Supplemental Figures

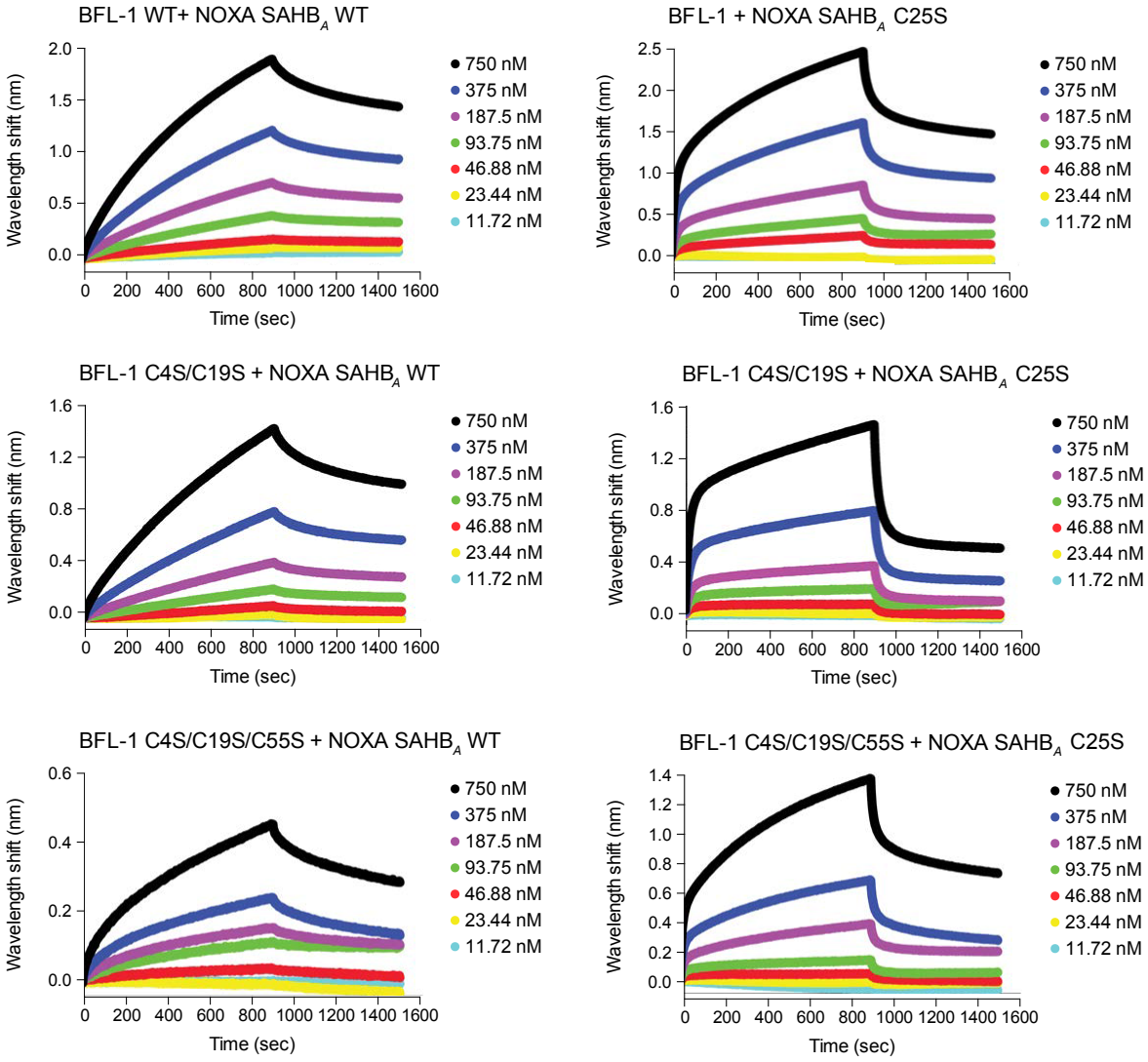

Figure S1, Related to Figure 1. BFL-1 Binding Activity of NOXA SAHBs.
The association and dissociation binding interactions between BFL-1ΔC constructs and biotin-PEG-NOXA SAHB$_A$ peptides bearing the indicated native cysteines and cysteine-to-serine mutations were measured by biolayer interferometry. Experiments were performed in technical and biological duplicate, with exemplary association and dissociation profiles shown.

