Supporting Information

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SI Methods

Immortalized Baby Mouse Kidney Epithelial Cell Transfection and Cell-Viability Assay. Immortalized baby mouse kidney epithelial (iBMK) cells deficient in B-cell lymphoma 2 (BCL-2)–associated X (BAX) and BCL-2 antagonist/killer (BAK) (1) were transfected with the pORF mammalian expression vector alone or with the corresponding plasmid containing wild-type or full-length (FL)-BAK using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The pORF plasmid containing wild-type murine BAK was purchased from Invivogen, and that containing FL-BAK was generated by PCR-based site-directed mutagenesis and confirmed by DNA sequencing. $Bax^{-/-}Bak^{-/-}$ iBMK cells were plated in 96-well plates (4×10^4 cells per well) in DMEM supplemented with penicillin/streptomycin, glutamine, 2 mM DTT, and 5% (vol/vol) FBS. A mixture of plasmid DNA (0.2 μg) and 0.5 μL Lipofectamine was added to each well, followed by 24 h incubation. The transfected cells and control wild-type and $Bax^{-/-}Bak^{-/-}$ iBMK cells were then treated with staurosporine (STS) (100 nM) or etoposide (10 μM), and cell viability was measured at 24 h by CellTiter-Glo assay (Promega) according to the manufacturer's instructions.

Fractionation of FL-BAK for Monitoring Oligomerization. FL-BAK (500 nM) was incubated with liposomes in the presence or absence of truncated BCL-2 homology domain 3 (BH3)-interacting domain death agonist (tBID) (100 nM) at room temperature for 30 min. After solubilization with 0.5% (wt/vol) CHAPS, the mixture was subjected to size-exclusion chromatography (SEC) at 4 °C in 20 mM Hepes (pH 7.8), 150 mM KCl, 0.5% (wt/vol) CHAPS. The fractions were collected, subjected to SDS/PAGE, and then analyzed by Western blot using BAK (BAK NT; Millipore) and BID (FL-195; Santa Cruz) antibodies.

1. Mathew R, Degenhardt K, Haramaty L, Karp CM, White E (2008) Immortalized mouse epithelial cell models to study the role of apoptosis in cancer. *Methods Enzymol* 446: 77–106.

Chemical Crosslinking Analysis of FL-BAK upon tBID-Induced Liposomal and Mitochondrial Release. FL-BAK (500 nM) was incubated with liposomes or $Bak^{-/-}$ mouse liver mitochondria in the presence or absence of tBID (100 nM) at room temperature for 30 min. Disuccinimidyl suberate (DSS) and bismaleimidohexane (BMH) were added to the liposomal and mitochondrial mixtures, respectively, at a final concentration of 100 μM and were incubated for 15 min. After the liposomal and mitochondrial crosslinking reactions were quenched with 1 M Tris base and 50 mM DTT, respectively, the mixtures were subjected to SDS/PAGE and anti-BAK Western analysis.

Generation and Characterization of FL-BAK Liposomes. 8-Aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (ANTS)/p-xylene-bis-pyridinium bromide (DPX)-loaded liposomes were generated as described in the liposomal-assay method, except that FL-BAK (20 μM) was added to the lipid film (1 mg) mixture in 200 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.0, and the freeze-thawing step was bypassed to preserve FL-BAK protein functionality. After extrusion (Avanti extruder) through a 100-nm nucleopore membrane, liposomes containing FL-BAK were purified by gravity-flow SEC, removing any nonincorporated FL-BAK from the preparation. Proteinase K digestion was performed at room temperature for the indicated durations. tBID treatment and Triton X-100 lysis were performed as described in the liposomal-assay method.

Photoaffinity Labeling and Mass Spectrometry Analysis of Membrane-Embedded FL-BAK. Site identification analysis using BID photoreactive stabilized α-helix of BCL-2 domain 1 (pSAHB-1) was performed as described for FL-BAK in solution except that (*i*) FL-BAK liposomes were used instead, and (*ii*) after UV irradiation, the mixture was incubated with 1% (wt/vol) CHAPS to solubilize the FL-BAK liposomes before the overnight dialysis step that removes unreacted pSAHB.

