Supplementary Material for

Constitutive BAK Activation as a Determinant of Drug Sensitivity in Malignant Lymphohematopoietic Cells

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SUPPLEMENTAL METHODS

In vitro GST pulldown assay

After purified GST or GST-MCL1ΔTM and His_6-tagged BAK 21-186 were incubated together in HEPES buffer (150 mM NaCl, 5 mM dithiothreitol, 20 mM HEPES, pH 7.4) lacking or containing 1% (w/v) Triton X-100 or 1% (w/v) CHAPS at 23 °C for 1 h, complexes were captured on GSH-agarose at 4 °C overnight. Following 4 washes with the corresponding buffer, bound polypeptides were solubilized in SDS sample buffer, subjected to SDS-PAGE, and stained with Coomassie blue or probed with antibodies that recognize BAK and GST.

In vitro cytochrome c release assay

Purified His_6-tagged BAKΔTM was dialyzed against HEPES/KCl buffer [20 mM HEPES-150 mM KCl (pH 7.5)] and diluted to a concentration of 0.5 μM in HEPES/KCl with 5 mM DTT. Purified proteins and the indicated peptides or compounds were incubated at 23 °C for 1 h with mitochondria from *Bax*$^{-/-}$*Bak*$^{-/-}$ double knockout MEFs (Goping et al. 1998; Sainski et al. 2014). After centrifugation at 10,000 g for 15 min, supernatants and pellets were analyzed for cytochrome c by immunoblotting.

Liposome release assay

Unilamellar vesicles (ULVs) containing MOM lipids encapsulating fluoresceinated dextran F-10 (F-d10) were prepared and separated from untrapped F-10 as described (Dai et al. 2011). Release of F-d10 from ULVs was monitored by fluorescence dequenching using a fluorimetric plate reader (Dai et al. 2011). % F-d10 release was quantified by the equation $(F_{sample}-F_{blank})/(F_{Triton}-F_{blank})\times100$, where F_{sample}, F_{blank} and F_{Triton} are fluorescence of reagent-, buffer-, and 1% Triton-treated LUVs.

Table S1 Sources of Lymphohematopoietic Cells		
Cell line	**Source**	**Institution**
HL-60, K562	R. T. Abraham[1]	Sanford Burnham Institute
Daudi	A. Fielding	University College London
KG1a, Molt3	R. J. Jones	Johns Hopkins University
ML-1	M. B. Kastan	Duke University
MV-4-11	A. Tefferi	Mayo Clinic, Rochester, MN
Jurkat	P. Leibson[2]	Mayo Clinic, Rochester, MN
CEM, H9, SKW6.4, U937, Hs445		American Type Culture Collection
SeAx	S. Ansell	Mayo Clinic, Rochester, MN
Nalm6	E. Hendrickson	University of Minnesota
Jeko, RL, HT, DoHH2,	T. Witzig	Mayo Clinic, Rochester, MN
SuDHL-4, SuDHL-6, WSU, Karpas422	G. Nowakowski	Mayo Clinic, Rochester, MN

[1]Current affiliation: Pfizer
[2]Deceased

Table S2 **siRNA sequences used**	
BIM siRNA	5’ gac cga gaa ggt aga caa t 3’
PUMA siRNA	5’ gga ggg tcc tgt aca atc t 3’
BID siRNA #1	5’ ctt gct ccg tga tgt ctt t 3’
BID siRNA #2	5’ ggg atg agt gca tca caa a 3’
BAK siRNA	5’ gta cga aga ttc ttc aaa t 3’
BAK shRNA	5’ ccc att cac tac agg tga a 3’

SUPPLEMENTAL FIGURES AND LEGENDS

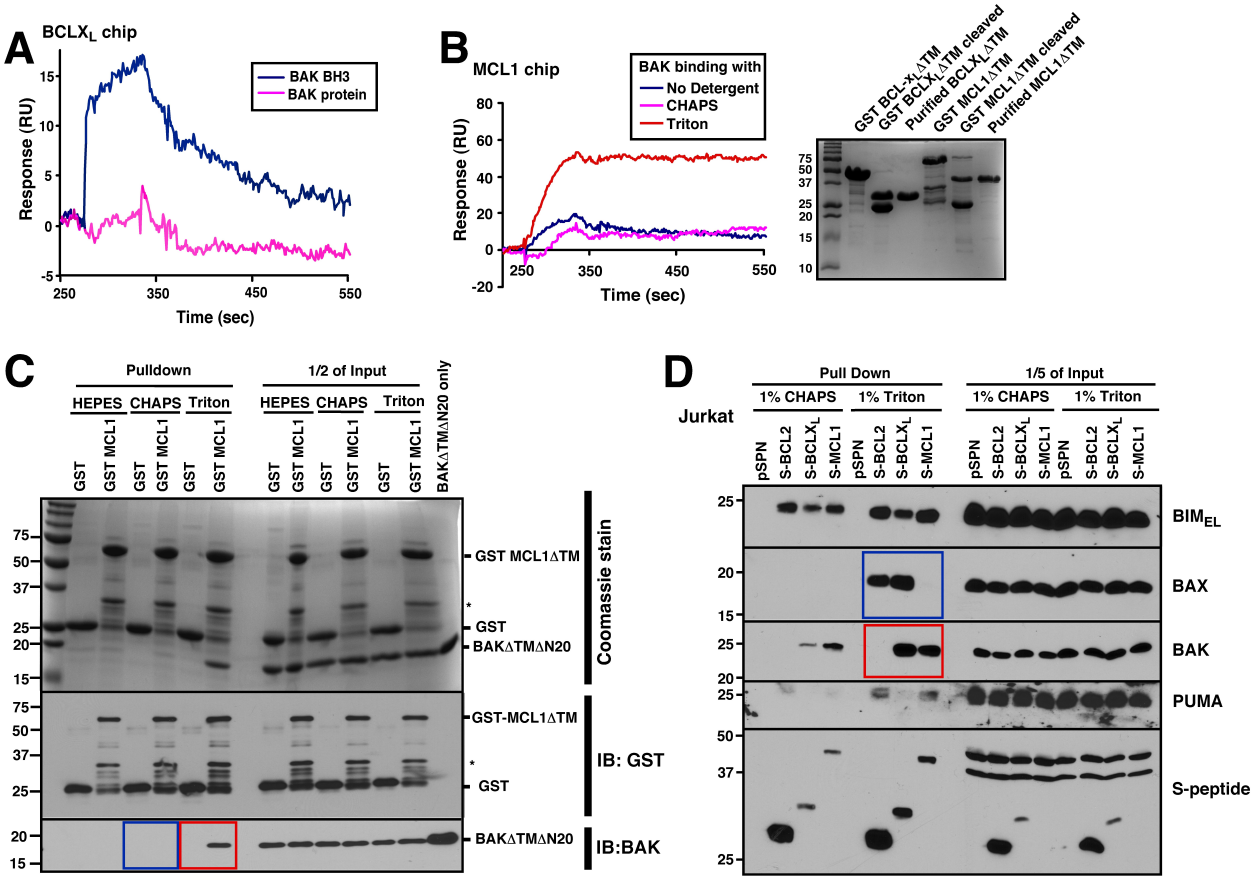

Figure S1. Triton X-100 alters BAK binding properties, related to Figure 1. A, direct comparison of BAKΔTM (1280 nM) or BAK BH3 peptide (1280 nM) binding to immobilized $BCLX_L$ΔTM. **B,** Left panel, BAKΔTM (640 nM) in HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) without or with 1% CHAPS or 1% Triton X-100 was passed over MCL1ΔTM immobilized on a CM5 chip. Right panel, Coomassie blue staining of purified $BCLX_L$ΔTM and MCL1ΔTM used for Biacore assay and GST-MCL1ΔTM used for pulldown assay in Figures 1 and S1. **C,** after GST or GST-MCL1ΔTM was incubated for 1 h with BAK ΔTMΔN20 (a variant used to clearly see the band distinct from GST in the Coomassie stained gels), the mixtures were pulled down with GSH-agarose for 4 h. The pulldowns and ½ of the inputs were separated by SDS-PAGE and stained with Coomassie blue or blotted with anti-GST and anti-BAK antibodies. Boxes, BAK binds to GST-MCL1 after incubation with buffer containing Triton X-100 (red box) but not CHAPS (blue box). **D,** after Jurkat cells stably expressing S peptide-tagged BCL2, $BCLX_L$ or MCL1 were lysed in buffer containing 1% CHAPS or 1% Triton X-100, the lysates were incubated with S protein-agarose and the pulldowns, along with 1/5 of the lysates ("Input"), were blotted with indicated antibodies. Boxes, BAX (blue box) binds predominantly to BCL2 and $BCLX_L$, whereas BAK (red box) binds predominantly to $BCLX_L$ and MCL1. Binding is absent (BAX) or diminished (BAK) in lysates prepared in CHAPS compared to Triton X-100.

