Supplementary Information

Biophysical Determinants for Cellular Uptake of Hydrocarbon-Stapled Peptide Helices

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Supplementary Results

Supplementary Tables

		Hydrophobicity	Retention Time	% Alpha-Helicity	Net Charge	pI
BIM BH3	IWIAQELRRIGDEFNAYYARR	0.337	9.2	29	1	9.14
BIM SAHB1	XWIAXELRRIGDEFNAYYARR	0.424	9.5	50	1	9.14
BIM SAHB2	IXIAQXLRRIGDEFNAYYARR	0.422	9.9	32	2	9.78
BIM SAHB3	IWXAQEXRRIGDEFNAYYARR	0.332	9.2	94	1	9.14
BIM SAHB4	IWIXQELXRIGDEFNAYYARR	0.532	11.3	86	0	8.73
BIM SAHB5	IWIAXELRXIGDEFNAYYARR	0.558	9.9	48	0	8.73
BIM SAHB6	IWIAQXLRRXGDEFNAYYARR	0.444	9.8	88	2	9.78
BIM SAHB7	IWIAQEXRRIXDEFNAYYARR	0.418	10.1	43	1	9.14
BIM SAHB8	IWIAQELXRIGXEFNAYYARR	0.584	10.9	60	1	9.41
BIM SAHB9*	IWIAQELRXIGDXFNAYYARR	0.578	10.1	63	1	9.39
BIM SAHB10	IWIAQELRRXGDEXNAYYARR	0.328	9.3	77	1	9.14
BIM SAHB11	IWIAQELRRIXDEFXAYYARR	0.528	10.7	100	1	9.14
BIM SAHB12	IWIAQELRRIGXEFNXYYARR	0.521	8.9	48	2	9.80
BIM SAHB13	IWIAQELRRIGDXFNAXYARR	0.484	9.8	84	2	9.73
BIM SAHB14	IWIAQELRRIGDEXNAYXARR	0.368	9.6	100	1	9.03
BIM SAHB15	IWIAQELRRIGDEFXAYYXRR	0.513	10.2	36	1	9.14
BIM SAHB16	IWIAQELRRIGDEFNXYYAXR	0.532	10.1	40	0	8.73
BIM SAHB17	IWIAQELRRIGDEFNAXYARX	0.501	10.5	88	0	8.53

*BIM $SAHB_{A1}$

Supplementary Table 1. Sequence composition and biophysical parameters of a BIM BH3 staple scanning library. Hydrophobicity was calculated as described[1], with X assigned the hydrophobicity value of Leu. Retention time in minutes reflects the peptide elution time from an HPLC C18 column (Agilent, 1200) run for 20 minutes using a 5-95% water:acetonitrile gradient (10 mM ammonium carbonate). Percent α-helicity of stapled peptides (50 μM, 10% acetonitrile in water) was derived from circular dichroism spectra using the mdeg value at 222 nm, as described[2]. Net charge was calculated by summing the positive and negative charges of Arg and Asp/Glu, respectively. The isoelectric point (pI) calculation employed the EMBOSS values for pKa.

BIM BH3 Staple Walk - Principal Components (Eigenvectors)

Variable	Comp1	Comp2	Comp3	Unexplained
pH7 retention time	0.6945	0.0137	0.1104	0.1305
percent helicity	0.1656	-0.7771	0.5604	0.01413
pI	-0.3947	0.4044	0.7843	0.01693
hydrophobicity	0.5783	0.4821	0.2422	0.0967

Component	Eigenvalue	Difference	Proportion	Cumulative
1	1.78087	0.672352	0.4452	0.4452
2	1.10852	0.256167	0.2771	0.7223
3	0.852352	0.594095	0.2131	0.9354

Supplementary Table 2. Principal component analysis of biophysical parameters impacting the variability of a staple scanning BIM BH3 library. The overall variability in the data can be explained by the cumulative contributions of hydrophobicity/HPLC retention time (component 1), percent α-helicity (component 2), and pI (component 3).

