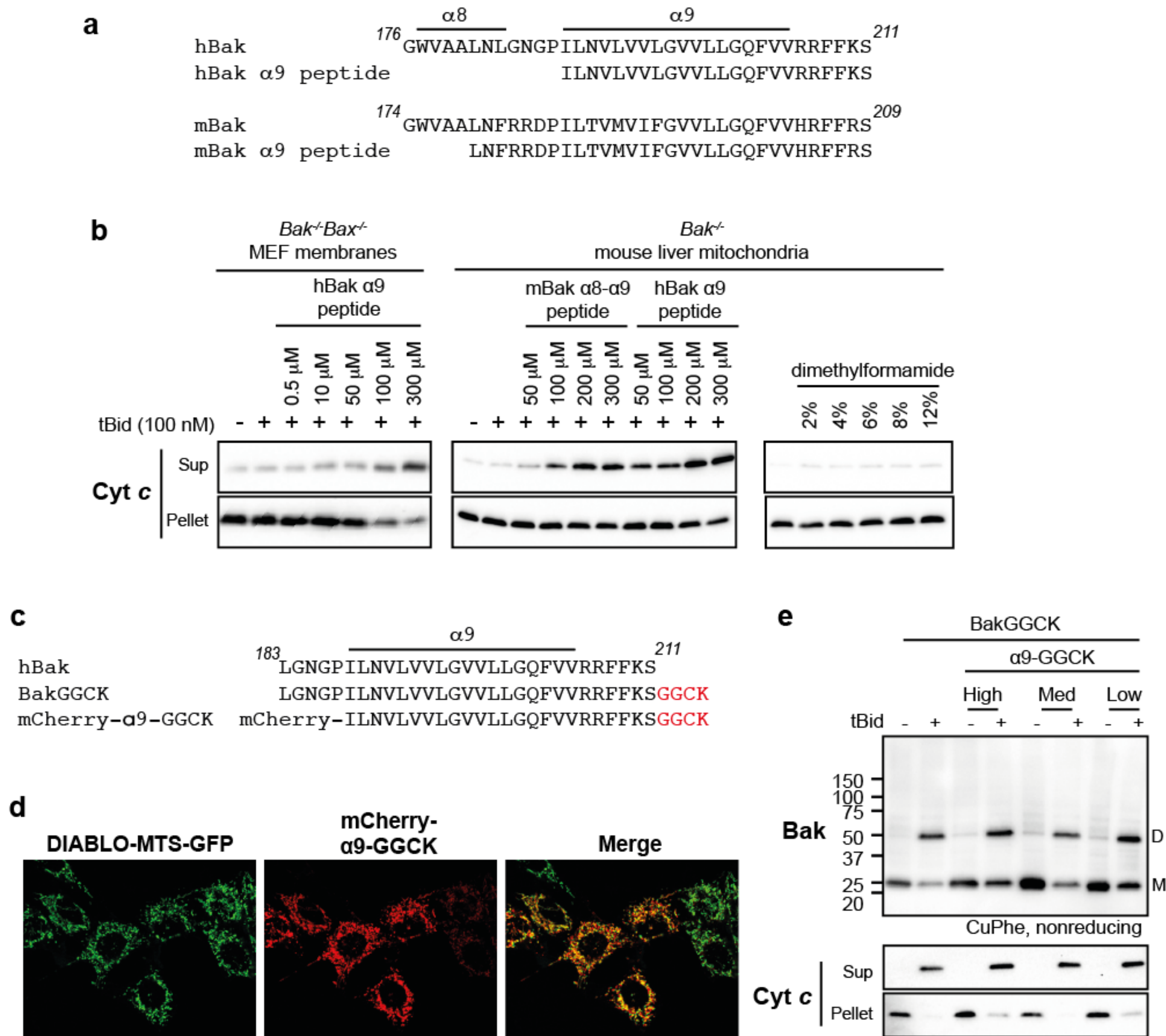

Figure S5. Bak α9 peptides or overexpressed Bak α9 in cells cannot block the α9: α9 interface

(a) Sequences of α9 peptides from human and mouse Bak.

(b) Bak α9 peptides induce cytochrome *c* release. Membrane fractions from *Bak*$^{-/-}$*Bax*$^{-/-}$ MEFs or mitochondria isolated from *Bak*$^{-/-}$ mouse liver as described previously [3] were incubated with α9 peptides at 30 °C for 20 min, followed by incubation with tBid for 30 min, as indicated. Aliquots were tested for cytochrome *c* release as in Figure 5. Note that peptides (Mimotopes, Victoria, Australia) were initially prepared as a 2 mM stock in 80% dimethylformamide, and that dimethylformamide alone did not release cytochrome *c*. Data are representative of two independent experiments.

(c) Sequence of mCherry-α9-GGCK.

(d) mCherry-α9-GGCK localizes to mitochondria. MEFs expressing the DIABLO mitochondrial targeting sequence fused to GFP (DIABLO-MTS-GFP [4]) were stably transduced with mCherry-α9-GGCK, and seeded (20,000 cells per well) on tissue culture-treated chamber slides (Ibidi). When analyzed by confocal microscopy (Zeiss LSM 5 LIVE), despite a range of mCherry-α9-GGCK expression levels, all protein becomes punctate, consistent with mitochondrial localization.

(d) mCherry-α9-GGCK fails to block either α9 linkage in oligomerized Bak or cytochrome *c* release. *Bak*$^{-/-}$*Bax*$^{-/-}$MEFs expressing BakGGCK and low, medium or high levels of mCherry-α9-GGCK (based on mCherry expression) were permeabilized and incubated with tBid. Samples were assessed for BakGGCK:BakGGCK linkage (D) and cytochrome *c* release as in Figure 6.

SUPPLEMENTARY REFERENCES

1. Satsoura D, Kucerka N, Shivakumar S, Pencer J, Griffiths C, Leber B*, et al.* Interaction of the full-length Bax protein with biomimetic mitochondrial liposomes: a small-angle neutron scattering and fluorescence study. *Biochim Biophys Acta* 2012 Mar; **1818**(3)**:** 384-401.

2. Moldoveanu T, Liu Q, Tocilj A, Watson MH, Shore G, Gehring K. The x-ray structure of a BAK homodimer reveals an inhibitory zinc binding site. *Mol Cell* 2006 December 8, 2006; **24**(5)**:** 677-688.

3. Dewson G, Kratina T, Sim HW, Puthalakath H, Adams JM, Colman PM*, et al.* To trigger apoptosis Bak exposes its BH3 domain and homo-dimerizes via BH3:grooove interactions. *Mol Cell* 2008 May 9; **30**(3)**:** 369-380.

4. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE*, et al.* Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000 Jul 7; **102**(1)**:** 43-53.