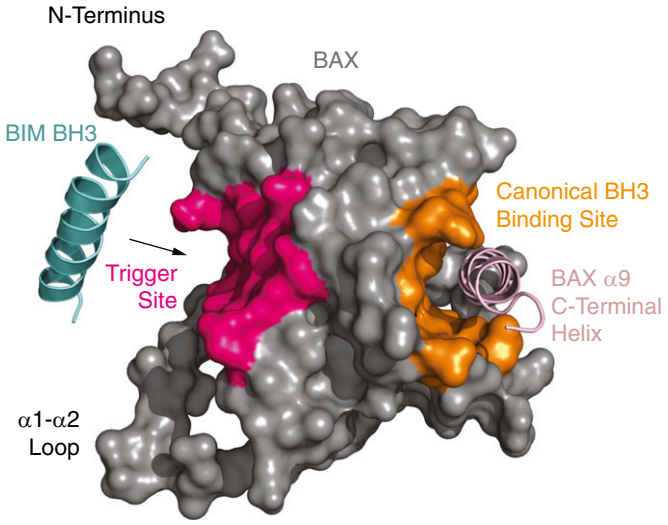

Fig. S1. A stapled BCL-2–interacting mediator of cell death (BIM) BH3 helix localized the trigger site for BH3-mediated direct BAX activation to the N-terminal face of BAX (1), as highlighted in pink. In contrast, the canonical BH3-binding pocket of BCL-2 family proteins (orange) maps to the opposite side of BAX and remains occupied by the C-terminal helix 9 (purple) when the protein is in the inactive, monomeric state.

1. Gavathiotis E, et al. (2008) BAX activation is initiated at a novel interaction site. *Nature* 455(7216):1076–1081.

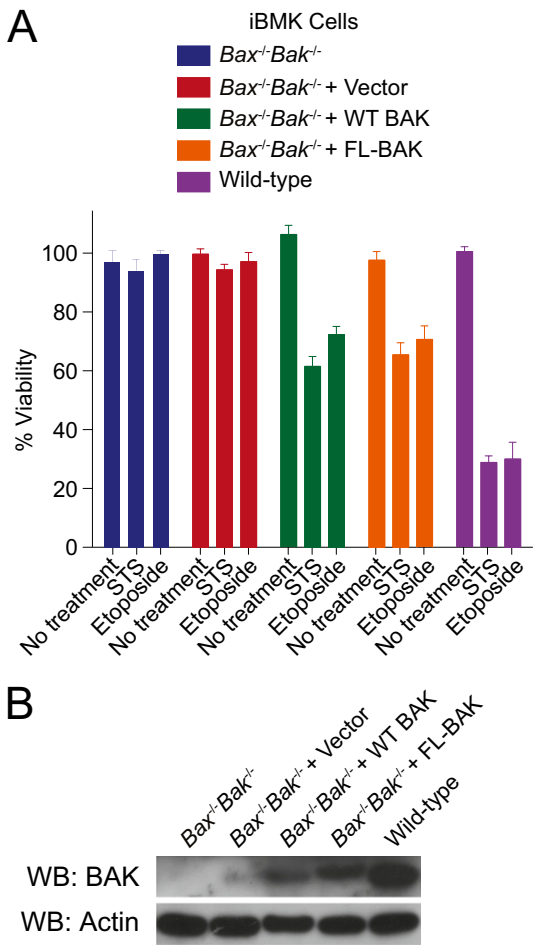

Fig. S2. Equivalent proapoptotic activity of wild-type and FL-BAK. (*A*) BAX/BAK-deficient iBMK cells reconstituted with wild-type BAK or FL-BAK were treated with etoposide (10 μM) or STS (100 nM), and cell viability was assessed by CellTiter-Glo (Promega) at 24 h. $Bax^{-/-}Bak^{-/-}$, $Bax^{-/-}Bak^{-/-}$ plus vector alone, and wild-type iBMK cells served as controls. Untreated controls also were included for all cell lines tested. Experiments were performed at least in triplicate. Data are mean ± SEM. (*B*) BAK expression levels in the indicated cell lines were determined by anti-BAK Western analysis. $Bax^{-/-}Bak^{-/-}$ and $Bax^{-/-}Bak^{-/-}$ plus vector alone iBMK cells showed no response to the etoposide or STS treatment, whereas cells with introduced wild-type and triply mutant BAK (FL-BAK), which express at similar levels, exhibited equivalent impairment of viability in response to the proapoptotic stimuli. Of note, wild-type iBMK cells demonstrated the most robust response to etoposide and STS treatment, consistent with their expression of relatively higher levels of native BAK (in addition to the presence of native BAX).