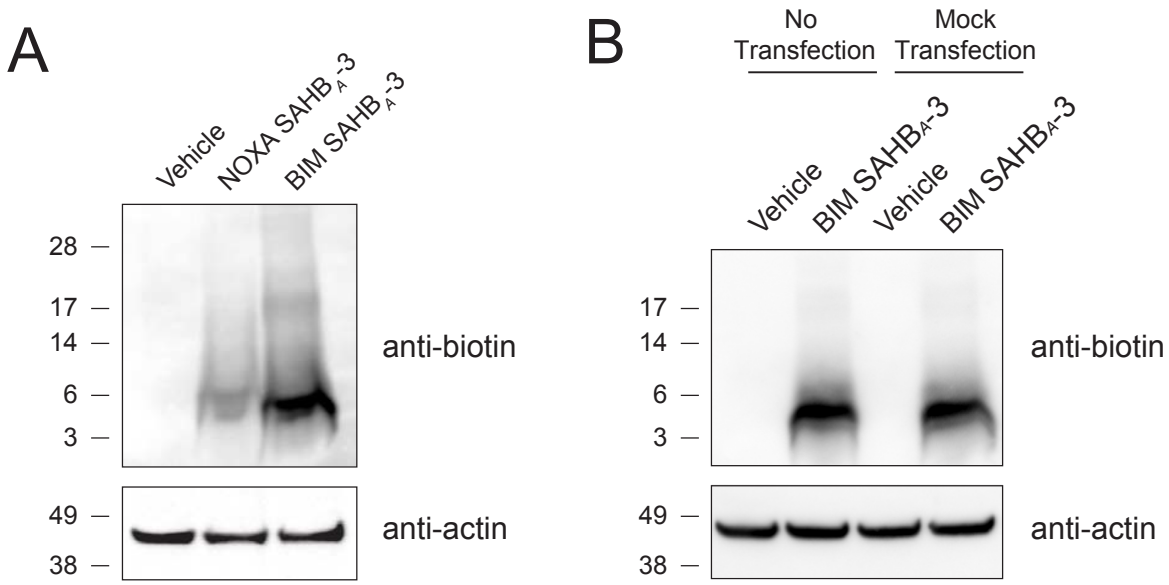

Figure S2, Related to Figure 4. Cellular Uptake of Biotinylated NOXA and BIM SAHB$_A$-3 Peptides.
(A) 293T cells were treated with biotinylated NOXA SAHB$_A$-3 or BIM SAHB$_A$-3 (20 μM) for 24 hr followed by washing, trypsinizing, rewashing and lysing the cells. Comparative stapled peptide uptake was assessed by electrophoresis of the cellular lysates and biotin western analysis.
(B) 293T cells were either mock transfected or not, and then 24 hr later treated with biotinylated BIM SAHB$_A$-3 (20 μM) for an additional 4 hr, and then processed as above for comparative biotin blotting of cellular lysates.

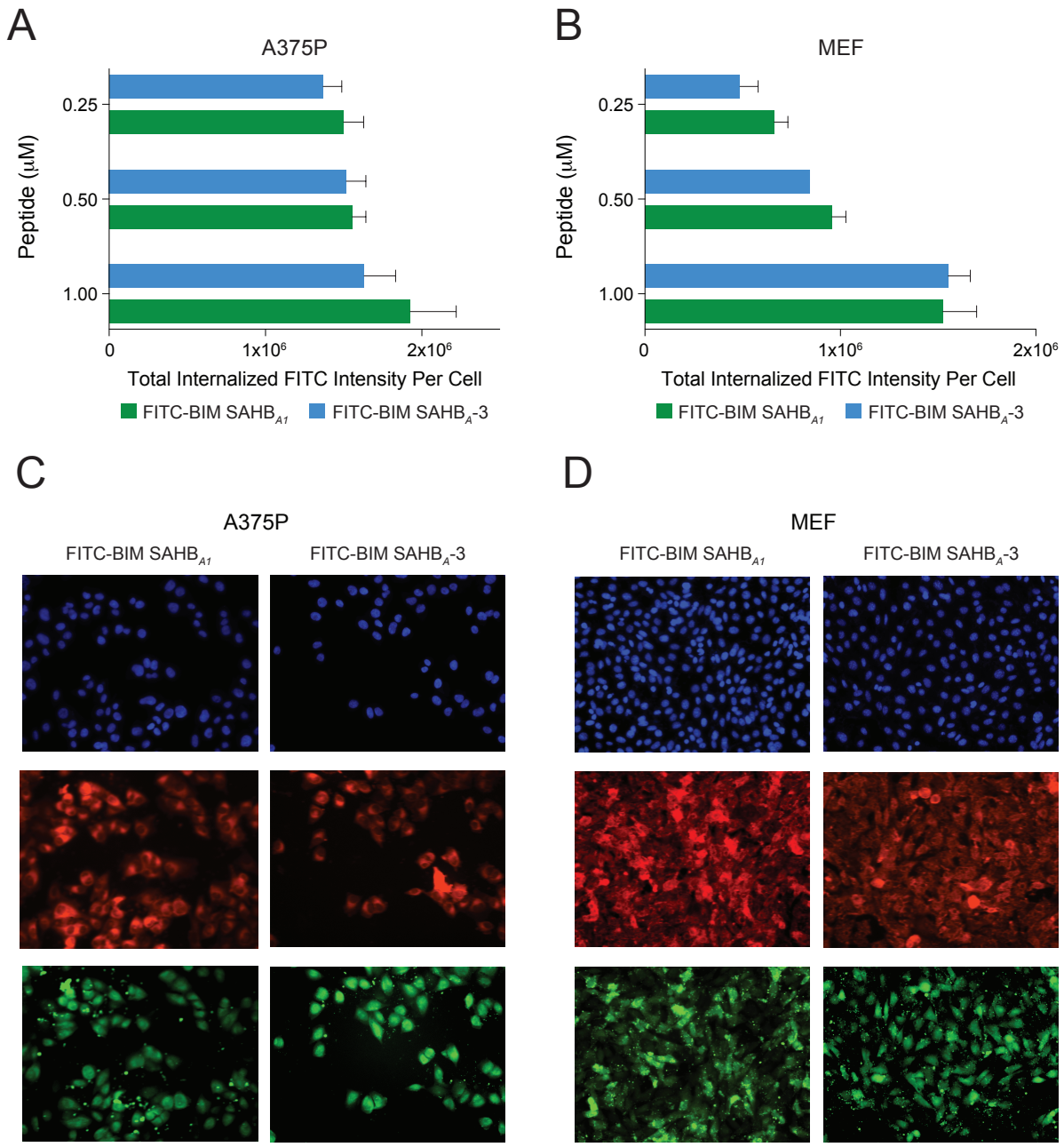

Figure S3, Related to Figure 5. Cellular Uptake of BIM SAHBs by A375P and MEF cells.
(A-B) TIFI values for A375P ($1x10^4$/well) and MEF ($1.5x10^4$/well) cells treated with the indicated doses of BIM SAHB peptides and measured by IXM (20x) after 4 hr. Data are mean ± SD for experiments performed in triplicate wells with 5 image acquisitions per well. Two biological replicates (independent cell cultures and platings) were performed with similar results.
(C-D) Representative IXM images of A375P (C) and MEF (D) cells treated with the indicated BIM SAHBs at 1 μM dosing.