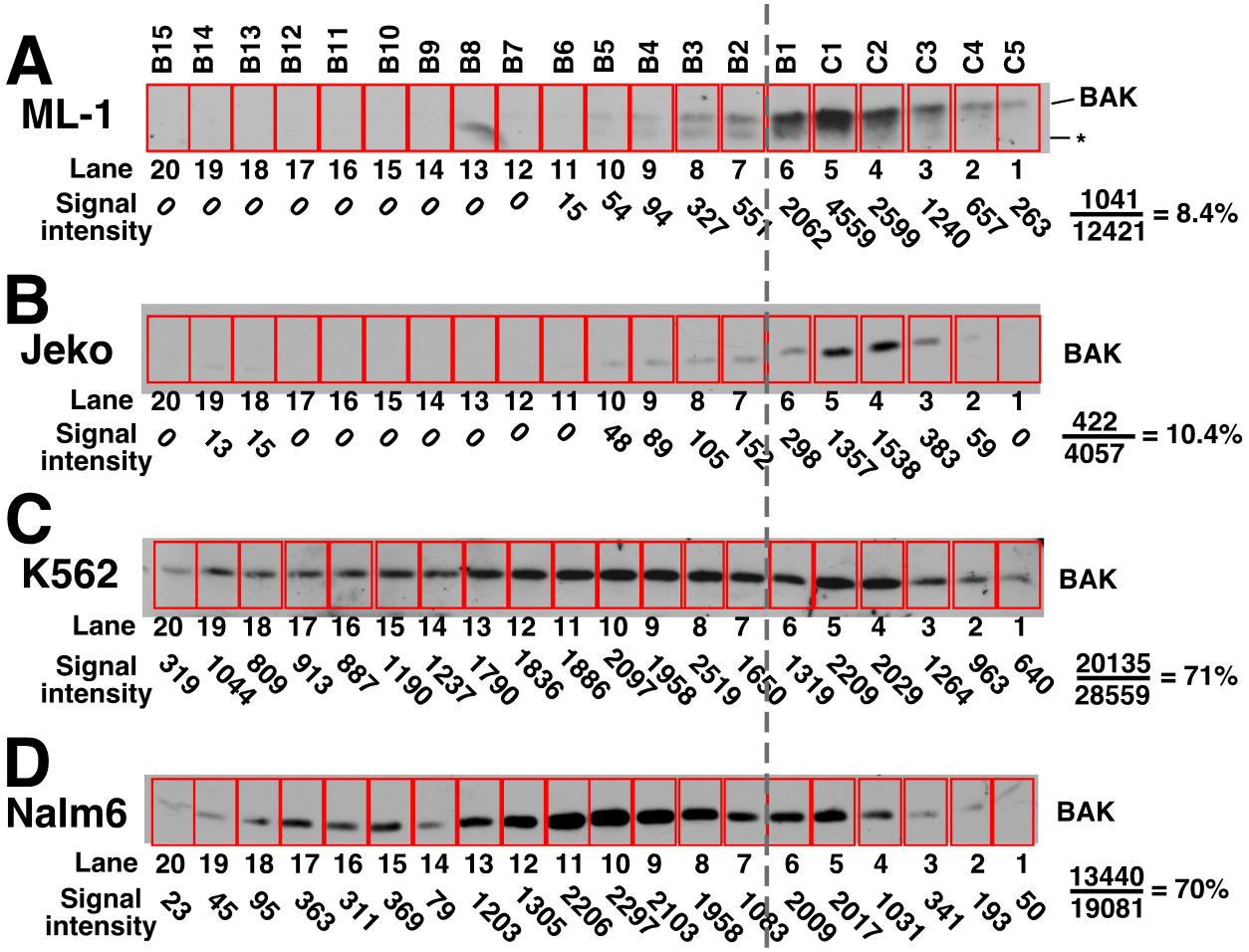

Figure S2. Calculation of % BAK oligomerized, related to Figure 2. Two cell lines with a low percentage of BAK in oligomers (ML-1 and Jeko) and two with a high percentage of total BAK in higher molecular weight fractions (K562 and Nalm6) are used as examples of the calculations summarized in Figure 2. After lysates were subjected to FPLC, fractions were subjected to SDS-PAGE and immunoblotting for BAK. After blots were scanned, the signal density [area x (absorbance – background)] in each lane was determined. Dashed line demarcates boundary between BAK monomer (fractions 1-6) and BAK oligomers (fractions 7-20) based on migration of recombinant human BAK shown in Figure 2A. The percentage of total BAK found in oligomers was estimated as the sum of signal densities in fractions 7-20 divided by the sum of signal densities in all lanes as shown at right of each blot.

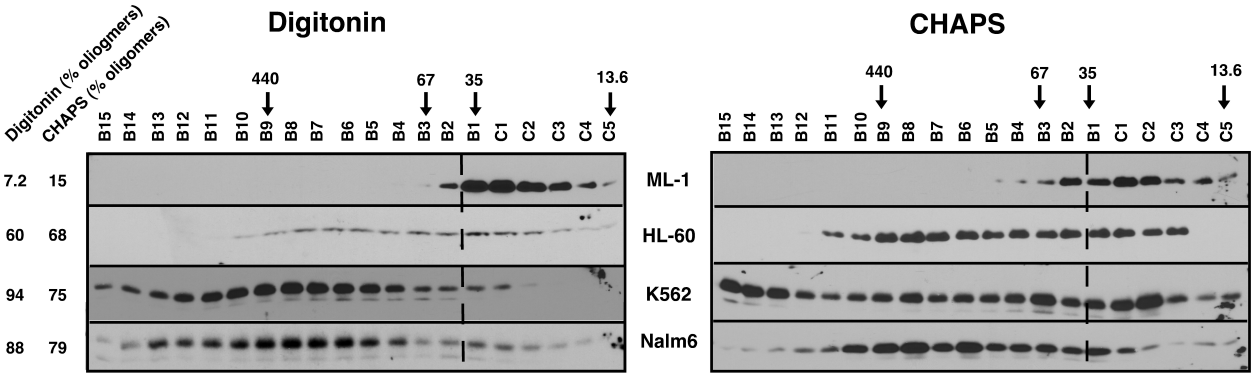

Figure S3. Comparison of BAK oligomerization in lysates prepared in digitonin versus CHAPS, related to Figure 2B. Lysates prepared from cells in digitonin lysis buffer (left panel) or CHAPS lysis buffer (right panel) were subjected to size exclusion chromatography by FPLC and blotted with anti-BAK antibody. Numbers at left indicate the percentage of BAK in oligomers (i.e., to left of the dashed line) in lysates prepared in digitonin vs. CHAPS, respectively. Calculations were performed as indicated in the legend to Figure S2. Arrows indicate size markers.