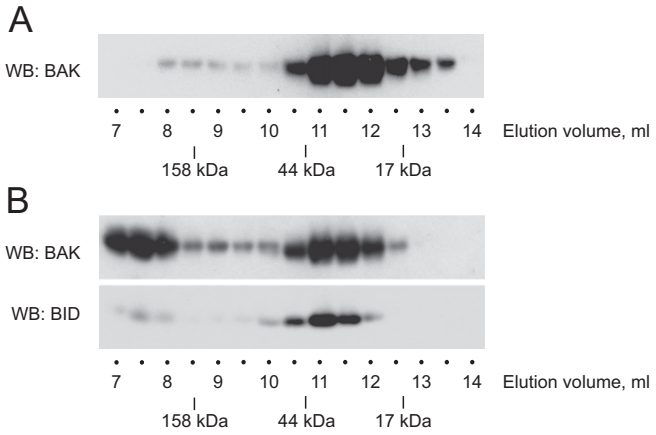

Fig. S3. SEC analysis of tBID-induced conversion of FL-BAK from monomer to oligomer. (*A*) FL-BAK (500 nM) was incubated with liposomes and analyzed by SEC after solubilization with CHAPS (0.5%). SEC fractions were collected, subjected to SDS/PAGE, and analyzed by anti-BAK Western blotting. The elution fractions for molecular weight marker proteins (17, 44, and 158 kDa) are indicated. (*B*) The experiment was repeated with the inclusion of tBID (100 nM) and analyzed as above. In the presence of tBID (100 nm), FL-BAK (500 nM) is partially converted from monomer to oligomer after a 30-min incubation. Of note, tBID remains predominantly in monomeric form, consistent with a hit-and-run mechanism for tBID-induced FL-BAK activation.

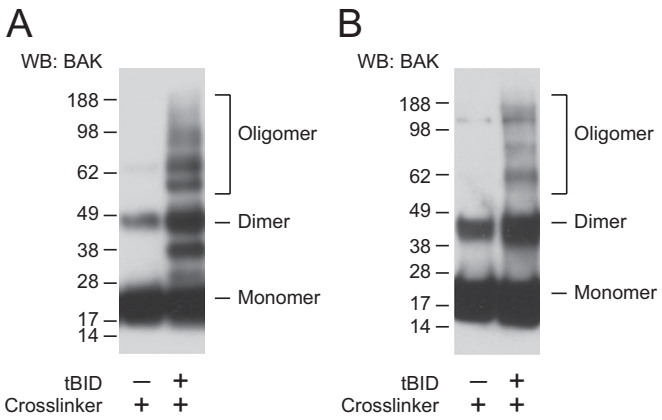

Fig. S4. tBID-induced and FL-BAK–mediated liposomal and mitochondrial release coincides with FL-BAK oligomerization. (*A*) FL-BAK (500 nM) was incubated with liposomes in the presence or absence of tBID (100 nM) for 30 min, followed by a 15-min incubation with 100 μM DSS. After quenching with Tris base, the reaction mixture was subjected to SDS/PAGE and anti-BAK Western analysis (BAK NT antibody; Millipore). (*B*) FL-BAK (500 nM) was incubated with *Bak*$^{-/-}$ mouse liver mitochondria in the presence or absence of tBID (100 nM), followed by a 15-min incubation with 100 μM BMH. After quenching with DTT, the reaction was subjected to SDS/PAGE and anti-BAK Western analysis.