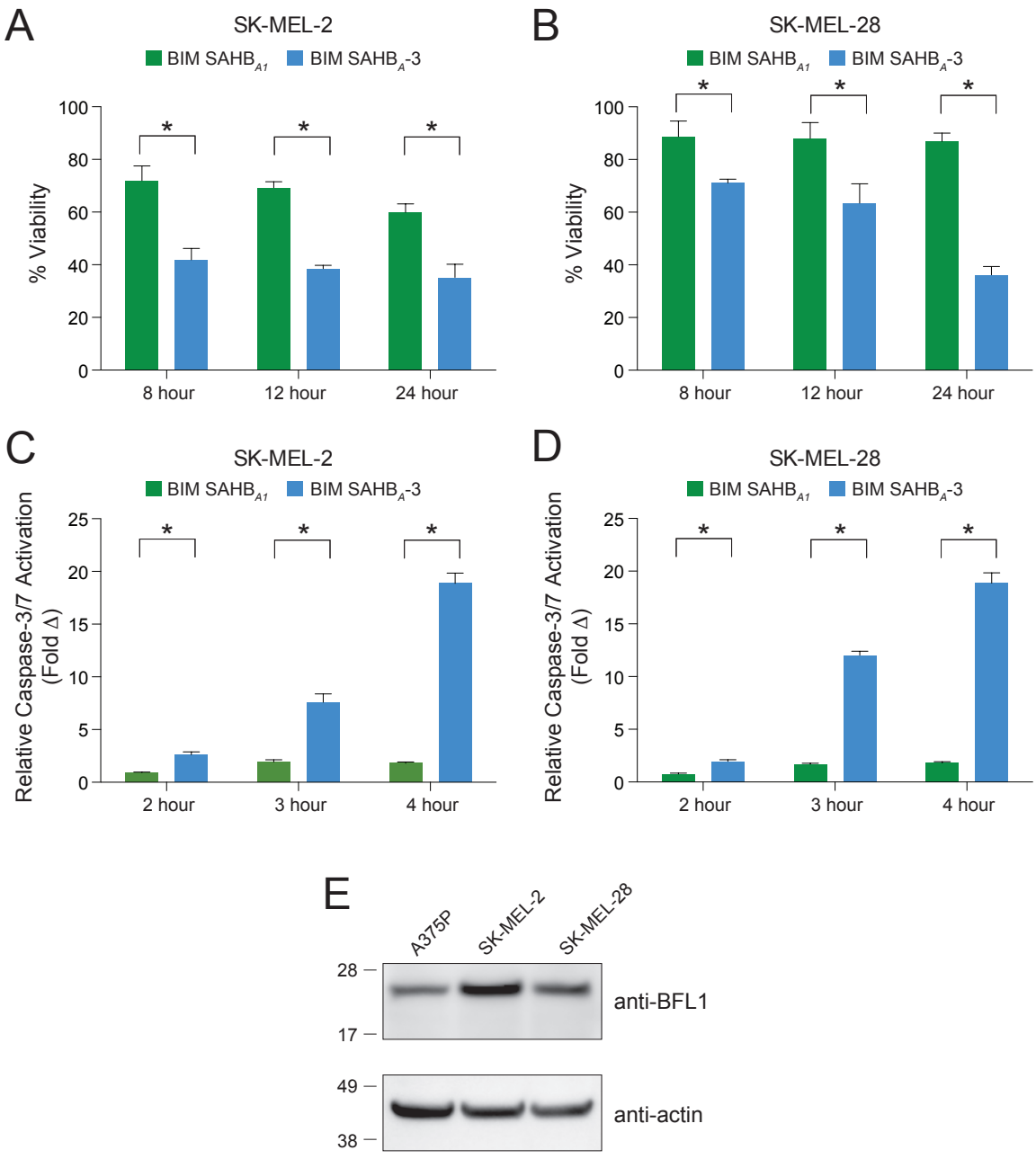

Figure S4, Related to Figure 5. Cell Viability and Caspase-3/7 Activation of BFL-1-Expressing Melanoma Cells Treated with BIM SAHBs.

(A,B) Cell viability of SK-MEL-2 and SK-MEL-28 cells treated with BIM SAHB$_{A1}$ or BIM SAHB$_A$-3 (40 μM), as measured by CellTiter-Glo assay at the indicated time points. Data are mean ± SD for experiments performed in technical sextuplicate.

(C, D) Caspase-3/7 activation in SK-MEL-2 and SK-MEL-28 cells treated with BIM SAHB$_{A1}$ or BIM SAHB$_A$-3 (40 μM), as monitored by CaspaseGlo at the indicated time points. Data are mean ± SD for experiments performed in technical sextuplicate.

(E) BFL-1 western analysis of electrophoresed lysates from A375P, SK-MEL-2, and SK-MEL-28 cells.

*, $p < 0.001$ by two-tailed Student's *t* test.