		Hydrophobicity	Retention Time	% Alpha-Helicity	Net Charge	pI
BIM SAHB9 (Q150L)	IWIALELRXIGDXFNAYYARR	0.669	10.7	64	1	9.39
BIM SAHB9 (E151L)	IWIAQLLRXIGDXFNAYYARR	0.689	12.0	67	2	10.27
BIM SAHB9 (R153L)	IWIAQELLXIGDXFNAYYARR	0.707	11.2	59	0	8.87
BIM SAHB9 (D157L)	IWIAQELRXIGLXFNAYYARR	0.695	10.0	54	2	10.30
BIM SAHB9 (W147L)	ILIAQELRXIGDXFNAYYARR	0.551	10.1	69	1	9.39
BIM SAHB9 (A161L)	IWIAQELRXIGDXFNLYYARR	0.644	10.3	61	1	9.39
BIM SAHB9 (Y162L)	IWIAQELRXIGDXFNALYARR	0.613	10.7	58	1	9.27
BIM SAHB9 (W147E)	IEIAQELRXIGDXFNAYYARR	0.440	9.6	53	0	8.73
BIM SAHB9 (Y162E)	IWIAQELRXIGDXFNAEYARR	0.501	10.1	51	0	8.53
BIM SAHB9 (A161R)	IWIAQELRXIGDXFNRYYARR	0.515	9.7	95	2	9.78
BIM SAHB9 (Y162R)	IWIAQELRXIGDXFNARYARR	0.484	9.3	50	2	9.73
BIM SAHB9 (R153D)	IWIAQELDXIGDXFNAYYARR	0.589	10.8	21	-1	8.16
BIM SAHB9 (I155E)	IWIAQELRXEGDXFNAYYARR	0.461	9.0	85	0	8.73
BIM SAHB9 (A164T)	IWIAQELRXIGDXFNAYYTRR	0.575	10.4	100	1	9.39
BIM SAHB9 (W147R)	IRIAQELRXIGDXFNAYYARR	0.422	8.8	66	2	9.78
BIM SAHB9 (A161E)	IWIAQELRXIGDXFNEYYARR	0.532	10.3	85	0	8.73
BIM SAHB9 (Y162T)	IWIAQELRXIGDXFNATYARR	0.544	10.2	52	1	9.27
BIM SAHB9 (G157E)	IWIAQELRXIEDXFNAYYARR	0.547	10.2	65	0	8.73
BIM SAHB9 (F159V)	IWIAQELRXIGDXVNAYYARR	0.550	10.3	50	1	9.39

Supplementary Table 3. Sequence composition and biophysical parameters of a BIM SAHB$_{A1}$ mutagenesis library. Parameters were calculated or experimentally-derived as described for Supplementary Table 1.

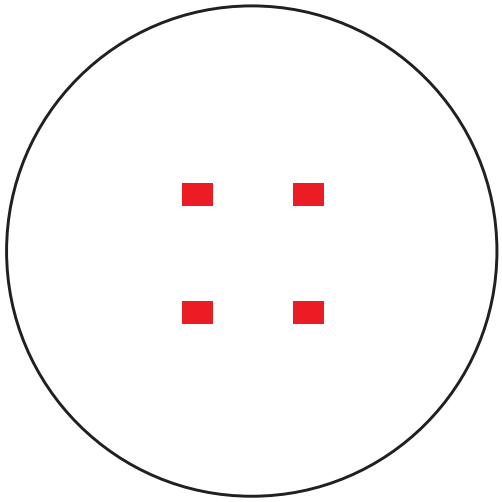
BIM BH3 Point Mutants - Principal Components (Eigenvectors)

Variable	Comp1	Comp2	Comp3	Unexplained
pH7 retention time	0.6692	-0.1098	0.3233	0.06539
percent helicity	-0.1788	0.6880	0.7017	0.00035
pI	0.1946	0.7125	-0.6332	0.08204
hydrophobicity	0.6946	0.0833	0.0465	0.07633

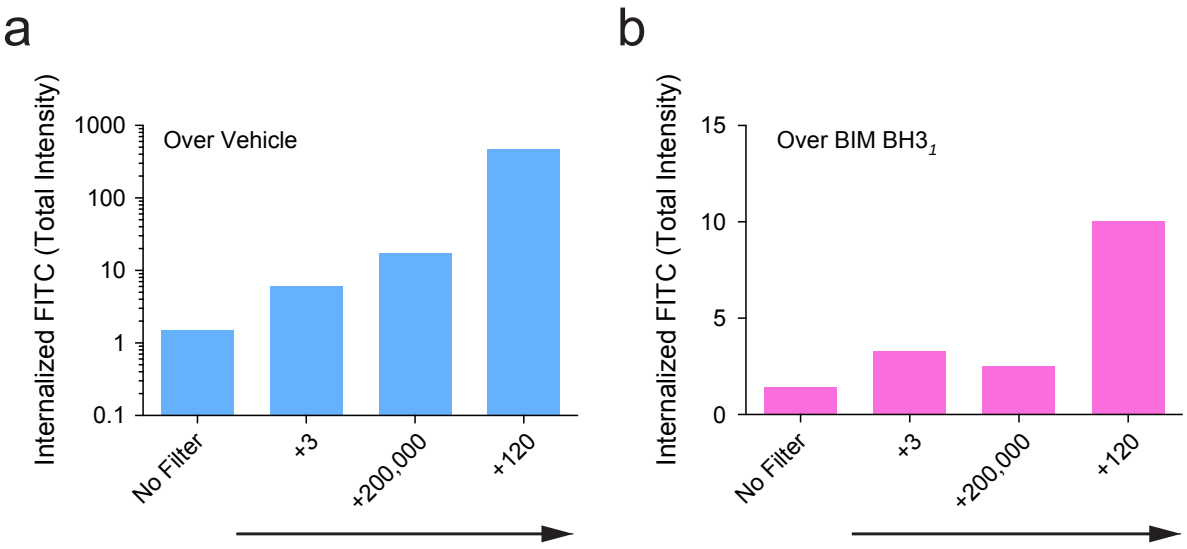
Component	Eigenvalue	Difference	Proportion	Cumulative
1	1.89333	0.620503	0.4733	0.4733
2	1.27283	0.589094	0.3182	0.7915
3	0.68373	0.533615	0.1709	0.9625

Supplementary Table 4. Principal component analysis of biophysical parameters impacting the variability of a BIM SAHB$_{A1}$ point mutant library. The overall variability in the data can be explained by the cumulative contributions of hydrophobicity/HPLC retention time (component 1), pI (component 2), and percent α-helicity (component 3).