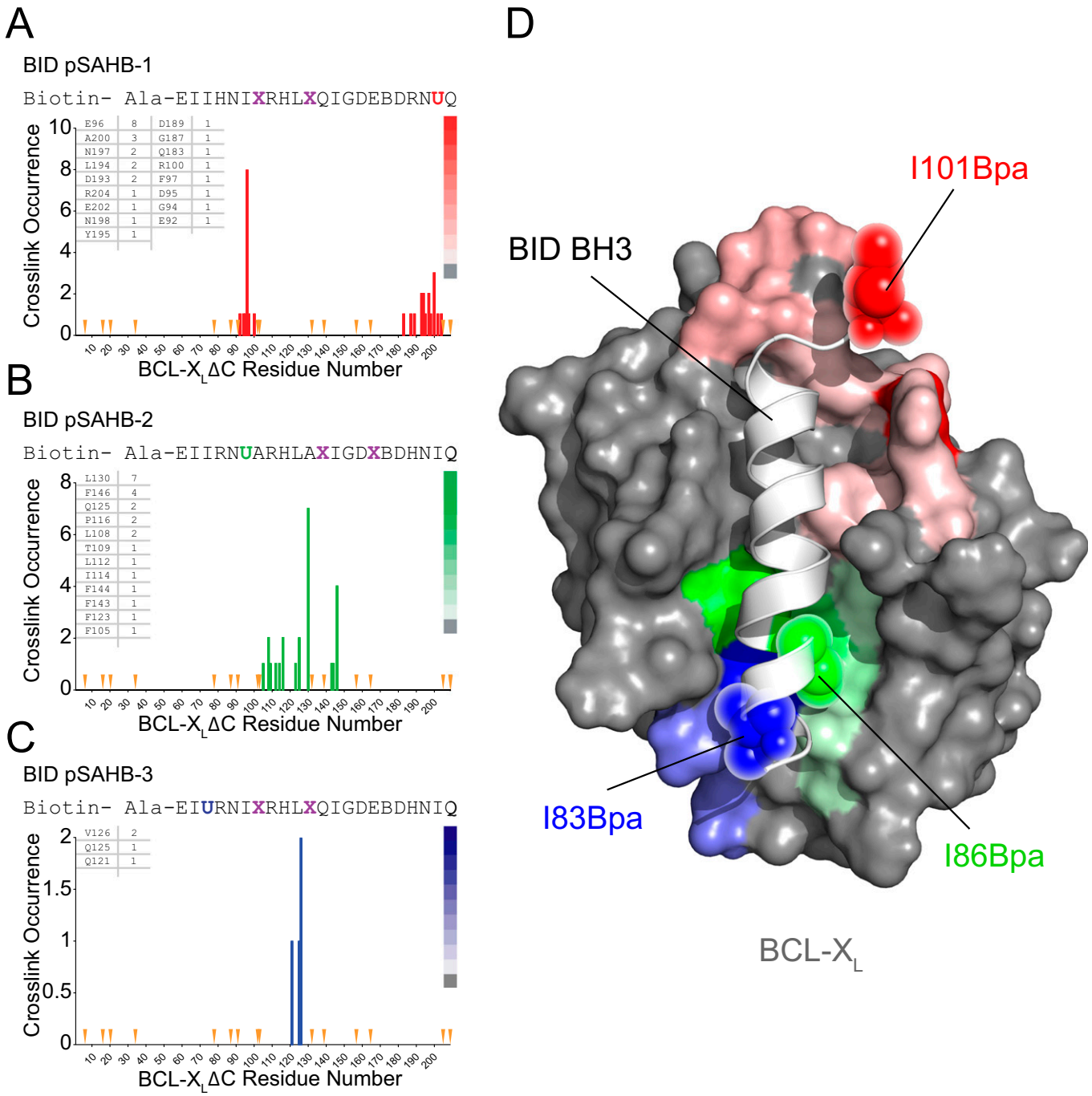

Fig. S5. Photoreactive BID SAHBs localize BH3-interaction sites with high fidelity. (*A–C*) BID pSAHBs 1–3 were incubated individually with the C-terminally deleted form of antiapoptotic BCL-X_L (BCL-X_LΔC), and the mixtures were subjected to UV irradiation, electrophoresis, excision of the crosslinked protein, trypsin proteolysis, and LC-MS/MS analysis. The plots depict the frequency of crosslinked sites identified across the BCL-X_LΔC polypeptide sequence. Orange arrowheads indicate trypsin digestion sites. (*D*) Mapping of BID pSAHB-crosslinked amino acids onto the BCL-X_LΔC structure [Protein Data Bank (PDB) ID 2BZW] highlighted the capacity of individual pSAHBs to localize sites of interaction to very circumscribed regions along the canonical BH3-binding pocket corresponding to the relative N-to-C terminal disposition of benzophenone residues within the pSAHB sequence. Docking was performed using crystallography and NMR system solve (CNS) within HADDOCK 2.0 (1), and results were displayed by PYMOL (2). The frequency of crosslinking occurrence is reflected on the BCL-X_LΔC structure by the color scale for each BID pSAHB (1, red; 2, green; 3, blue). X, stapling amino acid; B, norleucine; U, Bpa.