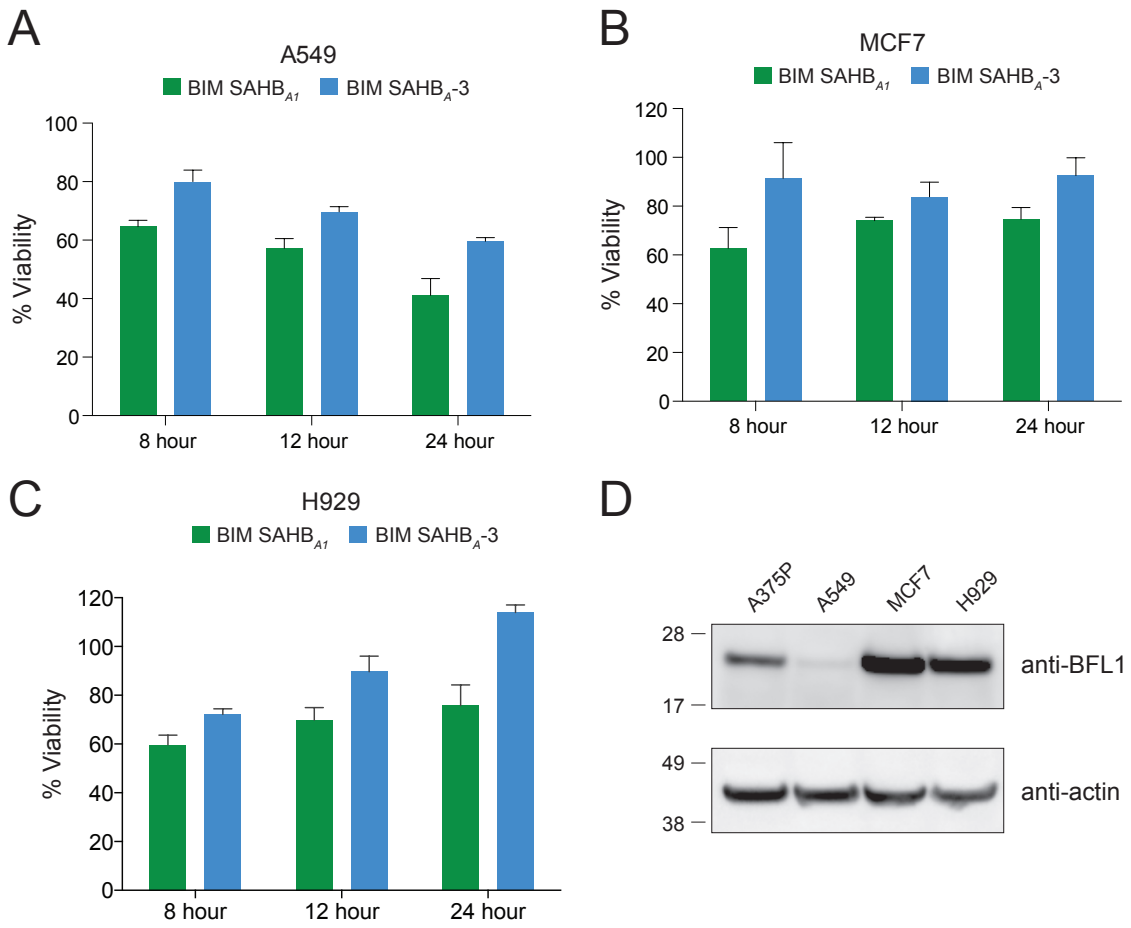

Figure S5, Related to Figure 5. Cell Viability of BIM SAHB-Treated Non-Melanoma Cancer Cells that Either Lack BFL-1 Expression or are Driven by Alternate Oncogenic Mechanisms.

(A-C) Cell viability of A549 (A), MCF7 (B), and H929 (C) cells treated with BIM SAHB$_{A1}$ or BIM SAHB$_A$-3 (40 μM for A549 and MCF7, 10 μM for H929), as measured by CellTiter-Glo assay at the indicated time points. Data are mean ± SD for experiments performed in technical sextuplet for A549 and MCF7 cells, and technical triplicate for H929 cells.

(D) BFL-1 western analysis of electrophoresed lysates from A375P, A549, MCF7, and H929 cells.