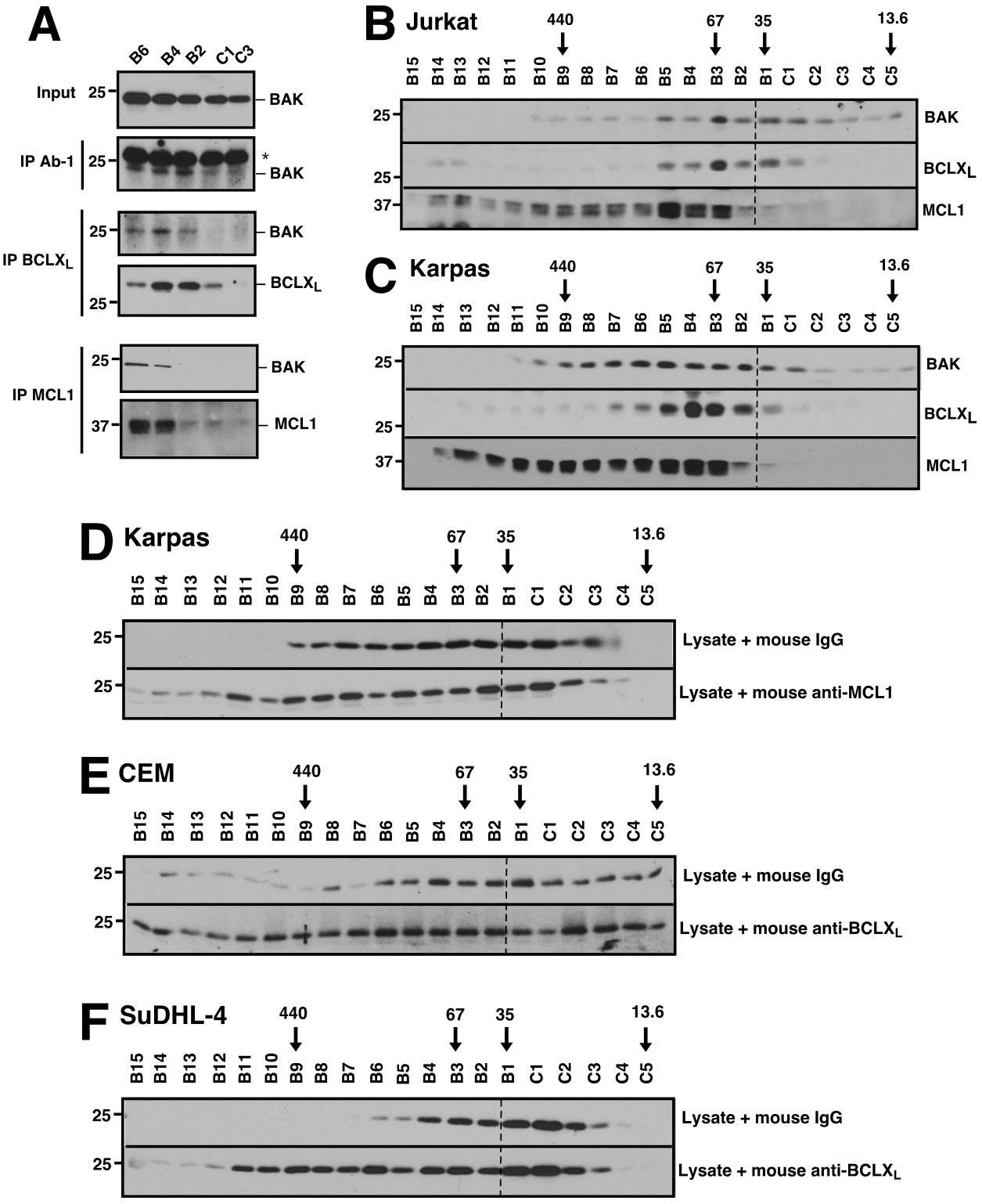

Figure S4. Ability of BAK oligomer fractions to react with Ab-1 and be supershifted by anti-MCL1 or anti-BCLX$_L$ antibodies, related to Figure 2. A, size exclusion chromatography fractions B6, B4, B2, C1, C3 (see Figure 2B) from Jurkat lysates were incubated with Ab-1 anti-BAK antibody, anti-BCLX$_L$ or anti-MCL1, respectively, and immunoprecipitates were blotted

with antibody to total BAK. *, IgG light chain. **B and C,** CHAPS lysates from Jurkat (B) and Karpas (C) cells, respectively, were subjected to size exclusion chromatography and blotted with antibodies to BAK, $BCLX_L$ and MCL1. **D-F,** CHAPS lysates from Karpas (D), CEM (E), or SuDHL-4 cells (F) were incubated with MCL1 antibody (D), $BCLX_L$ antibody (E and F) or control IgG for 2 hours as indicated before size exclusion chromatography and blotting with anti-BAK antibody.

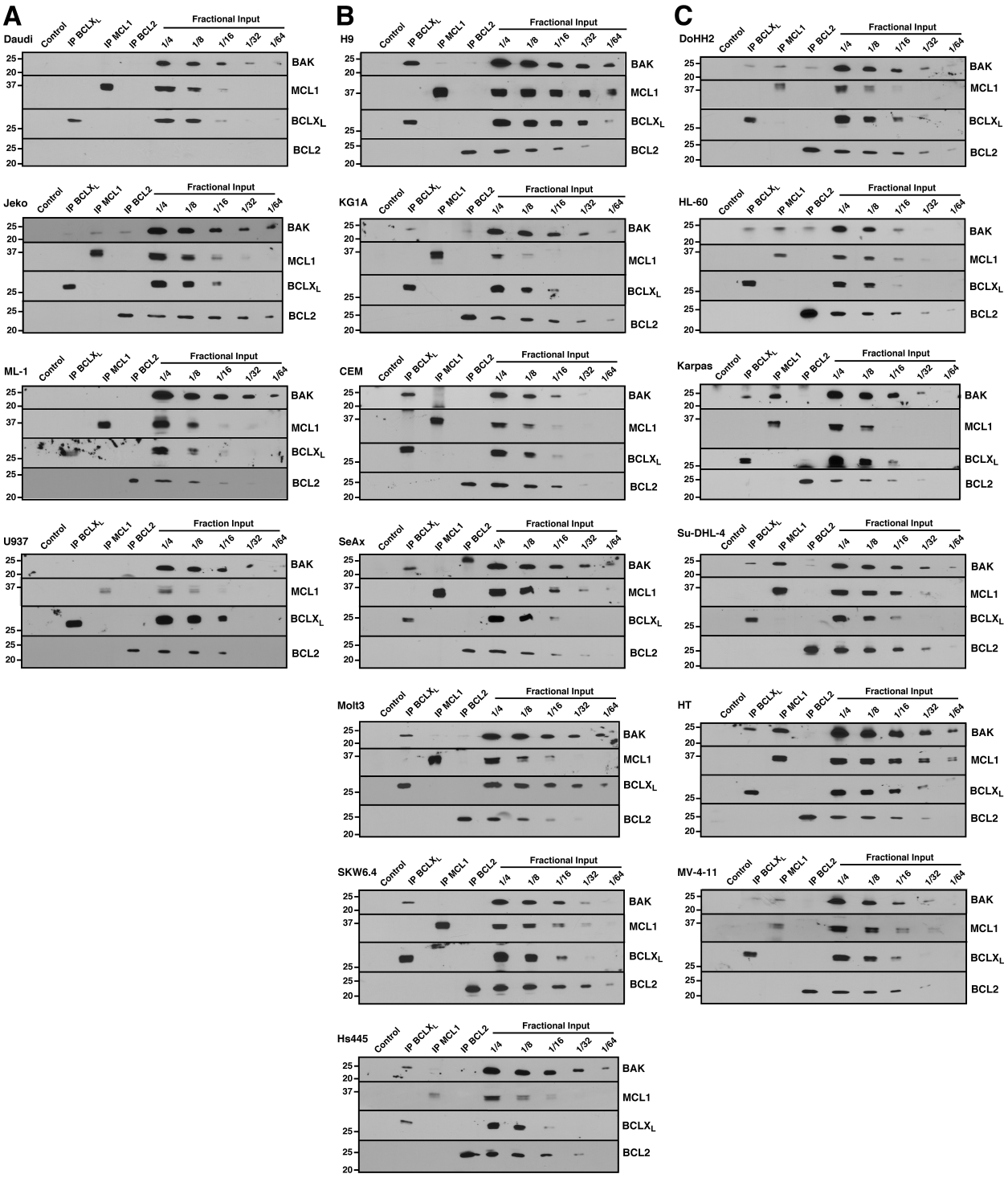

Figure S5. Constitutive binding of BAK to anti-apoptotic BCL2 family members varies among different cell lines, related to Figure 3. CHAPS lysates prepared from the indicated log phase cells were immunoprecipitated (IP) with antibodies to $BCLX_L$, MCL1 or BCL2, and compared to serial dilutions of the input. Normal rabbit IgG served as control for immunoprecipitations. **A,** cell lines in which BAK exhibits limited binding to $BCLX_L$, MCL1 or BCL2. **B,** cell lines in which BAK is predominantly bound to $BCLX_L$. **C,** cell lines in which BAK is bound to multiple partners.