1. de Vries SJ, van Dijk M, Bonvin AM (2010) The HADDOCK web server for data-driven biomolecular docking. *Nat Protoc* 5(5):883–897.
2. DeLano WL (2002) *The PyMOL Molecular Graphics System* (DeLano Scientific, San Carlos, CA) www.pymol.org. Accessed February 1, 2013.

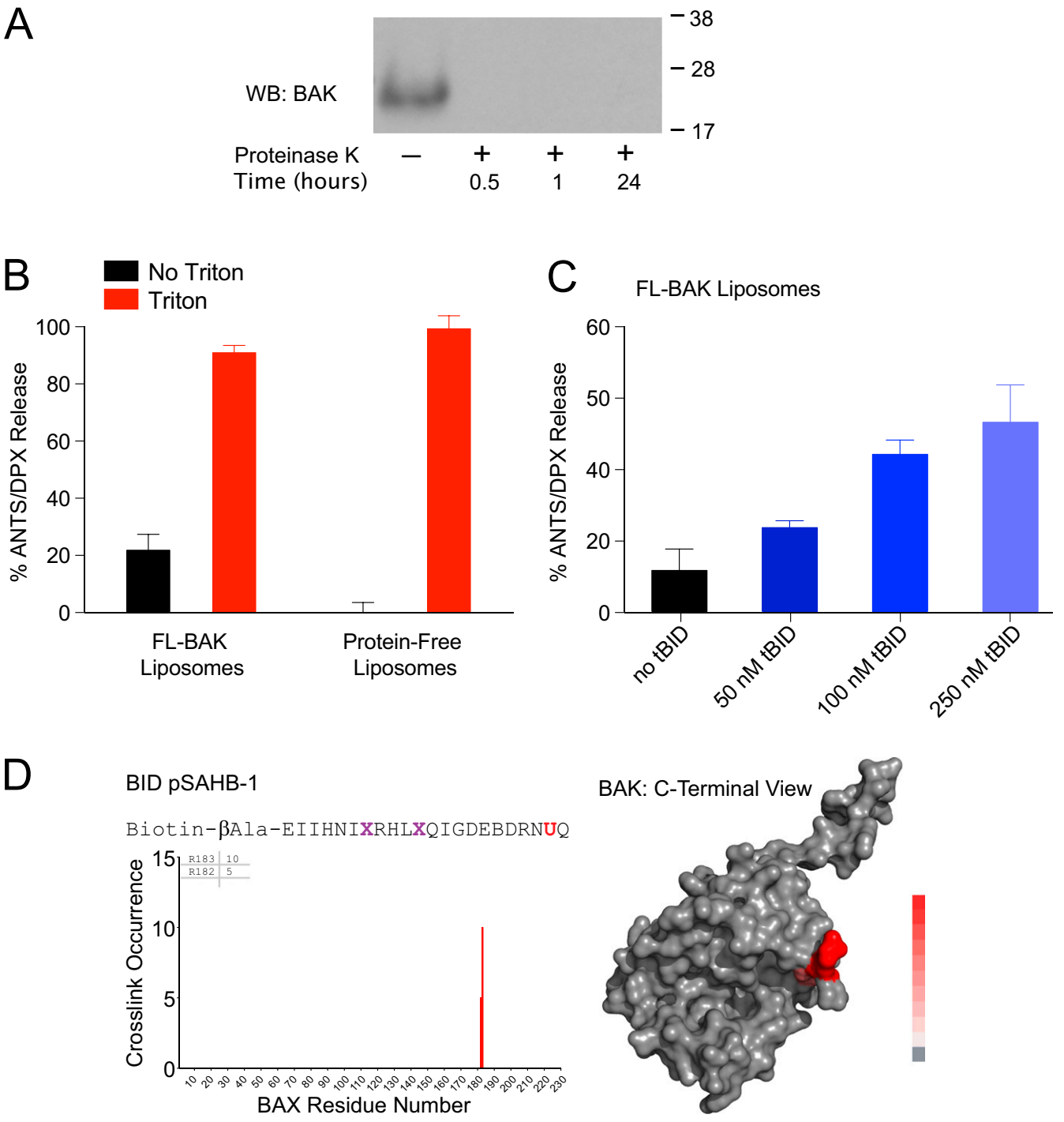

Fig. S6. BID pSAHB-1 crosslinks to discrete residues of the BH3-binding pocket of membrane-embedded FL-BAK. (*A*) Liposomes generated in the presence of FL-BAK and ANTS/DPX were purified by SEC, and the incorporation of FL-BAK was documented by anti-BAK Western analysis. Upon treatment with proteinase K, FL-BAK was proteolyzed efficiently, consistent with protein exposure at the liposomal surface. (*B*) Protein-free and FL-BAK–embedded liposomes manifested a similar degree of ANTS/DPX release upon lysis with Triton X-100, confirming that FL-BAK liposomes maintain membrane integrity and stably encapsulate ANTS/DPX. Experiments were performed at least in duplicate. Data are mean ± SEM and are representative of two independent experiments. (*C*) FL-BAK liposomes likewise maintained signaling functionality, as demonstrated by dose-responsive release of ANTS/DPX upon exposure to tBID. Experiments were performed at least in duplicate. Data are mean ± SEM and are representative of two independent experiments. (*D*) BID pSAHB-1 was incubated with FL-BAK liposomes, and the mixture was subjected to UV irradiation, solubilization, electrophoresis, excision of the crosslinked protein, trypsin proteolysis, and LC-MS/MS analysis. The plots depict the frequency of crosslinked sites identified across the FL-BAK polypeptide sequence (*Left*), with crosslinked residues mapped onto a calculated model structure of FL-BAK (based on sequence homology to BAX) and colored according to the frequency of occurrence as in Fig. S5 (*Right*). Because no crosslinks to residues of the C-terminal helix of FL-BAK were observed, α9 was removed from the structure to better visualize the crosslinked residues at the surface of the canonical BH3-binding pocket. Of note, residues of this same region were crosslinked by BID pSAHB-1 upon incubation with FL-BAK in solution (Fig. 4*B*). X, stapling amino acid; B, norleucine; U, Bpa.