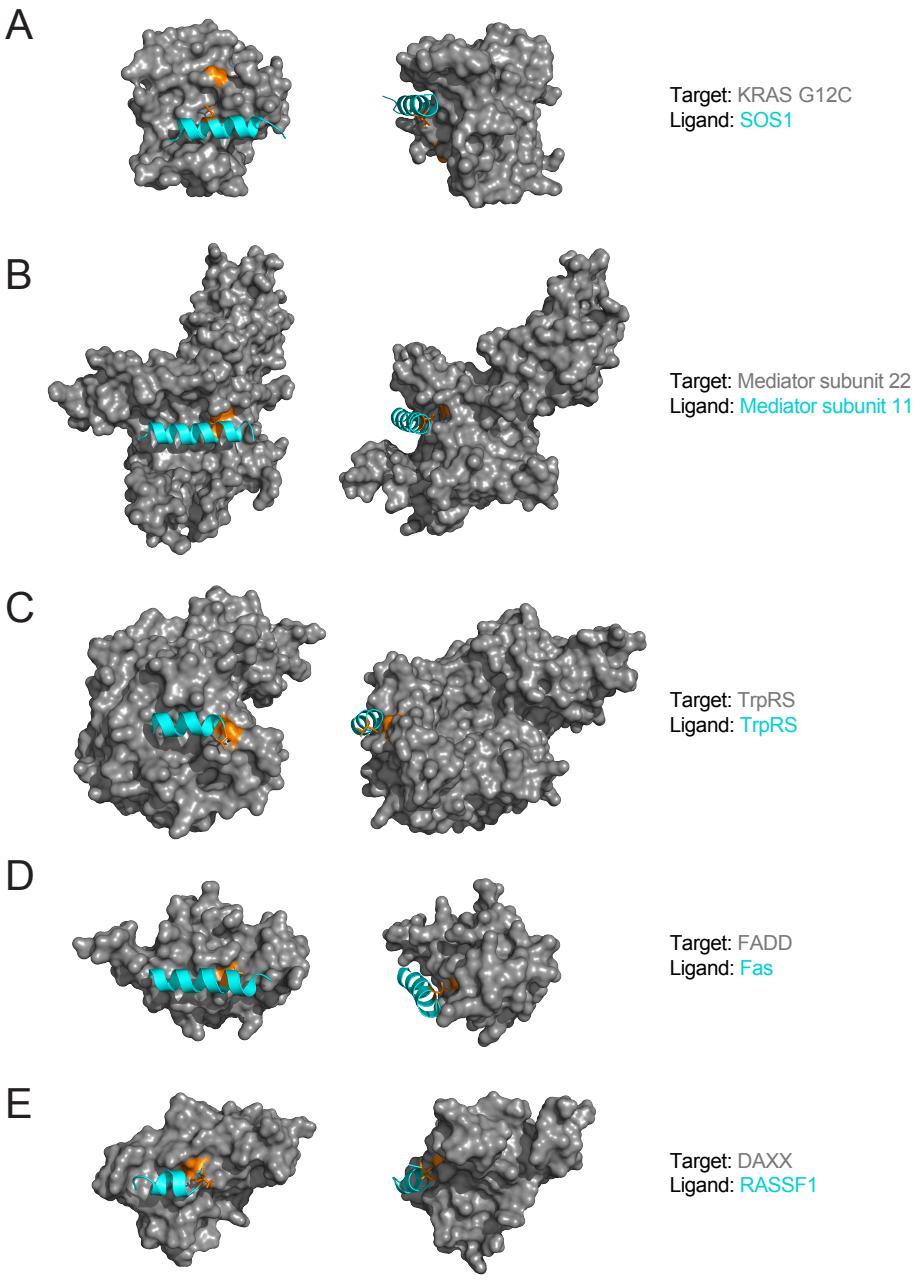

Figure S6, Related to Figures 1-6 and Discussion. Exemplary Helix-in-Groove Interactions Bearing Native Cysteines for Covalent Stapled Peptide Inhibitor Targeting.
(A-E) A diversity of regulatory binding surfaces that involve α-helical ligands inserting into surface grooves contain targetable cysteines, as exemplified by the (A) SOS1/KRAS G12C (PDB: 1NVU), (B) Mediator of RNA Polymerase II transcription subunit 11/Mediator of RNA polymerase II transcription subunit 22 (PDB: 4H62), (C) Tryptophanyl-tRNA synthetase homodimer (PDB: 1ULH), (D) FAS/FADD (PDB: 3EZQ), and (E) RASSF1C/ DAXX (PDB: 2KZU) interactions (α-helical ligand, cyan; cysteine-proximal residue on α-helix, orange sticks; protein target, grey; cysteine on protein target, orange surface).