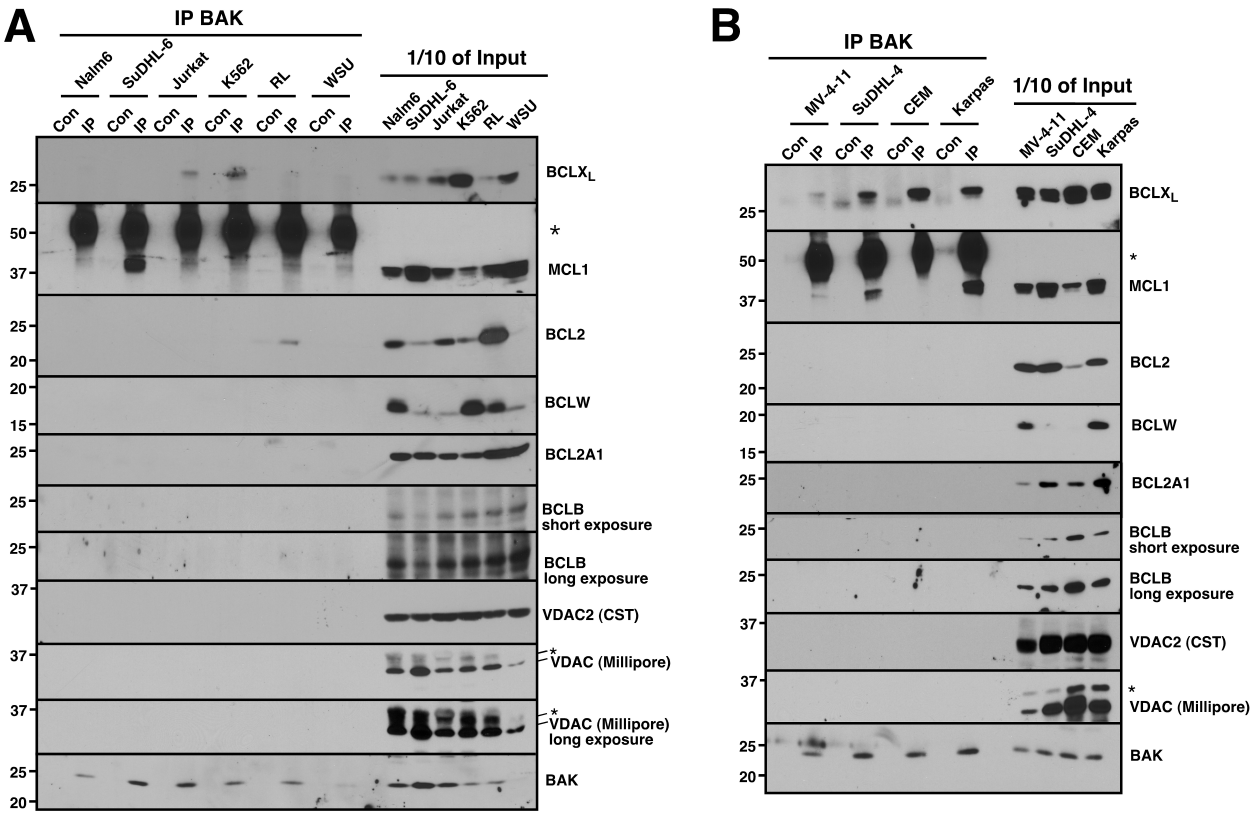

Figure S6. Identification of antiapoptotic proteins constitutively bound to BAK, related to Figure 3. After CHAPS lysates were prepared from the indicated cell lines, BAK was immunoprecipitated and blotted with the indicated antibodies. CHAPS lysates binding to the beads without anti-BAK antibody served as a control (Con). * indicates IgG heavy chain or nonspecific band.

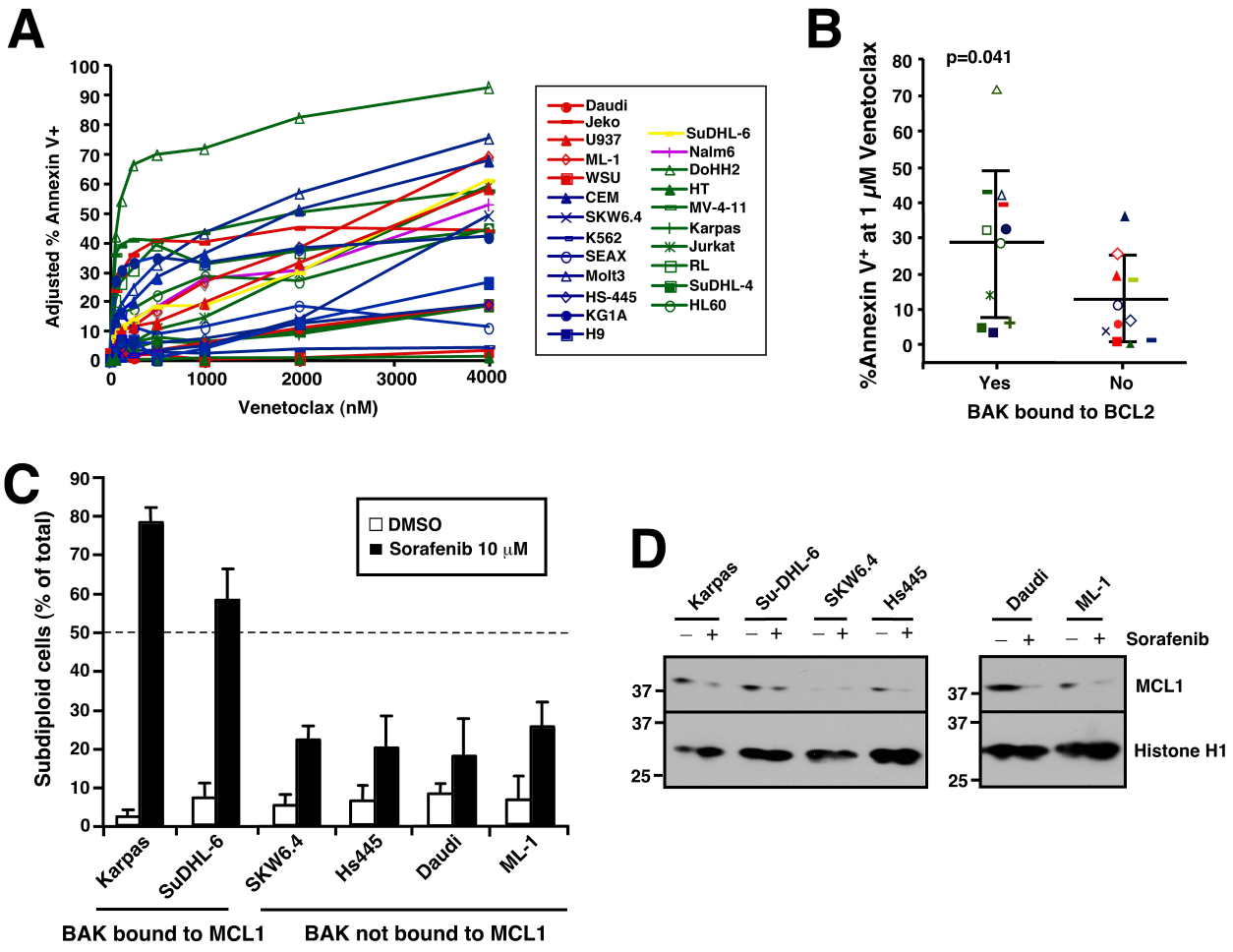

Figure S7. Sensitivities of cell lines to venetoclax and sorafenib correlate with BAK binding status, related to Figure 4. A, after cell lines were treated with the indicated concentrations of venetoclax for 48 h, the percentages of Annexin V positive cells were assessed by flow cytometry. Graph shows the average of three independent experiments. Symbol and line colors represent different BAK binding status in Figure 4: red, no binding; blue, BAK bound to $BCLX_L$ only; yellow, BAK bound to MCL1 only; purple, unknown binding partner; and green, BAK bound to multiple partners. **B,** for each of the cell lines, the mean percentage of apoptosis observed at 1 μM venetoclax was plotted as a function of BAK•BCL2 binding status determined in Figures 3 and S4. **C,** after the indicated cells were treated with 10 μM sorafenib or diluent for 48 hours, samples were extracted with sodium citrate, stained with propidium iodide and assayed by flow cytometry for subdiploid events. Error bars indicate ± SD from 4 independent experiments. **D,** after the indicated cells were treated with 10 μM sorafenib or diluent for 48 hours, whole cell lysates were subjected to immunoblotting for BAK and, as a loading control, Histone H1.