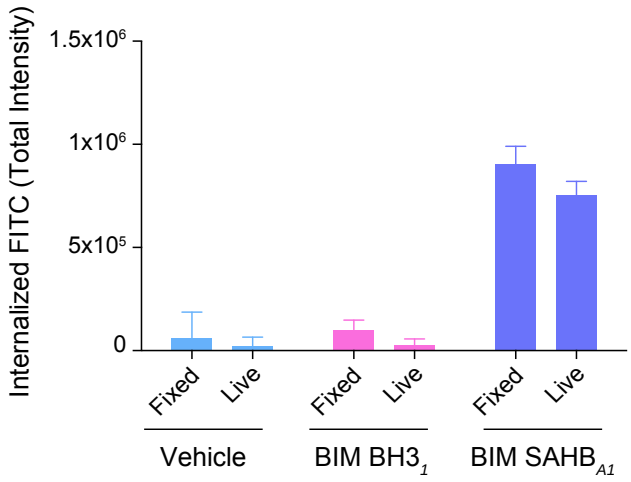
Supplementary Figures



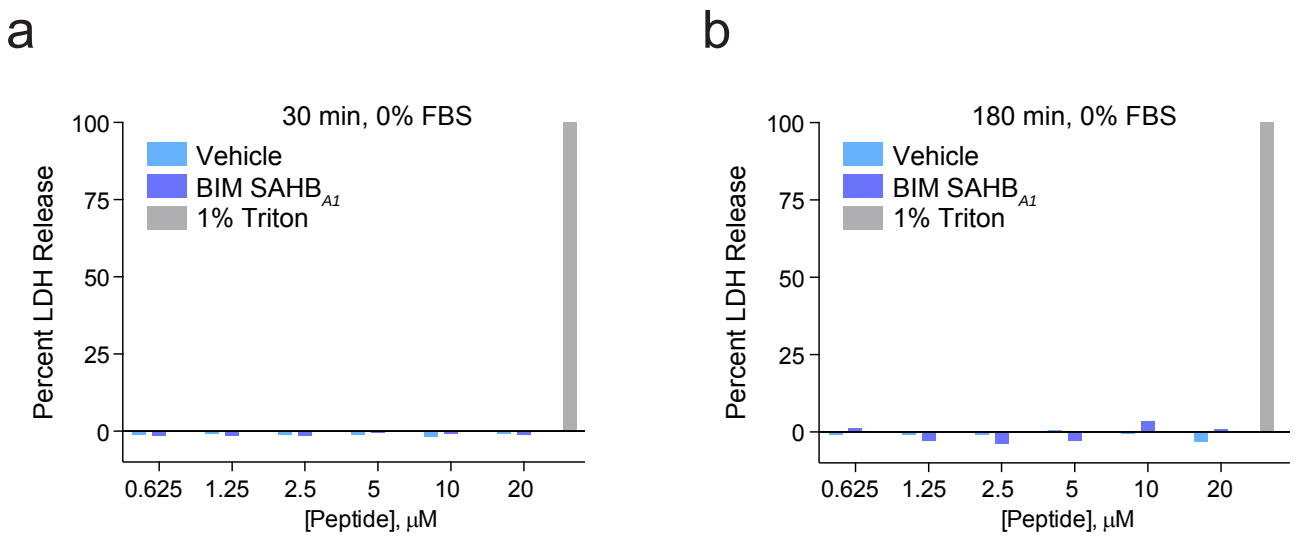
Supplementary Figure 1. Microscopic imaging field. Acquisition points (red) in the microscopic field (circle) for measuring total internalized FITC intensity on a per cell basis in 96 well format by IXM at 20x magnification.



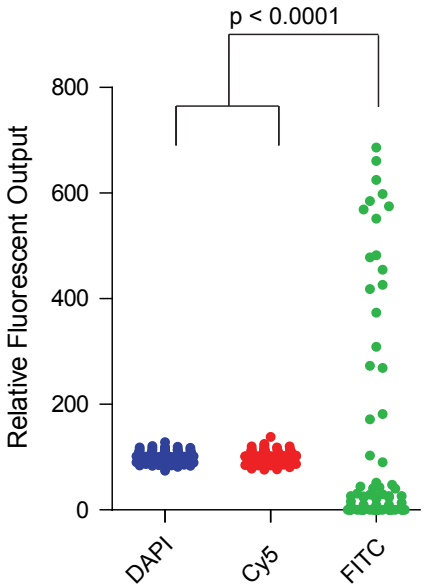
Supplementary Figure 2. Optimization of signal-to-noise ratios for fluorescence measurements. A custom module (CM) was designed to maximize signal-to-noise detection of FITC-stapled peptides for optimal sensitivity and specificity of internalized peptide measurement. From left to right, the first bar represents no filter followed by the cumulative addition of (1) a threshold intensity signal requirement of 3 over local background, (2) a threshold intensity requirement for objects (cells) having total intensity per cell of 200,000, and (3) a threshold intensity requirement for objects (cells) displaying an average intensity per cell of 120. Applying this CM, internalized FITC-stapled peptide was well discerned with respect to (**a**) DMSO and (**b**) unmodified template peptide.