Peptide	N-terminus	Sequence	C-terminus	MW	(M+3)/3	Figures
NOXA SAHB$_A$ WT (aa 19-43)	FITC-βAla-	AELEVECATQLR**X**FGD**X**LNFRQKLL		3403.1	1135.3	1
NOXA SAHB$_A$ WT	Btn-PEG-βAla-	AELEVECATQLR**X**FGD**X**LNFRQKLL		3556.0	1186.3	1, S1
NOXA SAHB$_A$ C25S	FITC-βAla-	AELEVESATQLR**X**FGD**X**LNFRQKLL		3387.0	1130.1	1
NOXA SAHB$_A$ C25S	Btn-PEG-βAla-	AELEVESATQLR**X**FGD**X**LNFRQKLL		3539.9	1181.1	1, S1
NOXA SAHB$_A$-1	1	EVESATQLR**X**FGD**X**LNFRQKLLK		2892.6	965.3	2
NOXA SAHB$_A$-2	2	EVESATQLR**X**FGD**X**LNFRQKLLK		2906.6	970.0	2
NOXA SAHB$_A$-3	3	EVESATQLR**X**FGD**X**LNFRQKLL		2778.4	927.2	2, 3, 4
NOXA SAHB$_A$-3	3	EVESATQLR**X**FGD**X**LNFRQKLL	Lys(biotin)	3132.6	1045.2	2, 3, 4, S2
NOXA SAHB$_A$-4	4	EVESATQLR**X**FGD**X**LNFRQKLLK		2906.6	969.9	2
NOXA SAHB$_A$-5	5	EVESATQLR**X**FGD**X**LNFRQKLLK		2892.6	965.2	2
NOXA SAHB$_A$-6	6	EVESATQLR**X**FGD**X**LNFRQKLLK		2892.6	965.3	2
NOXA SAHB$_A$-7	7	EVESATQLR**X**FGD**X**LNFRQKLLK		2852.5	951.8	2
NOXA SAHB$_A$-8	8	EVESATQLR**X**FGD**X**LNFRQKLLK		2795.4	932.9	2
NOXA SAHB$_A$ (aa 22-43)	Ac	EVESATQLR**X**FGD**X**LNFRQKLL		2655.3	886.0	4
NOXA SAHB$_A$	Ac	EVESATQLR**X**FGD**X**LNFRQKLL	Lys(biotin)	3009.4	1004.2	4
BIM SAHB$_A$-1	1	IAQELR**X**IGD**X**FNAYYARK		2428.0	810.5	2
BIM SAHB$_A$-2	2	IAQELR**X**IGD**X**FNAYYARK		2442.0	815.0	2
BIM SAHB$_A$-3	3	IAQELR**X**IGD**X**FNAYYARR		2469.3	824.4	2-5, S4, S5
BIM SAHB$_A$-3	3	IAQELR**X**IGD**X**FNAYYARR	Lys(biotin)	2823.5	942.3	2-4, 6, S2
BIM SAHB$_A$-4	4	IAQELR**X**IGD**X**FNAYYARK		2442.0	815.0	2
BIM SAHB$_A$-5	5	IAQELR**X**IGD**X**FNAYYARK		2428.0	810.3	2
BIM SAHB$_A$-6	6	IAQELR**X**IGD**X**FNAYYARK		2428.0	810.3	2
BIM SAHB$_A$-7	7	IAQELR**X**IGD**X**FNAYYARK		2388.0	797.1	2
BIM SAHB$_A$-8	8	IAQELR**X**IGD**X**FNAYYARK		2330.9	778.0	2
BIM SAHB$_A$ (aa 148-166)	Ac	IAQELR**X**IGD**X**FNAYYARR		2346.9	783.3	4
BIM SAHB$_A$	Ac	IAQELR**X**IGD**X**FNAYYARR	Lys(biotin)	2701.1	901.4	4, 6
BIM SAHB$_{A1}$ (aa 146-166)	FITC-βAla-	IWIAQELR**X**IGD**X**FNAYYARR		3064.3	1022.4	S3
BIM SAHB$_A$-3	FITC-Cyste-3-	IAQELR**X**IGD**X**FNAYYARR		2935.5	979.8	S3, 6
BIM SAHB$_{A1}$ (aa 146-166)	Ac	IWIAQELR**X**IGD**X**FNAYYARR		2646.3	883.1	5, S4, S5

1: (S)-1-acryloylpyrrolidine-3-carboxamide; **2**: 1-acryloylpiperidine-4-carboxamide; **3**. (R)-1 acryloylpiperidine-3-carboxamide; **4**: (S)-1-acryloylpiperidine-3- carboxamide;
5: (S)-1-acryloylpyrrolidine-2- carboxamide; **6**: (R)-1-acryloylpyrrolidine-2- carboxamide; **7**: (E)-4-(dimethylamino)but-2-enamide; **8**: acrylamide; FITC-Cyste: FITC-cysteamine.

Table S1, Related to Figures 1-6. Stapled Peptide Compositions.

Supplemental Procedures

FITC Derivatization of Acrylamide-Bearing Stapled Peptides
Cystamine dihydrochloride (1 eq) was dissolved in 10 mL DMSO, accompanied by 270 μL DIEA (3 eq), and then 400 mg (2 eq) of FITC was added. The reaction was monitored by LCMS and, after overnight stirring and reaction completion, 2 eq TCEP in 1 mL of water was added. The reduced product was purified on an Isco CombiFlash purification system equipped with a 40 g C18 reversed phase column using a water-acetonitrile gradient. The fractions containing product were lyophilized to afford 385 mg of FITC-labeled cysteamine. The subsequent conjugation reaction with acrylamide-containing stapled peptide was found to be pH dependent as expected, with no reaction occurring at pH 6 and pH 8, whereas the reaction in pH 10 borate buffer went to completion after overnight incubation in a 1:1:3 solution of 1 mM DMSO peptide stock, 5 mM DMSO stock of FITC-cysteamine, and 0.05 M borate buffer. The FITC-labeled peptide product, FITC-BIM SAHB$_A$-3, was then purified by HPLC.

Recombinant Protein Expression and Purification
cDNA encoding BFL-1ΔC (aa 1-153) was cloned into the pET19b expression vector (Novagen) followed by DNA sequencing to verify the construct. Constructs bearing cysteine to serine mutations were created by PCR-based site-directed mutagenesis (QuikChange Mutagenesis Kit, Stratagene). Transformed *Escherichia coli* BL21(DE3) LOBSTR(Andersen et al., 2013) (#EC1001, Kerafast) were cultured in ampicillin-containing Luria broth (LB) and protein expression induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 16°C. Bacterial pellets were resuspended in 20 mM Tris pH 7.5, 250 mM NaCl, and two complete protease inhibitor tablets (Roche), and then microfluidized (M-110L, Microfluidics) and centrifuged at 45,000 x g for 1 h. The supernatant was passed over a Ni-NTA (Qiagen) column equilibrated with 50 mM Tris pH 7.5, 250 mM NaCl. The column was sequentially washed with 25 mL of equilibration buffer containing 5 mM, 10 mM and 20 mM imidazole, and then His-BFL-1ΔC was eluted in equilibration buffer containing 300 mM imidazole. The fraction containing His-BFL-1ΔC was dialyzed against 50 mM Tris pH 8, 100 mM NaCl at 4°C and then concentrated and loaded onto a Superdex S-75 (GE Healthcare) gel filtration column equilibrated with 50 mM Tris pH 8.0, 100 mM NaCl. The column was washed with 30 mL equilibration buffer and fractions containing His-BFL-1ΔC were pooled and analyzed both by SDS-PAGE electrophoresis/Coomassie stain and anti-BFL-1 (Abcam, #125259) and anti-His (Abcam, #18184) western blotting. Purified protein was then concentrated, flash frozen using liquid nitrogen, and stored at -80°C until use.