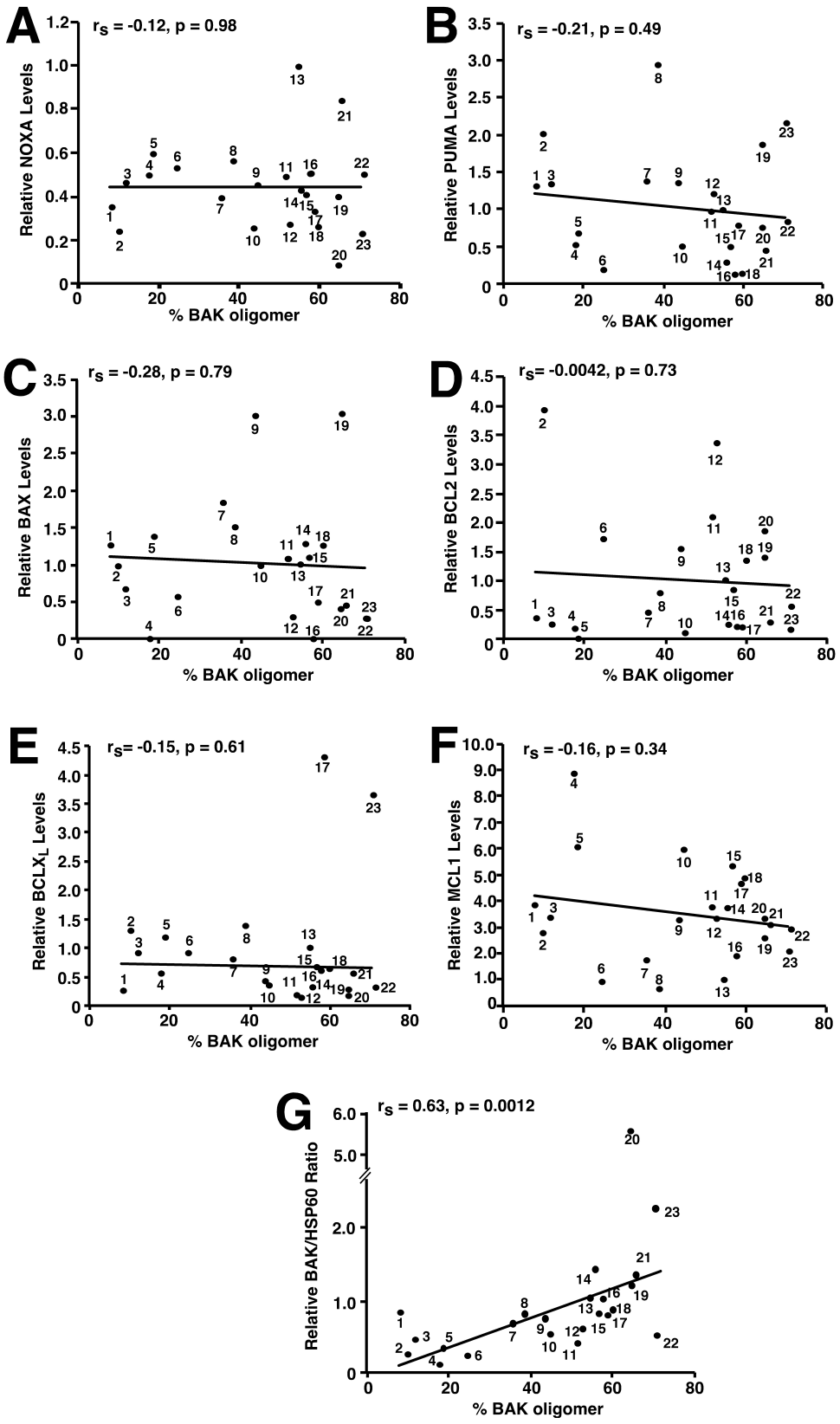

Figure S8. The percentage of constitutively oligomerized BAK correlates with relative levels of BAK protein but not NOXA, PUMA, BAX, BCLX$_L$, MCL1 or BCL2, related to Figure 5. The relative amounts of NOXA (A), PUMA (B), BAX (C), BCL2 (D), BCLX$_L$ (E) and

MCL1 (F) in the 23 cell lines (analyzed as illustrated in Figure 5A and normalized using β-ACTIN as a loading control) were compared with the percentage of constitutively oligomerized BAK. **G,** relative levels of BAK in the 23 cell lines normalized to HSP60 were compared with the percentage of constitutively oligomerized BAK. Numbers refer to individual cell lines as defined in the legend to Figure 3.

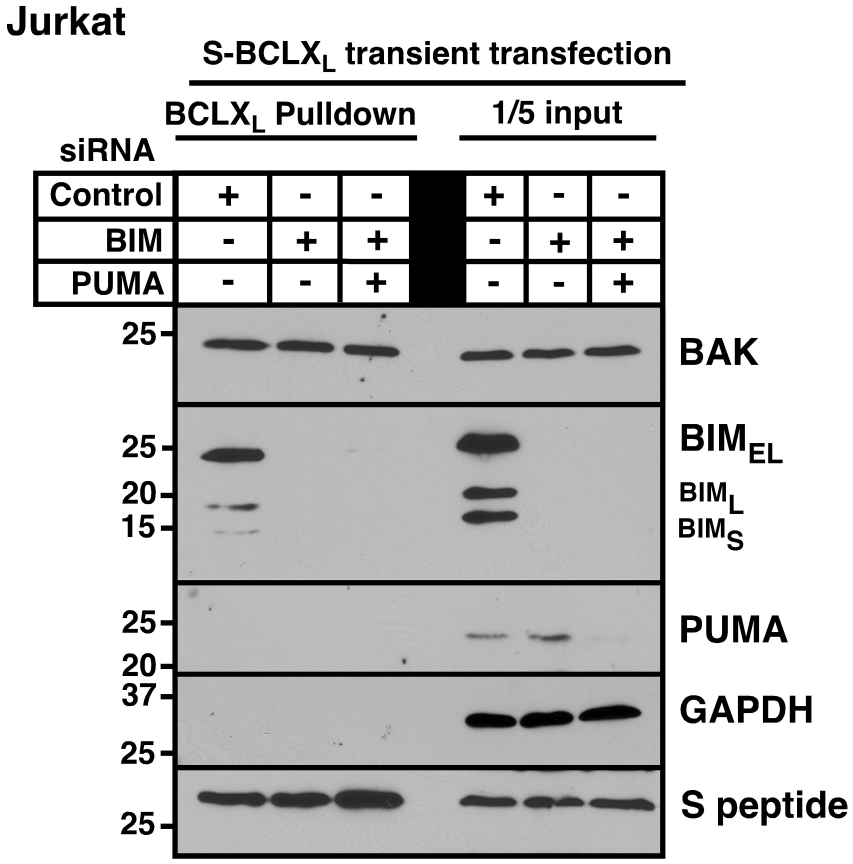

Figure S9. Constitutive activation of BAK is not inhibited by BIM or PUMA knockdown, related to Figure 6. 24 h after Jurkat cells were transiently transfected with S-$BCLX_L$, cells were transfected with BIM siRNA, BIM siRNA + PUMA siRNA, or nontargeting (control) siRNA for another 48 h. CHAPS lysates were then prepared and incubated with S protein-agarose overnight before the beads were washed. The pulldowns and lysates (1/5 of input) were blotted with indicated antibodies.