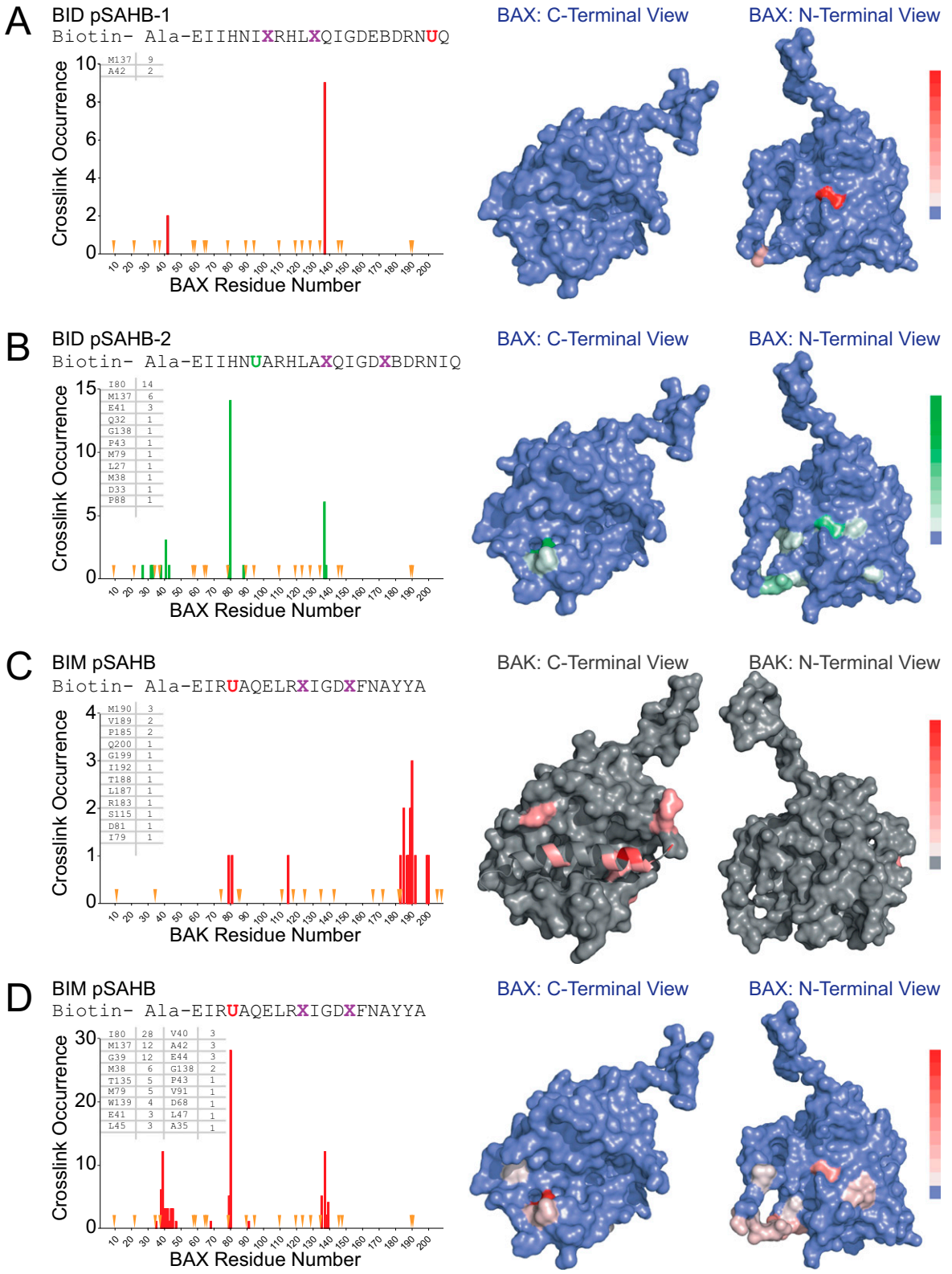

Fig. S7. BID and BCL-2–interacting mediator of cell death (BIM) pSAHBs crosslink to the α1/α6 trigger site on BAX but only to the C-terminal site on FL-BAK. (*A* and *B*) In contrast to the results obtained with FL-BAK (Fig. 4), BID pSAHBs crosslinked to a series of BAX surface residues located within the previously defined BH3 trigger site formed by the confluence of α-helices 1 and 6 at the N-terminal face of BAX. BID pSAHB-2 crosslinked to two additional amino acids of the canonical BH3-binding pocket of BAX. The plots depict the frequency of crosslinked sites identified across the BAX polypeptide sequence, with crosslinked residues mapped onto the solution structure of BAX and colored according to the frequency of occurrence for each pSAHB (1, red; 2, green). Because BID pSAHB crosslinks to BAX α9 were not evident, the C-terminal helix was removed from the BAX structure to better visualize the crosslinked residues at the surface of the canonical BH3-binding pocket. (*C* and *D*) To compare sites of interaction for another direct-activator BH3 helix, with a distinct sequence

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composition from BID BH3, a BIM pSAHB was used in crosslinking analyses with FL-BAK and BAX. BIM pSAHB crosslinked exclusively to residues at the C-terminal face of FL-BAK, including amino acids of the canonical BH3-binding pocket and α9 helix, as observed for the corresponding BID pSAHB with an N-terminally located benzophenone moiety (Fig. 4*D*). In contrast, the identical BIM pSAHB construct engaged a host of BAX surface residues at the α1/α6 trigger site on BAX in addition to select residues of the canonical groove. The plots depict the frequency of crosslinked sites identified across the FL-BAK and BAX polypeptide sequences, with crosslinked residues mapped onto the FL-BAK (gray) and BAX (blue) structures and colored according to the frequency of occurrence. Because BIM pSAHB-crosslinks to BAX α9 were not evident, the C-terminal helix was removed from the BAX structure to better visualize the crosslinked residues at the surface of the canonical BH3-binding pocket. X, stapling amino acid; B, norleucine; U, Bpa.