MCL-1ΔNΔC (aa 170-327) and BCL-X_LΔC (aa 1-212) constructs were cloned into pGEX-4T-1 (GE Healthcare) followed by DNA sequencing to verify the constructs. Transformed *Escherichia coli* BL21(DE3) (Sigma-Aldrich) were cultured in ampicillin-containing LB, and protein expression induced with 0.5 mM IPTG and grown for 4 hr at 37°C. Bacterial pellets were resuspended in phosphate-buffered saline (PBS), 0.1% Triton X-100, and complete protease inhibitor tablet (Roche), and then microfluidized and centrifuged at 45,000 x g for 1 hr. Supernatants were passed over a glutathione sepharose (GE Healthcare) column equilibrated with PBS containing 0.1% Triton X-100. The column was sequentially washed with 25 mL of PBS containing 0.1% Triton X-100 and PBS, and then GST cleaved on-resin with thrombin (Sigma) overnight at 25°C. The GST-free protein was eluted with PBS, concentrated, and loaded onto a Superdex S-75 (GE Healthcare) gel filtration column equilibrated with 50 mM Tris pH 7.4, 150 mM NaCl. The column was washed with 30 mL equilibration buffer and fractions containing MCL-1 ΔNΔC or BCL-X_LΔC were pooled and analyzed by SDS-PAGE electrophoresis and Coomassie staining. For GST-MCL-1ΔNΔC purification, protein was eluted from the column using 50 mM Tris, pH 8.0, 10 mM GSH, concentrated, and purified by size exclusion chromatography using a Superdex S-75 gel filtration column, as described above. Purified proteins were concentrated, flash frozen using liquid nitrogen, and stored at -80°C.

Biolayer Interferometry
Binding analyses of NOXA peptide interactions with BFL-1ΔC were performed on an Octet RED384 system (Fortebio, Menlo Park, CA) at 30°C. Super streptavidin (SSA) tips were prewetted in 1x kinetics buffer (PBS, pH 7.4, 0.01% BSA, 0.002% Tween-20) and then conjugated to NOXA SAHBs bearing an N-terminal biotin-PEG linker (10 μg/mL). Excess streptavidin was quenched by incubation with 2 μg/mL biocytin. The tips were then washed with kinetics buffer and soaked in a serial dilution of BFL-1ΔC for 10 min to measure association rate, followed by a 15 min incubation in kinetics buffer to measure dissociation rate. Dissociation constants were calculated using Octet Data Analysis version 9.0.

Liposomal Release Assay

Large unilamellar vesicles (LUVs) with encapsulated ANTS and DPX were generated and purified as described(Leshchiner et al., 2013; Lovell et al., 2008). The indicated combinations of BAX (400 nM), tBID (40 nM), and BFL-1ΔC or SAHB$_A$-3/BFL-1ΔC conjugates (1.5 μM), were added to liposomes (5 μL) in 384 well plates (final volume, 30 μL), and released fluorophore was measured over 120 min using an M1000 Infinite plate reader (Tecan) with excitation and emission wavelengths of 355 nm and 520 nm, respectively. SAHB$_A$-3/BFL-1ΔC conjugates were prepared by treating BFL-1ΔC (10 μM) with DTT (20 mM) for 30 min at 4°C, followed by sequential incubation with NOXA SAHB$_A$-3 or BIM SAHB$_A$-3 peptides at peptide:protein molar ratios of 1.2x, 0.75x, and 0.5x for 1 hr each at 4°C. Conjugation efficiency was confirmed by 12% Bis-Tris gel electrophoresis and Coomassie staining. The protein conjugate was then concentrated to 75 μM, loaded onto a Superdex S-75 (GE Healthcare) gel filtration column equilibrated with 20 mM HEPES pH 7.5, 300 mM NaCl, 1 mM DTT, washed with 30 mL equilibration buffer, and fractions collected, analyzed by SDS-PAGE electrophoresis, and used fresh in liposomal assays. Percent ANTS/DPX release was calculated as [(F-F0)/(F100-F0)] x 100, where F0 is baseline fluorescence at time 0, F is the fluorescence recorded for each time point, and F100 is the maximum amount of ANTS/DPX release based on liposomal treatment with 1% Triton X-100.