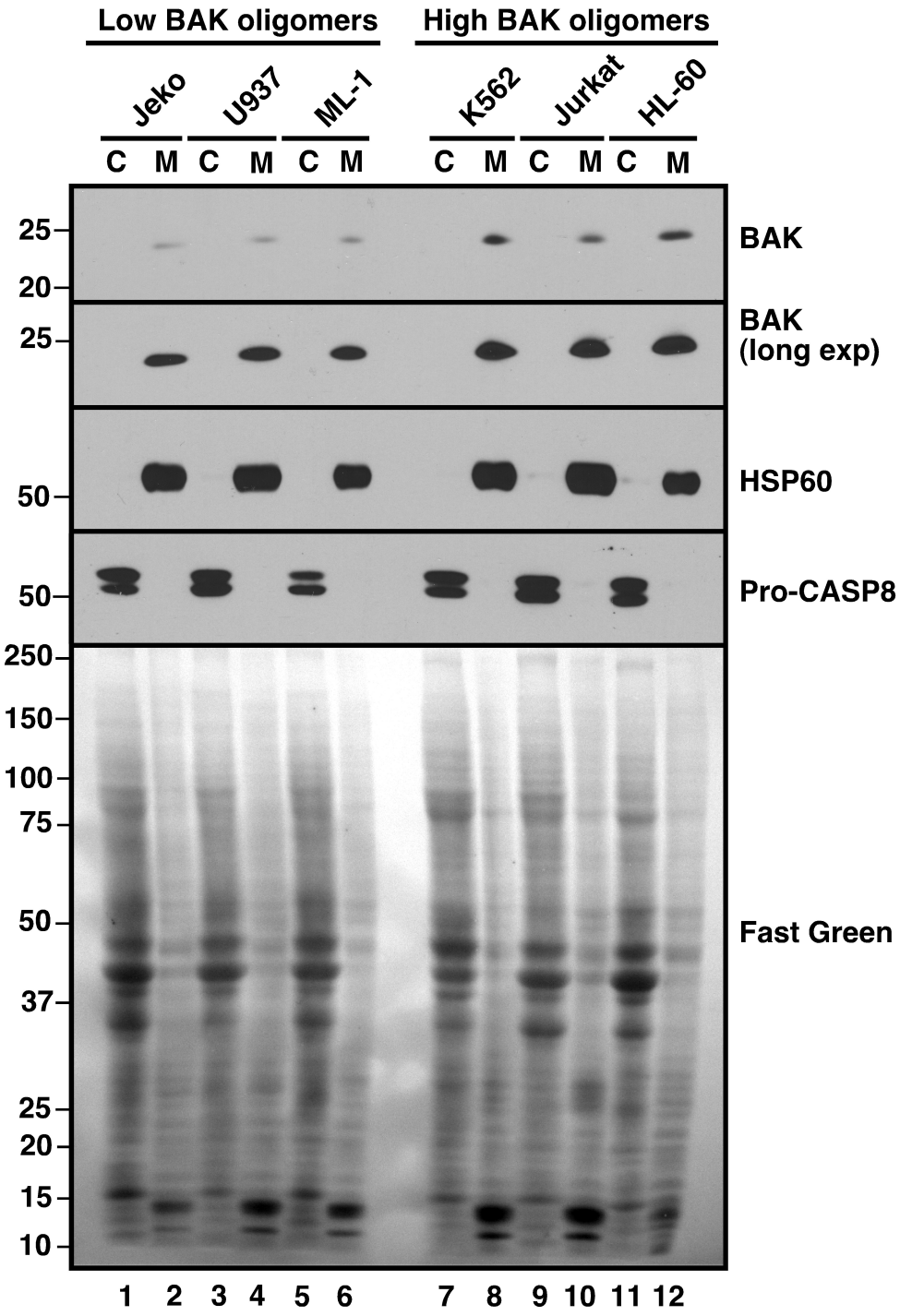

Figure S10. BAK expression in mitochondria. Fractions containing 45 μg of cytosolic protein (C) or 15 μg of mitochondrial protein (M) from 3 cell lines with low BAK oligomerization (Jeko, U937 and ML-1) and 3 with high levels of BAK oligomers (K562, Jurkat and HL-60) were separated by SDS-PAGE and blotted with antibodies to BAK. HSP60 and Procaspase 8 served as markers of mitochondria and cytosol, respectively.

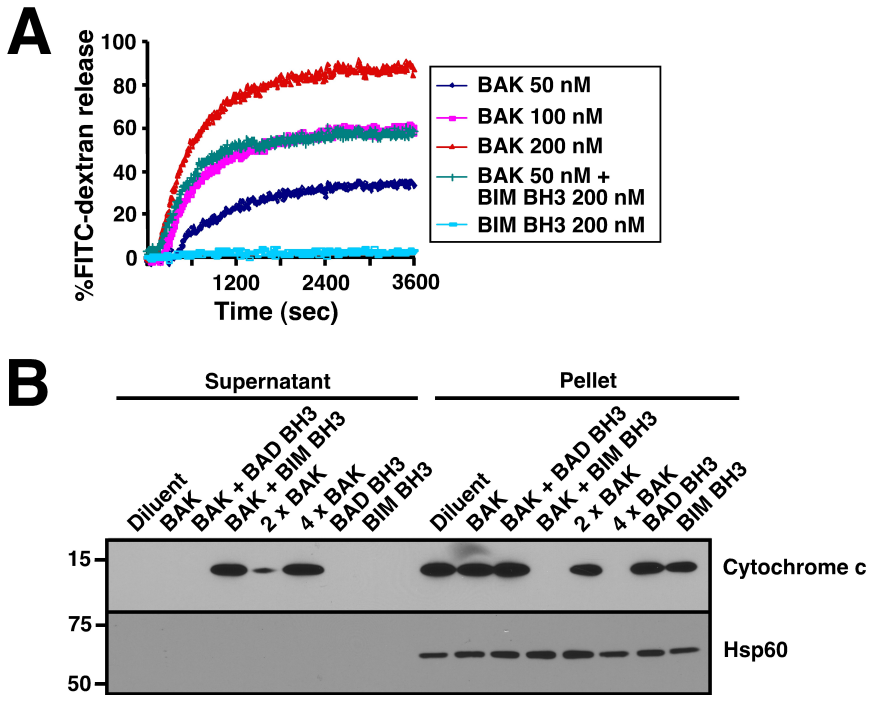

Figure S11. BAK undergoes auto-activation at high concentrations, related to Figure 6. A, FITC-dextran 10 encapsulating LUVs containing 5% DGS-NTA-Ni were incubated with the indicated concentrations of proteins and peptides. FITC-dextran release was measured as a function of time. Assay is representative experiment of 3 independent experiments, which are summarized in Figure 6D. **B,** mitochondria isolated from *Bax*$^{-/-}$/*Bak*$^{-/-}$ double knockout mouse embryo fibroblasts were incubated with the indicated concentrations of proteins and peptides at 37 °C for 1 h. After centrifugation at 10,000 x g for 15 min, the supernatants and pellets were blotted with antibodies to cytochrome c and heat shock protein 60 (Hsp60).