Supplementary Figure 3. Effect of cell fixation on TIFI. Cell fixation with 4% paraformaldehyde had no effect on TIFI when compared to acquisition and analysis using live cells treated with DMSO, BIM $BH3_1$, or BIM $SAHB_{A1}$. Data are mean ± s.d. for experiments performed in technical quadruplicate for vehicle and BIM $BH3_1$, and octuplicate for BIM $SAHB_{A1}$, with 4 image acquisitions per well. Three biological replicates were performed for each experiment with similar results.

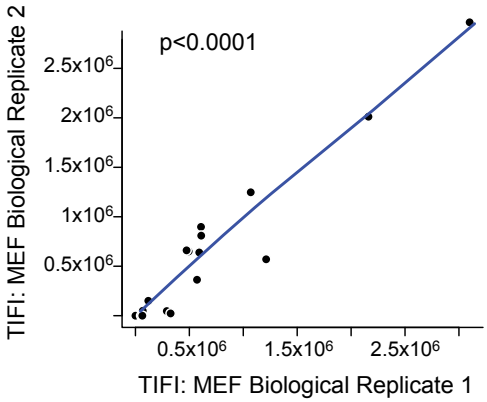


Supplementary Figure 4. Effect of BIM SAHB$_{A1}$ on plasma membrane integrity. MEFs ($2x10^4$ cells/well) were subjected to a serial dilution of BIM SAHB$_{A1}$ for (**a**) 30 min and (**b**) 180 min in media lacking serum. No cellular lysis was observed, as measured by LDH release assay. Treatment with 1% Triton X-100 served as the positive control for maximal release. BIM SAHB$_{A1}$ treatments were performed in technical duplicate and the experiment was repeated twice.

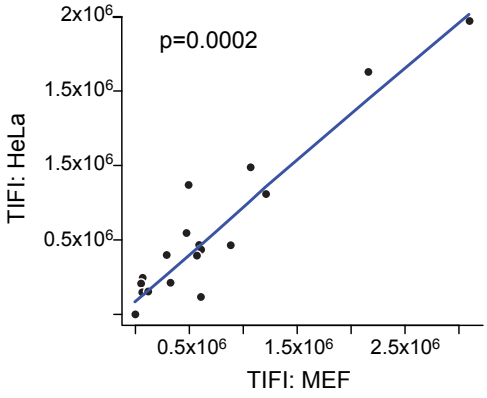


Supplementary Figure 5. Variation in detected fluorescence signals upon stapled peptide treatment. There was no change in acquired DAPI and Cy5 channels upon treating MEFs with fluorescent stapled peptides. Variation was only observed in the FITC channel, consistent with expected differences in cellular uptake propensity for stapled peptides bearing alternatively placed staples and point mutations. Intensities were scaled such that the mean of each channel was set to 100. Each dot within a given cluster represents the fluorescence from a distinct well of a 96-well plate. p-value was determined by Kruskal-Wallis test.