BFL-1 Targeting in Lysates and Cells

293T cells were maintained in DMEM containing 10% FBS and penicillin/streptomycin, and transfections performed with 2 μg pCMV plasmid containing HA-BFL-1ΔC C4S/C19S using X-tremeGENE 9 (Roche). For lysate experiments, cells were trypsinized 24 hr post-transfection, washed with PBS, and lysed by incubation with 1% CHAPS lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 100 mM DTT). Protein concentration of the soluble fraction was measured using a BCA kit according to manufacturer's instructions (Thermo Scientific). Biotinylated NOXA SAHB$_A$-3 or BIM SAHB$_A$-3 (10 μM) was added to 100 μg of lysate and incubated at RT for 2 hr. Samples were then boiled in LDS buffer and subjected to western analysis using 1:1000 dilutions of HA (Sigma-Aldrich, #12CA5) and biotin (Abcam, #53494) antibodies. To evaluate the capacity of biotinylated SAHBs to compete with tBID for interactions with BFL-1 and MCL-1, 293T cells were transfected with either HA-BFL-1ΔC C4S/C19S or FLAG-MCL-1 in the p3XFLAG-CMV-10 vector (Sigma) as above. After 24 hr, cells were trypsinized, washed with PBS, lysed in 1% CHAPS buffer, and the supernatant collected for protein concentration determination by BCA kit. Lysate samples (0.5 mg) were incubated with 0.25 μM recombinant tBID (R&D Systems) and 5 μM biotinylated BIM SAHB$_A$ or BIM SAHB$_A$-3 for 6 h at RT. The mixtures were then subjected to HA or FLAG (Sigma-Aldrich, F7425) immunoprecipitation, followed by western analysis using 1:1000 dilutions of HA, FLAG, and BID (Santa Cruz sc-11423) antibodies. For HA-immunoprecipitation from 293T cells treated with biotinylated peptides, cells were transfected with HA-BFL-1ΔC C4S/C19S as above and, after 24 hr, incubated with 20 μM biotinylated BIM SAHB$_A$ or BIM SAHB$_A$-3 in DMEM containing 5% FBS for 6 hr. Cells were harvested and lysed as above, and incubated overnight with anti-HA agarose beads (Pierce). The beads were washed 3 times with lysis buffer, eluted by boiling in LDS buffer, and subjected to western analysis with HA and biotin antibodies. For 293T treatment with non-biotinylated SAHBs, cells were transfected with HA-BFL-1ΔC C4S/C19S as above, incubated with 20 μM BIM SAHB$_A$ or BIM SAHB$_A$-3 in DMEM containing 5% FBS, and lysates harvested as above at the indicated time points for western analysis using the HA and actin antibodies. For A375P melanoma studies, cells were maintained in DMEM containing 10% FBS and penicillin/streptomycin, and biotinylated NOXA SAHB$_A$-3 or BIM SAHB$_A$-3 (30 μM) was added to 1 mg of lysate, followed by overnight incubation in CHAPS lysis buffer at 4°C. Biotin capture was accomplished by incubating the mixture with high-capacity SA agarose (Thermo Scientific) for 2 hr at 4°C, followed by centrifugation and washing the pelleted beads with 3 x 1 mL lysis buffer. Bead-bound proteins were eluted by boiling in 10% SDS containing 10 mg/mL biotin for 10 min and then subjected to electrophoresis and western blotting using BFL-1 (Abcam, #125259) and MCL-1 (Rockland, #600-401-394S) antibodies.

Celullar Uptake of Stapled Peptides

To evaluate cellular uptake of biotinylated SAHBs by biotin western analysis of electrophoresed lysates from treated cells, 293T cells were plated in 6-well Corning plates (2 x 10^5 cells/well) in DMEM containing 10% FBS and penicillin/streptomycin. After 24 h, biotinylated NOXA SAHB$_A$-3 or BIM SAHB$_A$-3 peptides (20 μM) were added to the cells in DMEM containing 5% FBS for an additional 24 h incubation. The cells were then trypsinized to remove any surface-bound peptide, washed with PBS, lysed as above in 1% CHAPS lysis buffer, and the supernatant collected for protein concentration determination by BCA kit according to manufacturer's instructions (Thermo Scientific). Cellular lysate samples (50 μg) were boiled in LDS buffer and subjected to western analysis

using a 1:1000 dilution of anti-biotin (Abcam, #53494) and 1:2000 dilution of anti-actin (Sigma-Aldrich, # A1978) antibodies. To evaluate the potential effect of transfection conditions on stapled peptide uptake, 293T cells were plated in 6-well Corning plates (2 x 10^5 cells/well) and cultured as above. After 24 h, a mock transfection was performed with X-tremeGENE 9 (Roche) and no plasmid alongside control cells that were not transfected. After an addition 24 hour incubation, 20 μM biotinylated BIM $SAHB_A$-3 peptide was added to the cells in DMEM containing 5% FBS and incubated for 4 h. Cells were then washed, trypsinized, and lysed as above, and lysates subjected to biotin and actin western analyses. For cellular uptake analysis by ImageXpress high-content epifluorescence microscopy, the indicated cell lines were plated in black, clear bottom 96-well plates overnight at a density of 1.5×10^4 cells per well for MEFs or 1×10^4 cells per well for A375P cells in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% glutamine. The following day, cells were treated with the FITC-labeled peptides or the equivalent amount of vehicle (0.1% DMSO) for 4 h in DMEM supplemented with 5% FBS, and then stained with Hoechst 33342 and CellMask Deep Red (CMDR, Invitrogen) for 10 min. The media was then aspirated and cells fixed with 4% (wt/vol) paraformaldehyde for 10 min, followed by washing three times with PBS and an imaging by ImageXpress Microscopy (Molecular Devices). Data were collected for five sites per well at 20x magnification, with each treatment performed in triplicate, and then analyzed and quantified using MetaXpress software. The CMDR stain was used to visualize the boundaries of the cell and to create a mask for measuring FITC-peptide inside the cell, thereby excluding fluorescent debris from the analysis. A custom module in MetaXpress was applied to incrementally recede the CMDR image mask from the cellular border, further restricting the analyzed FITC signal to internalized peptide. The measurement of Total Internalized Fluorescence Intensity (TIFI) represents the level of absolute fluorescence detected per cell, per peptide construct. Maximum and minimum thresholding was utilized to exclude FITC and Cy5 outliers that were much larger and brighter than average, and total intensity and average intensity per cell thresholds were set such that vehicle-treated cells scored negative by the analysis.

Supplemental References

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