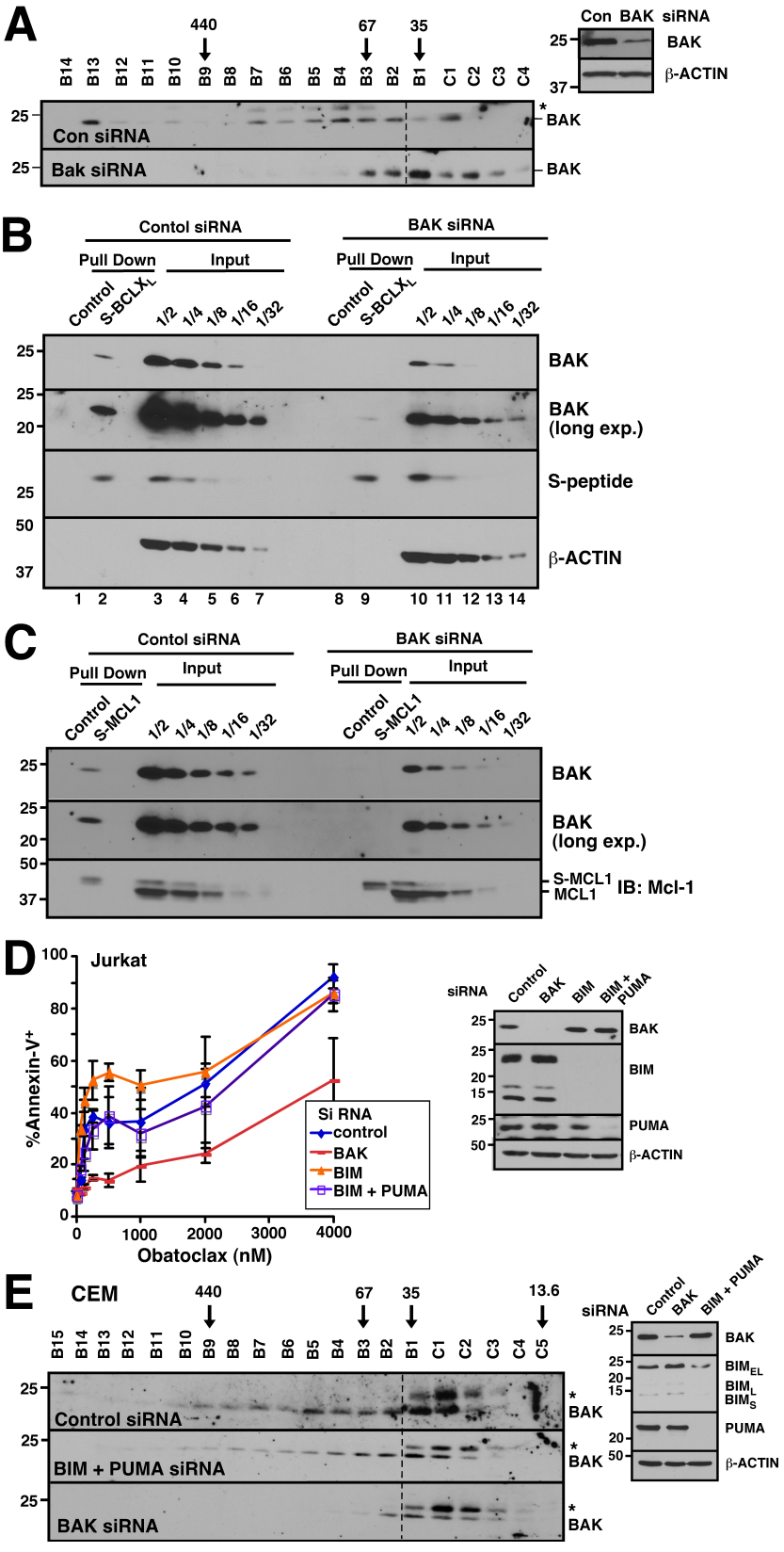

Figure S12. Constitutive BAK oligomerization is inhibited by BAK knockdown, related to Figure 6. A, 48 h after Jurkat cells were transfected with BAK siRNA or nontargeting control, CHAPS lysates were subjected to size exclusion chromatography and blotted with BAK

antibody. **Inset,** western blots of whole cell lysates showing the knockdown of BAK. **B, C,** 48 h after Jurkat cells stably expressing S peptide-$BCLX_L$ (B) or S peptide-MCL1 (C) were transfected with BAK siRNA or nontargeting control, CHAPS lysates were prepared and incubated with S protein-agarose. Pull-downs and serial dilutions of the lysates ("Input") were probed with the indicated antibodies. Untransfected Jurkat cells subjected to pulldown with S protein-agarose served as a control. **D,** beginning 24 h after Jurkat cells were transfected with the indicated siRNAs together with plasmid encoding EGFP-Histone H2B, cells were treated with obatoclax for another 24 h and stained with APC-annexin V. The percentage of $EGFP^+$ cells that stain positive for Annexin V is shown. Error bars, mean ± SD of 3 independent experiments. Inset, immunoblot of whole cell lysates showing effects of siRNA. **E,** 48 h after CEM cells were transfected with nontargeting (control) siRNA, BIM siRNA + PUMA siRNA, or BAK siRNA, whole cell lysates were probed for the indicated proteins (inset) or CHAPS lysates were separated by FPLC and blotted for BAK. * in A and E, nonspecific bands.

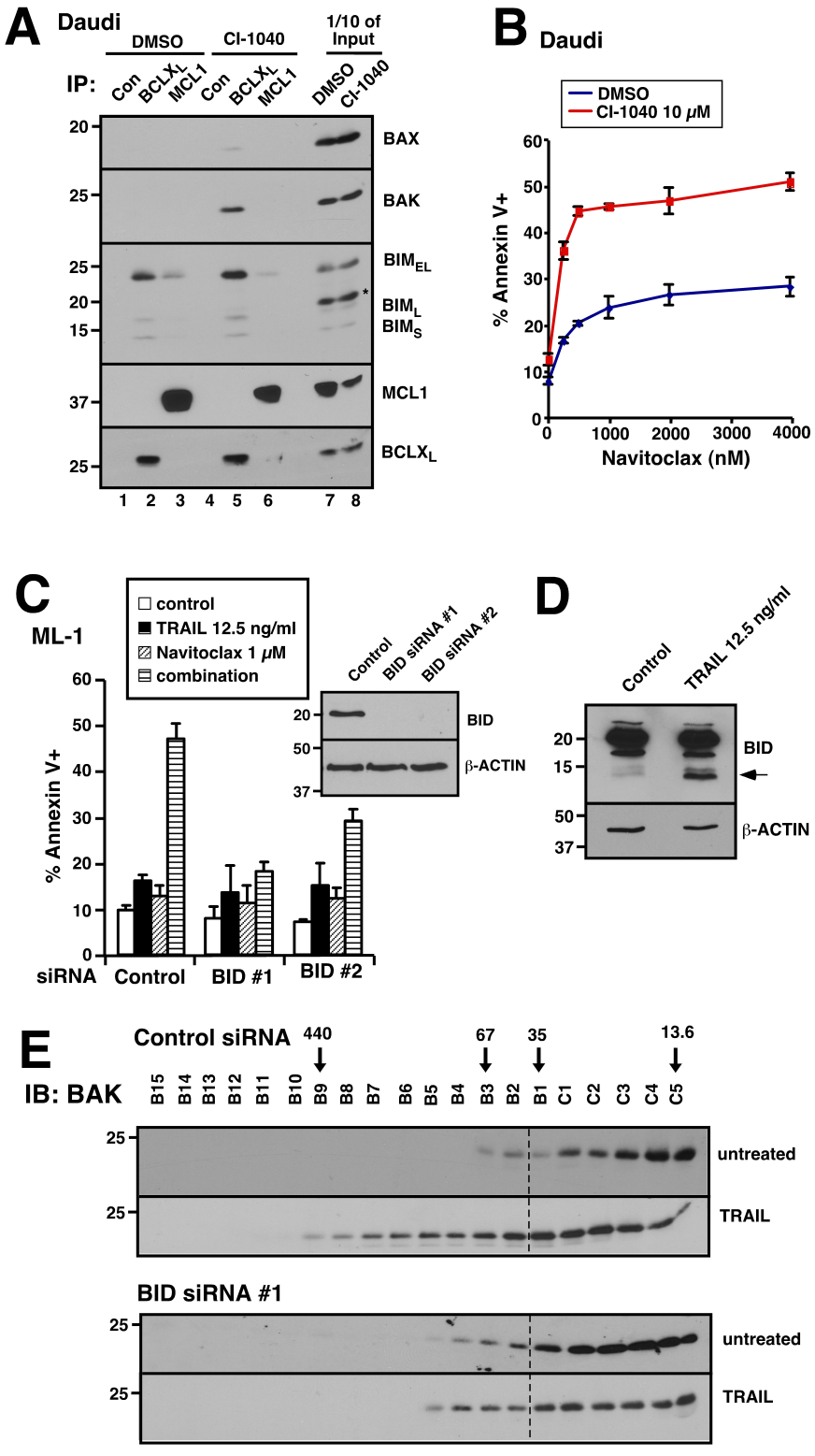

Figure S13. Drug-induced BAK oligomerization, related to Figure 7. A, after Daudi cells were treated with 10 μM CI-1040 for 24 h, $BCLX_L$ and MCL1 were immunoprecipitated and compared to 1/10 of input. Normal rabbit IgG antibody served as an IP control. **B,** after Daudi were treated with the indicated drugs for 24 h, apoptosis was detected by Annexin V staining. **C,** 24 h after ML-1 cells were transfected with indicated siRNAs, cells were treated with 12.5 ng/ml

TRAIL or control for another 24 h and apoptosis was detected by Annexin V staining. **Inset,** western blots show BID knockdown. **D,** whole cell lysates of ML-1 cells treated for 24 h with TRAIL or control were blotted with BID or, as a loading control, β-ACTIN. Arrow indicates cleaved BID. **E,** CHAPS lysates from panel C were subjected to size exclusion FPLC and blotted for BAK. Error bars in panels B and C, ± SD of 3 independent assays.