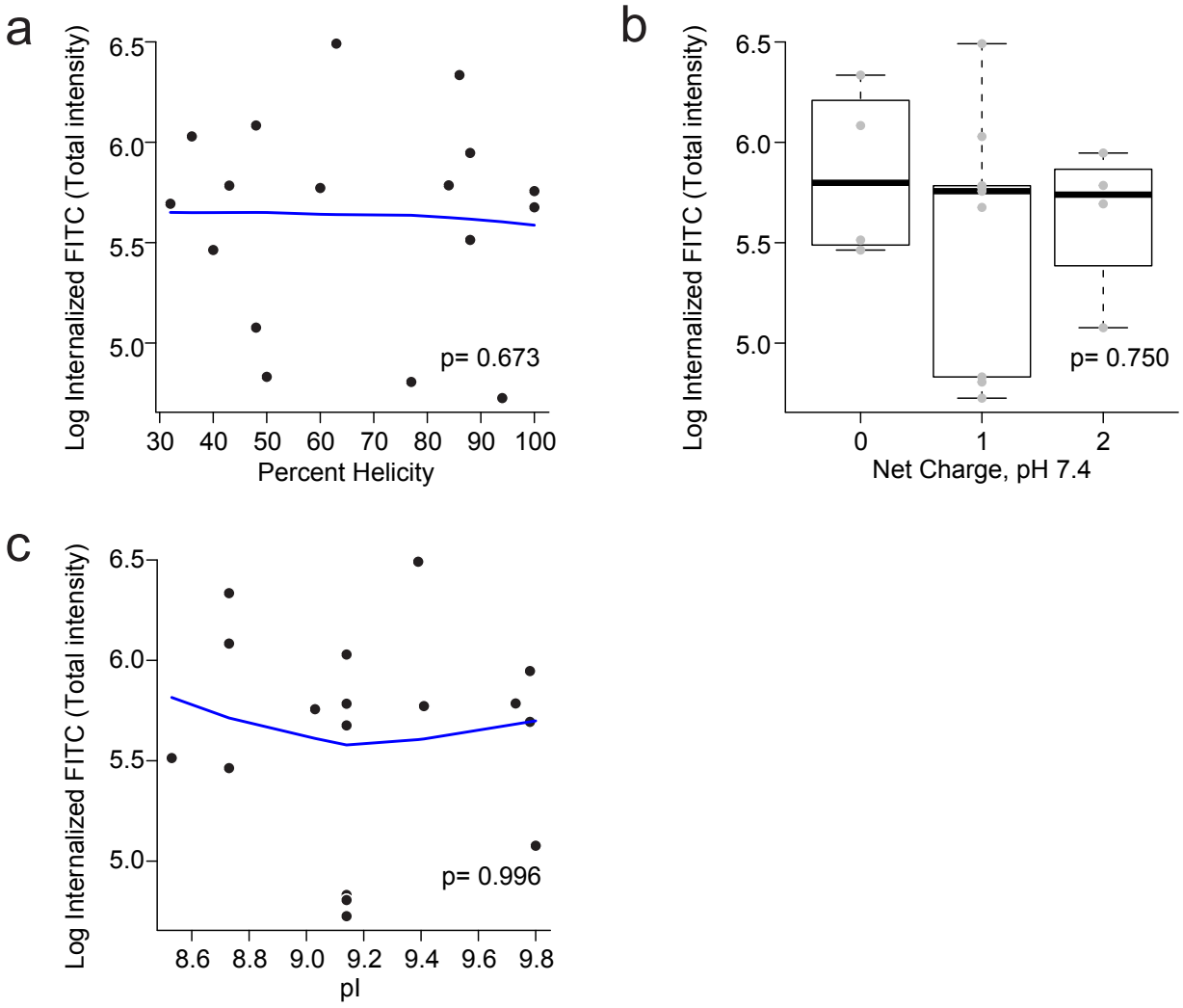
a



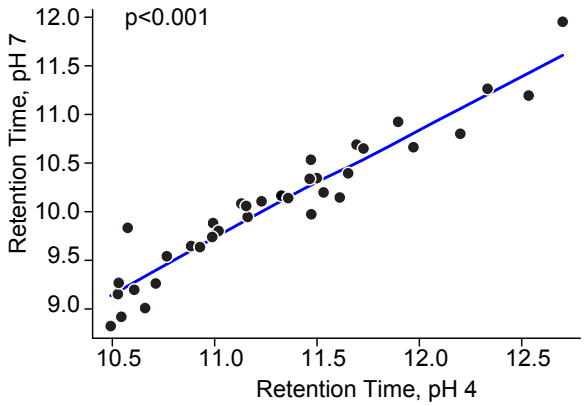
b



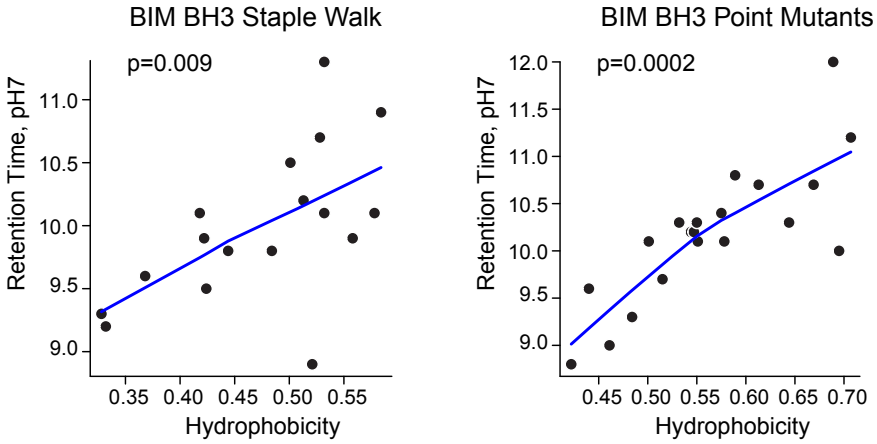
Supplementary Figure 6. Association between TIFI values measured in biological replicates and in distinct cell lines. The TIFI values for cellular uptake of BIM SAHB peptides between (a) biological replicates and (b) MEF vs. HeLa cells are strongly associated, as assessed by Spearman's rank correlation. Each dot represents a distinct stapled peptide from the staple walk library. p-values were calculated using the permutation test included in the R package pvrank, and line fitting was performed using a loess smoother.