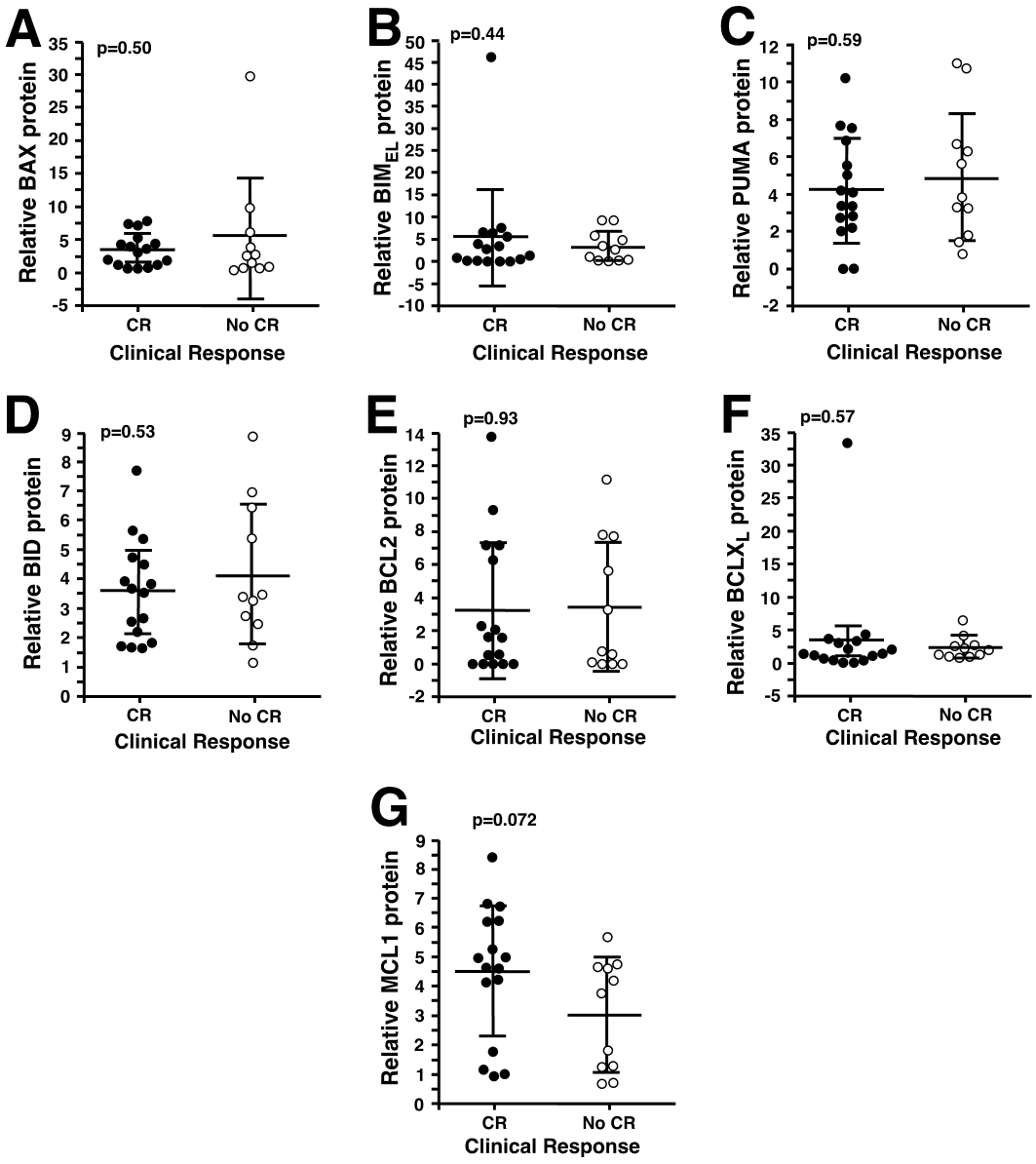

Figure S14. Comparison of the relative levels of BCL2 family proteins in samples from patients with different responses, related to Figure 7. A-G, the relative amounts of BAX (A), BIM_{EL} (B), PUMA (C), BID (D), BCL2 (E), $BCLX_L$ (F) and MCL1 (G) in pretreatment samples from patients with newly diagnosed AML (>80% blasts) were compared in two groups accordingly to patient outcome. CR, complete remission.

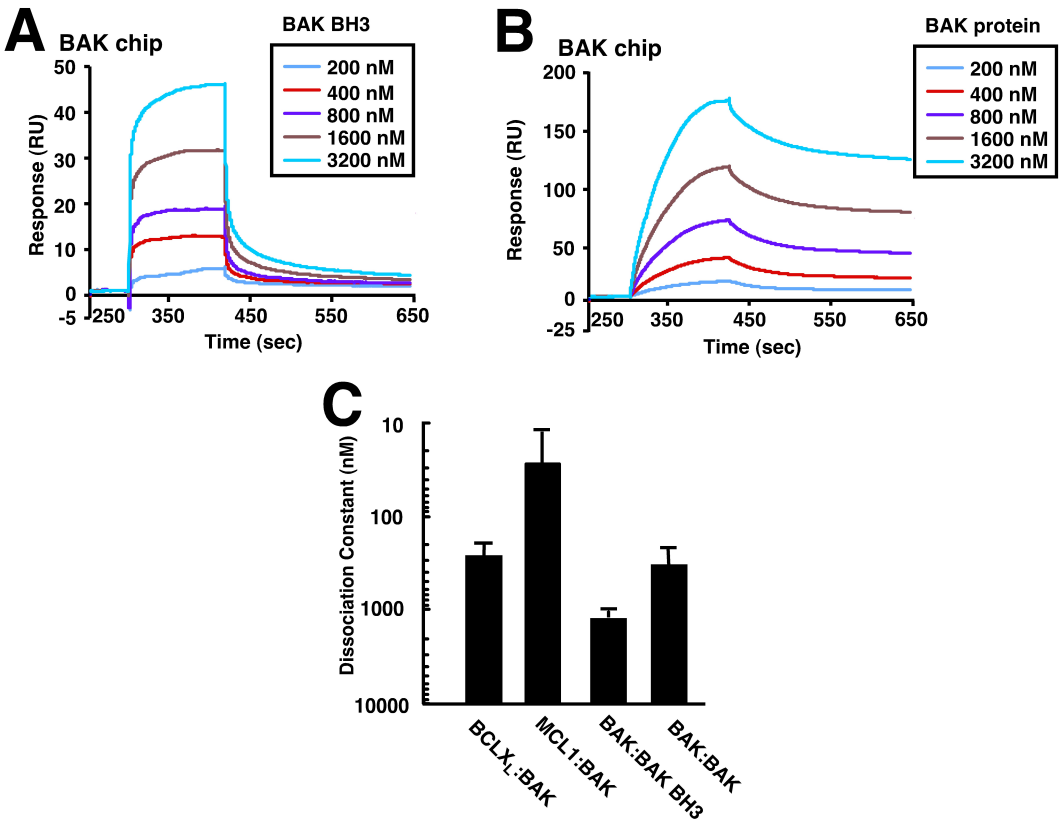

Figure S15. Assessment of BAK•BAK interactions by SPR, related to Figure 7. A, B, binding of various concentrations of BAK BH3 peptide (A) or BAKΔTM (B) to BAK immobilized on a CM5 SPR chip. **C,** calculated equilibrium dissociation constants for the indicated complexes. Each bar graph reflects mean ± SD from three independent experiments.