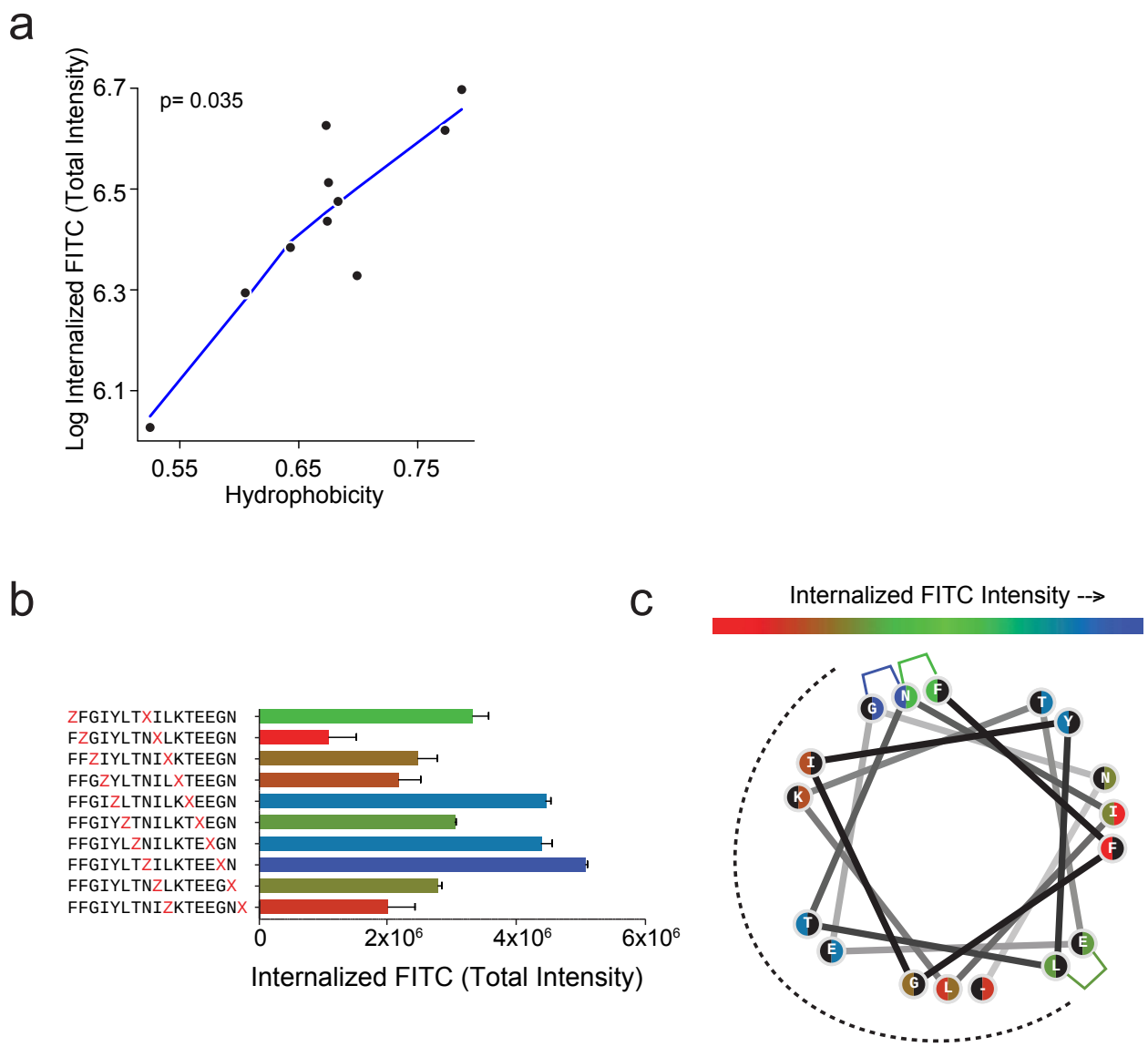
Supplementary Figure 7. Determinants of cellular uptake for differentially stapled BIM BH3 peptides. Single variable plots for TIFI vs. (**a**) percent α-helicity, (**b**) net charge at pH 7.4, and (**c**) pI, as assessed by Spearman's rank correlation (**a, c**) or Kruskal-Wallis test (**b**). p-values were calculated using the permutation test included in the R package pvrank, and line fitting was performed using a loess smoother (**a, c**).



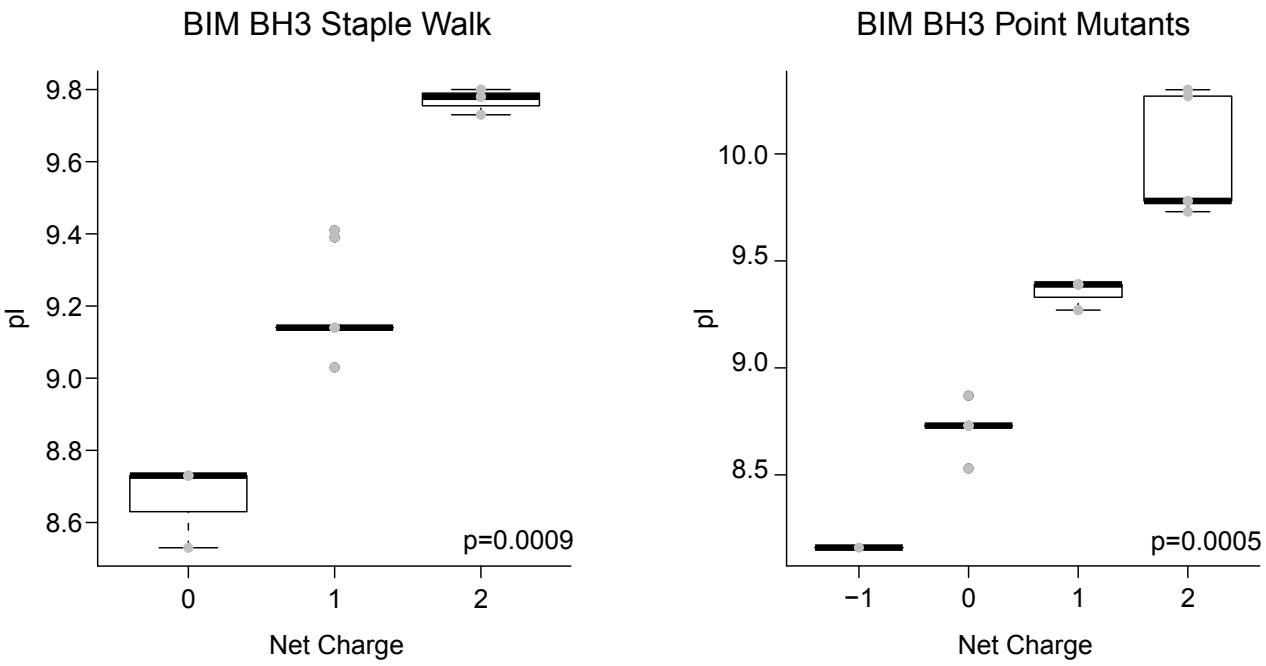
Supplementary Figure 8. Association between peptide retention time measurements at pH 4 and 7. Retention times were experimentally determined for each stapled peptide using pH 4 and pH 7 HPLC buffer conditions. The retention time values for both conditions are highly associated, as assessed by Spearman's rank correlation. Each dot represents a distinct stapled peptide from the staple walk and point mutant libraries. p-values were calculated using the permutation test included in the R package pvrank, and line fitting was performed using a loess smoother.



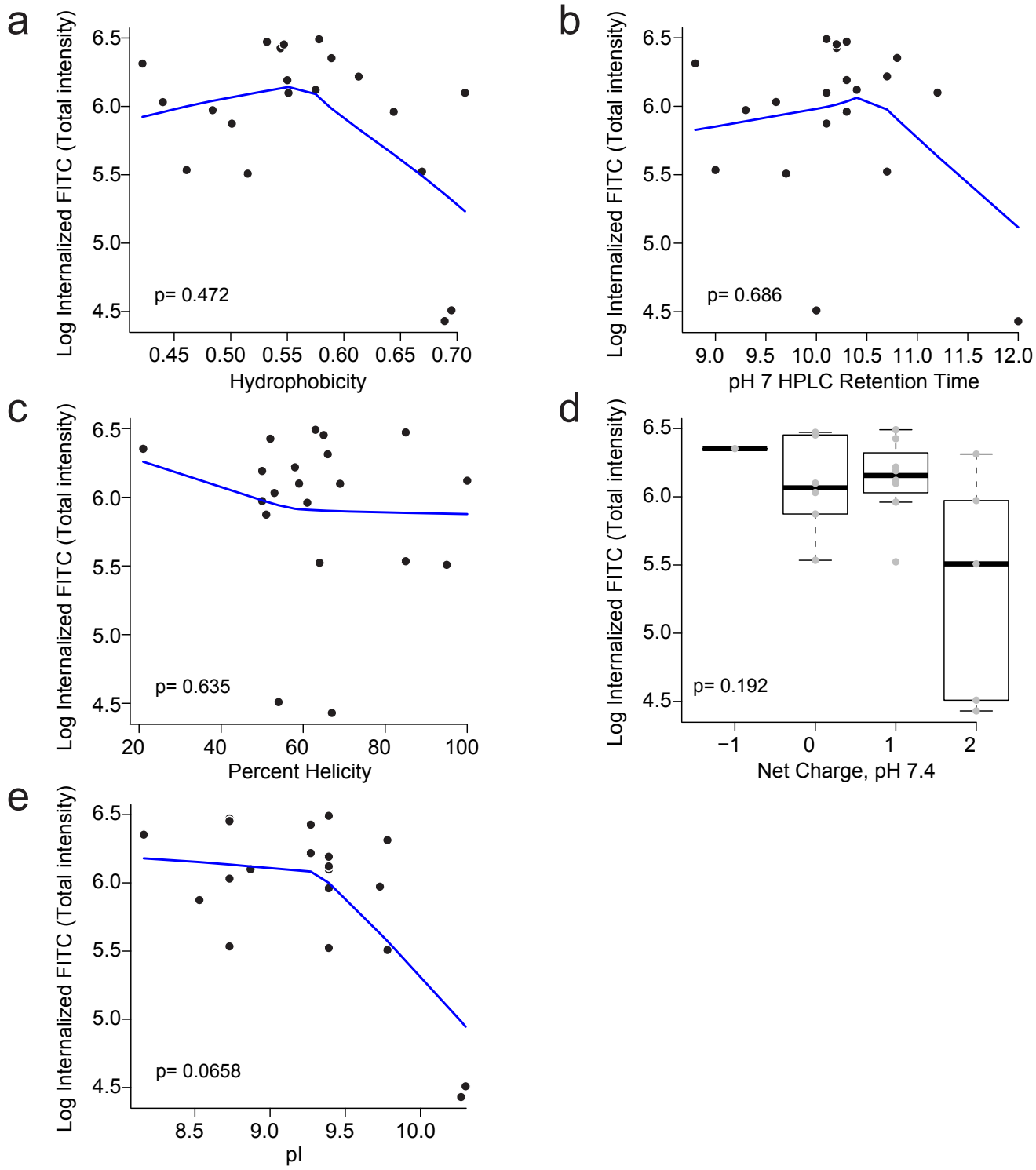
Supplementary Figure 9. Relationship between peptide hydrophobicity and retention time at pH 7. The calculated hydrophobicity and experimentally-determined HPLC retention time (pH 7) are strongly associated by Spearman's rank correlation for BIM BH3 peptides in both the staple walk (left) and point mutant (right) libraries. p-values were calculated using the permutation test included in the R package pvrank, and line fitting was performed using a loess smoother.



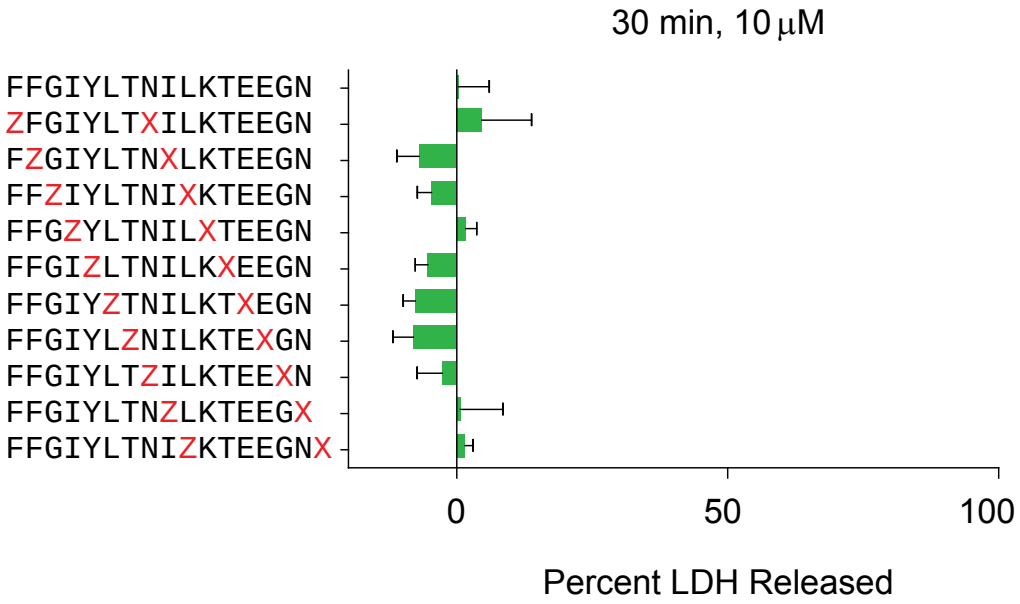
Supplementary Figure 10. Cellular uptake of differentially stapled SOS1 peptides. (**a**) Single variable plot for TIFI vs. calculated hydrophobicity of SOS1 peptides (aa 929-944) bearing sequentially placed *i, i+7* staples, as assessed by Spearman's rank correlation. p-value was calculated using the permutation test included in the R package pvrank, and line fitting was performed using a loess smoother. (**b**) Range of TIFI for MEFs ($2x10^4$/well) treated with 500 nM peptides and measured by IXM (20x) at 4 hours. Data are mean ± s.d. for experiments performed in duplicate wells with 4 image acquisitions per well. Three biological replicates were performed with similar results. Z, R-octenyl alanine; X, S-pentenyl alanine. (**c**) Wheel depiction of the SOS1 α-helix, with the KRAS-interaction face indicated by the dotted surface and stapling amino acid pairs color-coded based on level of measured TIFI. Residues that participate in two distinct staples are shown as split circles, with the left and right colors corresponding to their roles in N- vs. C-terminal staples, respectively.



Supplementary Figure 11. Relationship between peptide net charge and isoelectric point. The calculated net charge and pI parameters are highly associated based on Kruskal-Wallis test for BIM BH3 peptides in both the staple walk (left) and point mutant (right) libraries.



Supplementary Figure 12. **Impact of point mutagenesis on the cellular uptake of BIM SAHB$_{A1}$.** (**a-e**) Single variable plots for TIFI vs. (**a**) calculated hydrophobicity, (**b**) HPLC retention time (pH 7), (**c**) percent α-helicity, (**d**) net charge at pH 7.4, and (**e**) pI, as assessed by Spearman's rank correlation (**a-c, e**) or Kruskal-Wallis test (**d**). p-values were calculated using the permutation test included in the R package pvrank, and line fitting was performed using a loess smoother (**a-c, e**).



Supplementary Figure 13. Cellular LDH release testing of SAH-SOS1 peptides. A staple scanning SOS1 peptide library was screened for membrane lytic properties by LDH release assay, performed on Jurkat T-cells ($2x10^4$ cells/well) treated with 10 μM peptide for 30 minutes in the absence of serum. Data are normalized based on the response to treatment with 1% Triton X-100 (100% release) and media alone (0% LDH release). Data are mean ± s.d. for experiments performed in technical triplicate and repeated twice.

Supplementary References

1. Eisenberg, D. & McLachlan, A.D. Solvation energy in protein folding and binding. *Nature* **319**, 199-203 (1986).
2. Bird, G.H., Bernal, F., Pitter, K. & Walensky, L.D. Synthesis and biophysical characterization of stabilized alpha-helices of BCL-2 domains. *Methods Enzymol* **446**, 369-86 (2008).